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Pharmacology, Biochemistry and Behavior 70 (2001) 55–63

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Lack of allosteric modulation of striatal $GABA_A$ receptor binding and function after cocaine sensitization

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Received 5 July 2000; received in revised form 13 April 2001; accepted 27 April 2001

Abstract

$GABA_A$ receptor binding after repeated cocaine has been shown to be either increased as indicated by benzodiazepine binding or decreased as indicated by convulsant-site binding. We measured the GABA binding site with [^{3}H]-muscimol binding to $GABA_A$ receptors and found no differences between saline- and cocaine-sensitized rats. Allosteric modulation of [^{3}H]-muscimol binding with flunitrazepam was also unchanged after cocaine sensitization. In addition, [^{3}H]-flunitrazepam binding and allosteric modulation of [^{3}H]-flunitrazepam binding with GABA was unchanged after 1 day withdrawal from repeated cocaine. $GABA_A$ receptor function and allosteric modulation of $GABA_A$ receptor function measured by GABA-stimulated Cl^- uptake was also unchanged after withdrawal from repeated cocaine. Finally, in vitro cocaine reduced $GABA_A$ receptor function in striatal microsacs of saline- and cocaine-treated rats. In conclusion, repeated cocaine did not change the coupling of the $GABA_A$ receptor between the GABA and benzodiazepine (BZD) binding site after 1 day withdrawal.

Keywords: Striatum; Cocaine; Behavioral sensitization; Allosteric modulation; Cl uptake

1. Introduction

$GABA_A$ receptors possess multiple ligand binding sites that are both clinically and functionally relevant such as the benzodiazepine (BZD) and convulsant sites (Sieghart, 1992). There exists multiple $GABA_A$ receptor subpopulations because $GABA_A$ receptor subunit expression and composition influence the structural and pharmacologic properties, where there are multiple subunits and different combinations of subunits (Sieghart, 1995). Consequently ligands can bind to one $GABA_A$ receptor subpopulation without binding to the entire $GABA_A$ receptor population. The specific $GABA_A$ receptor agonist, muscimol, binds to a GABA recognition site on the interface of the $\alpha\beta$ subunit combination of the $GABA_A$ receptor (Zezula et al., 1996). Flunitrazepam and clonazepam bind to the allosteric BZD site on the interface of the $\alpha\gamma$ subunit combination of the $GABA_A$ receptor and allosterically modulate receptor function (Sieghart, 1995). Allosteric ligand sites confer additional regulatory control on receptor function, which can be measured both by changes in receptor binding and changes in agonist stimulated $^{36}Cl^-$ uptake. The convulsant site is located near the chloride ionophore on the $GABA_A$ receptor and can bind *t*-butyl bicyclophos phorothionate (TBPS) (Olsen et al., 1990). After repeated cocaine it is not clear if there are changes in $GABA_A$ receptor number because there are reported increases in BZD binding (Goeders, 1991) and decreases in convulsant binding (Pecins-Thompson and Peris, 1993). Although there were variances between these studies in the length of repeated cocaine and withdrawal, another critical difference was that different noncompetitive sites on the $GABA_A$ receptor were measured. Since the site at which GABA binds to the $GABA_A$ receptor was not measured and this site interacts with the BZD binding site, the effect of repeated cocaine on these binding sites was examined in this study.

Binding to the $GABA_A$ receptor can be affected after repeated cocaine by a variety of receptor changes. First, the absolute number of receptors may have changed but, as stated previously, changes in both directions have been

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0091-3057/01/$ – see front matter
PII: S0091-3057(01)00580-9

reported (Goeders, 1991; Pecins-Thompson and Peris, 1993). Second, changes in the affinity of the receptor at either the main or the allosteric ligand binding site could affect ligand binding. However, this does not occur with either BZD or ethanol tolerance (Allan et al., 1992; Buck and Harris, 1990). Third, changes in the number of allosteric binding sites, but not the main agonist binding site could indicate allosteric receptor uncoupling (Roca et al., 1990), which occurs between the $GABA_A$ receptor and the BZD allosteric site with BZD tolerance (Gallager et al., 1984). Receptor uncoupling was also demonstrated by decreased $GABA_A$ receptor $^{36}Cl^-$ uptake in the presence of a BZD compound (Allan et al., 1992; Buck and Harris, 1990; Li et al., 1993; Yu et al., 1988). After repeated cocaine, the increase in BZD binding (Goeders, 1991) and decrease in convulsant binding (Pecins-Thompson and Peris, 1993) could be explained if in this $GABA_A$ receptor subpopulation that the $GABA_A$ receptor ionophore was uncoupled from the allosteric BZD site. Since GABA-stimulated $^{36}Cl^-$ uptake was decreased in cocaine-sensitized rats (Peris, 1996), we hypothesized that the functional binding sites of the $GABA_A$ receptor were uncoupled after cocaine sensitization. If the BZD site is uncoupled from the $GABA_A$ receptor after repeated cocaine, this could further strengthen the role for decreased striatal GABAergic neurotransmission after cocaine sensitization (Jung et al., 1999; Peris, 1996) and possibly explain the discrepancies in binding to the $GABA_A$ receptor after cocaine sensitization.

In this study, binding to both the GABA and BZD site was measured after repeated cocaine by quantitative autoradiography in various brain regions. Since the previous reports (Goeders, 1991; Pecins-Thompson and Peris, 1993; Peris, 1996) did not measure the number of the main agonist binding site on the $GABA_A$ receptor, it would be important to determine if there are differences in this site after repeated cocaine. In addition, $GABA_A$ receptor number has not been evaluated for allosterically modulated binding after repeated cocaine, which could be relevant if receptor bindings were uncoupled. The region of primary interest was the striatum (STR) because of the hypothesized effects of repeated cocaine in the maintenance of cocaine sensitization in this brain area (see Kalivas et al., 1993; Koob, 1992). In addition, the STR has regional differences between its head and tail regions, thus the STR was measured at two levels in the brain. The cortex (CTX) was measured because $GABA_A$ receptor composition in this brain area is different and in vivo cocaine does not affect this brain area. Nucleus accumbens (NAc) was also measured to determine if changes in $GABA_A$ receptor binding occur after repeated cocaine, which may be related to cocaine addiction.

The effects of in vitro cocaine have been examined on GABA-stimulated $^{36}Cl^-$ uptake in microsacs, but without the inclusion of protease inhibitors (Peris, 1996). The purpose of that previous study was to show that the presence of cocaine in tissue, which should be little to none after 1 day withdrawal, was not causing the decrease in striatal GABA-stimulated $^{36}Cl^-$ uptake after cocaine withdrawal. Originally, we observed that there was no change in $GABA_A$ receptor function with the addition of in vitro cocaine. However, from preliminary data obtained with the inclusion of protease inhibitors in the assay buffer, an effect of in vitro cocaine has been observed. Hence, we were interested in determining if in vitro cocaine had a greater effect on striatal GABA-stimulated $^{36}Cl^-$ uptake after repeated cocaine compared to control. Additionally, allosteric modulation of stimulated $GABA_A$ receptor function after repeated cocaine was measured to determine if changes occurred in the coupling of the $GABA_A$ receptor.

2. Methods

2.1. Animals and treatment

Male Sprague–Dawley rats (Harlan) were used and housed in the University of Florida Animal Care Facility with a 12-h light/dark cycle and allowed rat chow and water ad libitum. Rats care and all experimental procedures were approved by the University of Florida's Institutional Animal Care and Use Committee and meet criteria for the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments for $GABA_A$ receptor binding and function were carried out on rats treated in two separate groups. For receptor binding, rats received either 1.0 ml kg^{-1} saline intraperitoneally (Group S, $n=11$) or 15 mg kg^{-1} cocaine intraperitoneally (Group C15, $n=11$). For cocaine treatment, the rats were treated as follows. After two or three daily saline injections (1.0 ml kg^{-1} ip) in the home cages, rats were treated with cocaine once daily for 14 days. Rats were killed 1 day after their last injection to obtain brain hemispheres. Right and left brain hemispheres were rapidly frozen on powdered dry ice. Brains were stored at $-80°C$ until sectioning which was between 3 and 5 months.

For $GABA_A$ receptor function, rats received either 1.0 ml kg^{-1} saline intraperitoneally (Group S, $n=11$) or 20 mg kg^{-1} cocaine intraperitoneally (Group C20, $n=10$). We increased the dose of cocaine used previously to increase the number of sensitized rats and the range of behavioral scores. After three daily saline injections (1.0 ml kg^{-1} ip) in the home cages, rats were treated with saline or cocaine once daily for 14 days. In this study, two separate groups of treated rats were prepared at different times. In the first set of treated rats, one of the cocaine-treated rats was sick and subsequently euthanized. Rats were killed 1 day after their last injection to obtain striatal and cortical tissue.

2.2. Behavioral rating

Locomotor and stereotyped behaviors were quantified using a modified five-point rating scale as described previously (Ernst, 1967). Behavior was rated for 10 s every

2.5 min for 60 min on Days 1, 7, and 14 depending on the experiment. The total behavioral score was calculated by summing the individual scores into a cumulative total. Rats were determined to be sensitized if there was a significant increase in behavioral scores when Day 14 was compared to Day 1.

2.3. [^{3}H]-muscimol binding

Frozen brains were cut into 15 μm coronal sections on a cryrostat. Duplicate sections were thaw-mounted onto gelatin-coated glass slides. Two regions of the brain were identified and isolated according to coordinates by Paxinos and Watson (1993). The first slice contained the head of the STR, CTX, and NAc (rostral). The second slice contained the body–tail of the STR and the CTX (caudal). Slide-mounted sections were brought to room temperature and allowed to completely dry. The buffer for this experiment was 0.31 M Tris–citrate (TC, pH 7.1). TC buffer was used for the following [^{3}H]-muscimol binding experiments unless stated otherwise. The wash incubation consisted of two 20-min incubations at 4°C; 30 nM [^{3}H]-muscimol (12.05 Ci mmol^{-1}, NEN Life Sciences) binding was for 90 min at 4°C. Nonspecific [^{3}H]-muscimol binding was determined in the presence of GABA (200 μM). On adjacent sections in a separate experiment, 30 nM [^{3}H]-muscimol was coincubated with 1 μM flunitrazepam for 90 min at 4°C. Nonspecific [^{3}H]-muscimol binding, which also contained 1 μM flunitrazepam, was determined in the presence of GABA (200 μM). The reported K_d for GABA is 360.3 nM (Bristow and Martin, 1989). After total and nonspecific binding, slides were washed for 1 min at 4°C and then dipped in distilled H_2O for 1 s. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm (Amersham) for 6 weeks. Film was developed using D-19 (Kodak) for 4 min, stop bath for 1 min, and fix bath for 4 min. Finally, the film was washed for 10 min in H_2O and then allowed to dry.

2.4. [^{3}H]-flunitrazepam binding

Additional adjacent slide-mounted sections, previously described, were brought to room temperature and allowed to completely dry. The buffer for this experiment was Ca^{2+} buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.5 with Tris–base). The wash incubation consisted of one 20-min incubation at 4°C. The K_d for flunitrazepam has been reported to be 3.0 nM in the presence of GABA (Villar et al., 1989). 3 nM [^{3}H]-Flunitrazepam (82 Ci mmol^{-1}, NEN Life Science Products) binding was determined for 30 min at 4°C. Nonspecific [^{3}H]-flunitrazepam binding was determined in the presence of 3 μM clonazepam. The K_i of clonazepam for [^{3}H]-flunitrazepam binding has been reported to be 0.51 nM (Greenblatt et al., 1987). On adjacent sections in a separate experiment, 3 nM [^{3}H]-flunitrazepam was coincubated with 200 μM GABA for 30 min at 4°C. Nonspecific [^{3}H]-flunitrazepam binding was also determined with both 200 μM GABA and 3 μM clonazepam. After total and nonspecific binding, slides were washed in buffer for 6 min at 4°C and then dipped in distilled H_2O for 1 s. Slides were then quickly dried by a flow of warm air. Slides were apposed to Hyperfilm for 4 weeks. Film was developed as described above.

2.5. Film analysis

Film analysis was performed on brain images that were visualized using a light box (Northern Light, Imaging Research, Canada) and digitized using a video camera (Sierra Scientific 4030 solid state video camera) with a Nikon 55 mm Micro lens (Nikon, USA). The image was then captured onto a personal computer and analyzed with quantitative imaging software (BRS2, Imaging Research). Film density was normalized using [^{3}H]-Microscales (Amersham) against a known concentration of protein. Specific binding was determined by subtracting nonspecific binding from total binding.

2.6. GABA-stimulated $^{36}Cl^-$ uptake in brain microsacs

The method for microsac preparation and $^{36}Cl^-$ uptake for brain microsacs from the STR and CTX has been previously described (Peris, 1996). Briefly, brains were homogenized using a Potter–Elvehjem tissue grinder in ice-cold Ca^{2+} buffer containing protease inhibitors (10 μM antipain, 10 μg/μl aprotinin, and 10 μM leupeptin). Homogenates were centrifuged at $900 \times g$ for 15 min using a Sorvall SA600 rotor; supernatants were discarded; pellets were resuspended and centrifuged again. The resulting pellets (microsacs) were suspended in Ca^{2+} buffer with protease inhibitors to yield 800–1600 μg of protein per milliliter.

Next, microsacs were preincubated for 5 min at 34°C. The subsequent 3-s incubation (uptake period) contained both $^{36}Cl^-$ and treatment drugs. Assays with drug pretreatment included the drug in the 5-min preincubation period as well as during the uptake period. Uptake was terminated by addition of Ca^{2+} buffer with 100 μM picrotoxin and followed by vacuum filtration onto a glass fiber filter (Whatman). Two additional washes of Ca^{2+} buffer with picrotoxin were applied onto the filter. The amount of $^{36}Cl^-$ retained on the filter was determined by liquid spectrophotometry. Stimulated $^{36}Cl^-$ uptake was defined as the amount of $^{36}Cl^-$ taken up while agonist was present minus the amount of $^{36}Cl^-$ taken up when agonist was not present. GABA (20 μM) was used as the $GABA_A$ receptor agonist. Cocaine (10 μM) was added during both preincubation and uptake periods. Clonazepam is a BZD that increases $GABA_A$ receptor $^{36}Cl^-$ uptake in the presence of GABA (Francis et al., 1994; Li et al., 1993). Clonazepam (20 μM) was included only during the uptake period (3 s) in the assays.

2.7. Data analysis

The data are expressed as the mean ± S.E.M.; n = number of experiments or subjects. For statistical analysis, Wilcoxon signed-rank test was group behavioral data across days. Mann–Whitney U was used to determine within-group differences. When appropriate for binding results, ANOVA was used to analyze quantitative autoradiography binding. ANOVA was used on $^{36}Cl^-$ uptake data. When appropriate, the Bonferroni–Dunn analysis was used as the post hoc follow-up test. A P value less than .05 was considered to be significant.

3. Results

3.1. Behavior

Rats were treated with either repeated saline or cocaine injections for studies by quantitative autoradiography and GABA-stimulated $^{36}Cl^-$ uptake. For quantitative autoradiography studies, rat behavior was rated on Days 1 and 14 (Fig. 1A). Group S did not have a significant change in behavior across days. Group C15 treatment resulted in a significant increase in behavior when Day 14 was compared to Day 1 ($P<.05$). In this study, two separate groups of treated rats were prepared. In the first group of cocaine-treated rats, only three of the six rats showed higher behavioral scores when Day 14 was compared to Day 1 (data not shown). In the second group of cocaine-treated rats, all five rats showed higher behavioral scores when Day 14 was compared to Day 1 (data not shown). The behavioral scores for Day 1 or Day 14 between the separately cocaine-treated rats were not significantly different and combined for ease of comparison.

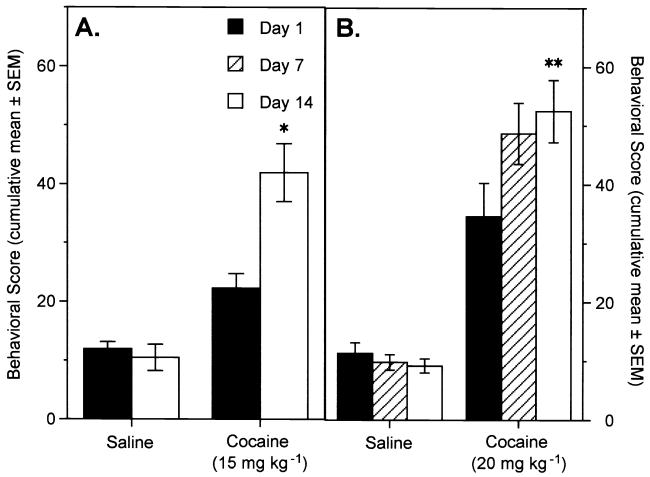

Fig. 1. Behavioral scores for saline- and cocaine-treated rats. (A) Cumulative behavior scores of saline-treated (0.1 ml kg^{-1}) and cocaine-treated (15 mg kg^{-1}) rats on Days 1 and 14. Daily cocaine treatment led to a significant greater behavior on Day 14 compared to Day 1 ($P<.05$). (B) Cumulative behavioral scores of saline-treated (0.1 ml kg^{-1}) and cocaine-treated (20 mg kg^{-1}) rats on Days 1, 7, and 14. Daily cocaine treatment led to a significantly greater behavior on Day 14 compared to Day 1 ($P<.01$). Behavior was rated for 10 s every 2.5 min for 60 min. The cumulative behavioral scores within treatment groups were compared across days using the Wilcoxon signed-rank test. Data are expressed as the cumulative mean ± S.E.M. for $n=10–11$ per group. * $P<.05$ vs. Day 1 cocaine, ** $P<.01$ vs. Day 1 cocaine.

For GABA-stimulated $^{36}Cl^-$ uptake studies, rat behavior was rated on Days 1, 7, and 14 (Fig. 1B). Again, Group S did not have in a significant change in behavior across days. Group C20 treatment resulted in a significant increase in behavior when Day 14 was compared to Day 1 ($P<.01$). In this study again, two separate groups of treated rats were prepared. In the first group, only four of the five surviving cocaine-treated rats showed a higher behavioral scores when Day 14 was compared to Day 1 (data not shown). In the second group of cocaine-treated rats, all five rats showed higher behavioral scores when Day 14 was compared to Day 1 (data not shown). The behavioral scores for Day 1 or Day 14 between the separate cocaine-treated rats were not significantly different and combined for ease of comparison.

3.2. Quantitative autoradiography of [^{3}H]-muscimol binding

The repeated administration of cocaine resulted in little or no effect either on binding of [^{3}H]-muscimol (Fig. 2A) or on [^{3}H]-muscimol binding modulated by flunitrazepam (Fig. 2B) in any of the regions measured. The CTX had greater [^{3}H]-muscimol binding relative to the STR and NAc in both saline- and cocaine-treated rats for both rostral [$F(2,60)=9.08$, $P<.01$] and caudal slices [$F(1,40)=29.32$, $P<.0001$]. Modulation of [^{3}H]-muscimol binding with flunitrazepam was dependent on the region and treatment (Fig. 2B). In modulated [^{3}H]-muscimol binding, the CTX again had greater binding relative to the STR and NAc for both rostral slices [$F(2,60)=7.49$, $P<.01$] and caudal slices [$F(1,40)=13.35$, $P<.001$]. In the saline-treated rats, there was a trend towards enhancement of [^{3}H]-muscimol binding with flunitrazepam (23–53%) in all regions measured. In cocaine-treated rats, the NAc showed a trend towards a decrease in enhancement of [^{3}H]-muscimol binding with flunitrazepam (24%). Variability obscured other possible changes in modulation of [^{3}H]-muscimol binding with flunitrazepam in saline- and cocaine-treated rats.

3.3. Quantitative autoradiography of [^{3}H]-flunitrazepam binding

The repeated administration of cocaine resulted in little or no effects on the number of $GABA_A$ receptors as measured by either [^{3}H]-flunitrazepam binding (Fig. 3A) or [^{3}H]-flunitrazepam binding modulated by GABA (Fig. 3B) in any of the regions measured. The CTX of both saline- and cocaine-treated rats had greater [^{3}H]-flunitra-

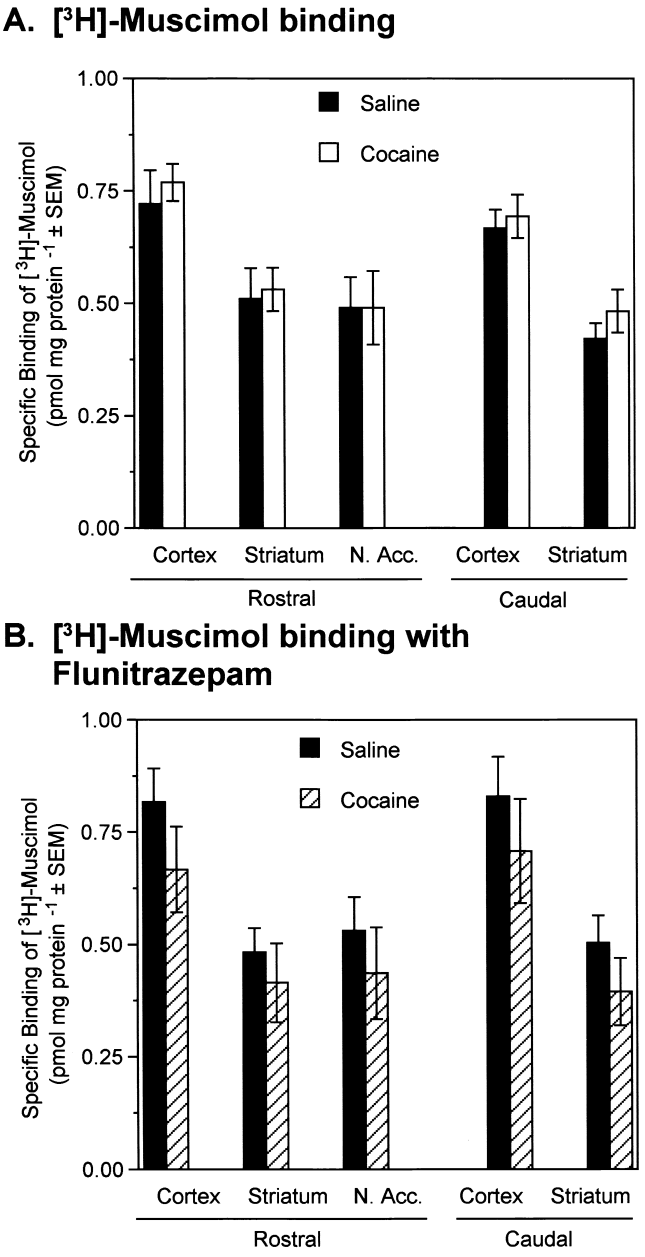

Fig. 2. Binding of [^{3}H]-muscimol without and with flunitrazepam. (A) Effects of repeated cocaine on specific [^{3}H]-muscimol binding. There was no significant difference between the two treatment groups. The CTX in both the rostral and caudal slices had significantly higher [^{3}H]-muscimol binding (rostral, $P<.01$; caudal, $P<.0001$). (B) Effects of repeated cocaine on specific [^{3}H]-muscimol binding modulated by flunitrazepam. There was no significant difference between the two treatment groups. The CTX in both the rostral and caudal slices again had significantly higher [^{3}H]-muscimol binding (rostral, $P<.01$; caudal, $P<.001$). Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 1 day after the last treatment. Data are expressed as the mean ± S.E.M. for $n=11$.

zepam binding relative to the STR and NAc for both rostral slices [$F(2,60)=39.32$, $P<.0001$] and caudal slices [$F(1,40)=36.92$, $P<.0001$]. In GABA-enhanced [^{3}H]-flunitrazepam binding, the CTX again had greater [^{3}H]-flunitrazepam binding relative to the STR and NAc for rostral slices [$F(2,60)=78.37$, $P<.0001$] and for caudal slices [$F(1,36)=252.17$, $P<.0001$]. GABA enhanced [^{3}H]-flunitrazepam binding between 61% and 99%. There was a significant interaction between enhanced GABA-

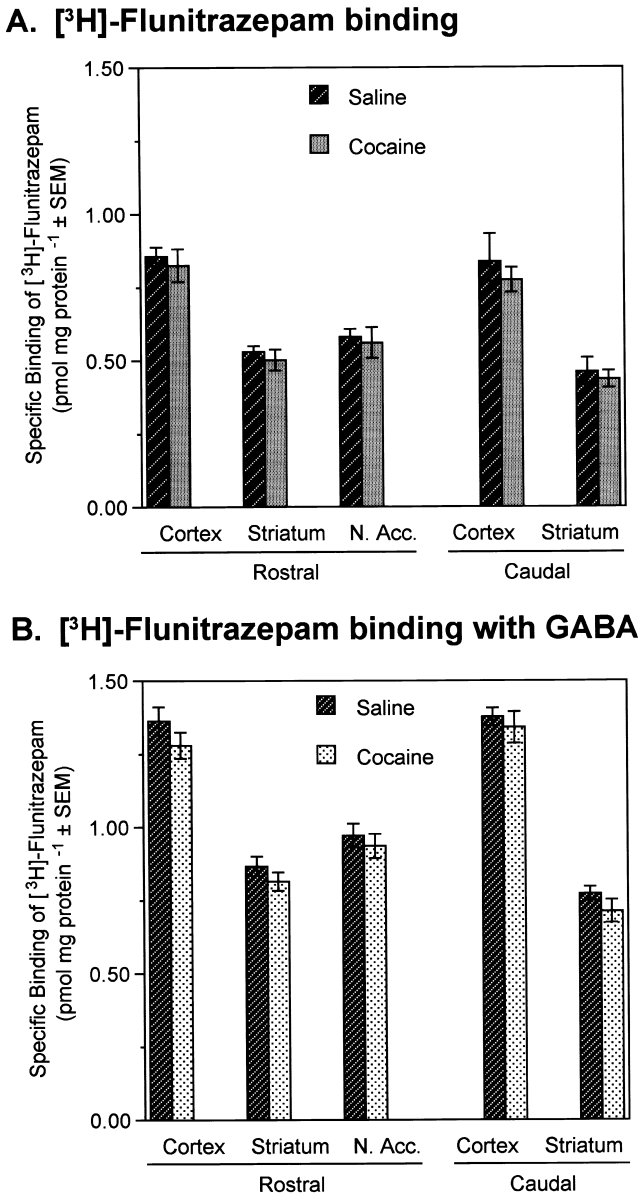

Fig. 3. Binding of [^{3}H]-flunitrazepam without and with GABA. (A) Effects of repeated cocaine on specific [^{3}H]-flunitrazepam binding. There was no significant difference between the two treatment groups. The CTX in both the rostral and caudal slices had significantly higher [^{3}H]-flunitrazepam binding (rostral, $P<.0001$; caudal, $P<.0001$). (B) Effects of repeated cocaine on specific [^{3}H]-flunitrazepam binding modulated by GABA. There was no significant difference between the two treatment groups. The CTX in both the rostral and caudal slices had significantly higher [^{3}H]-muscimol binding (rostral, $P<.0001$; caudal, $P<.0001$). Rats were treated for 14 days with either saline (0.1 ml kg^{-1}) or cocaine (15 mg kg^{-1}) and were killed 1 day after the last treatment. Data are expressed as the mean percentage change ± S.E.M. for rostral ($n=11$) and caudal ($n=10-11$).

modulated [^{3}H]-flunitrazepam binding and brain regions for both rostral slices [$F(2,120)=3.84$, $P<.05$] and caudal slices [$F(1,76)=12.85$, $P<.001$]. However, there were no significant differences in enhancement between treatment groups.

3.4. Striatal and cortical microsac $^{36}Cl^-$ uptake after cocaine sensitization

After treatment with either repeated saline or cocaine, brain microsacs were prepared from both STR and CTX from these rats. When striatal GABA-stimulated $^{36}Cl^-$ uptake was measured, there were no differences between repeated saline- and cocaine-treated rats (Fig. 4). Next, functional modulation of striatal $GABA_A$ receptor function was examined with clonazepam and/or cocaine. ANOVA revealed that clonazepam significantly enhanced striatal GABA-stimulated $^{36}Cl^-$ uptake in both the saline- and cocaine-treated rats [$F(1,68)=72.64$, $P<.0001$], but there were no differences between repeated saline and cocaine groups. ANOVA also revealed that in vitro cocaine significantly decreased striatal GABA-stimulated $^{36}Cl^-$ uptake in both saline- and cocaine-treated rats [$F(1,68)=4.68$, $P<.05$], but again there were no differences between the treatment groups.

When cortical GABA-stimulated $^{36}Cl^-$ uptake was measured, there were no differences between repeated saline- and cocaine-treated rats (Fig. 5). Again, clonazepam modulation of $GABA_A$ receptor function significantly enhanced cortical GABA-stimulated $^{36}Cl^-$ uptake [$F(1,76)=63.53$, $P<.0001$], but there were no differences between saline- and cocaine-treated rats. In contrast to the STR, in vitro cocaine did not have a significant effect on cortical $GABA_A$ receptor function. When the effects of both clonazepam and in vitro cocaine were examined, clonazepam affected cortical GABA-stimulated $^{36}Cl^-$ uptake similarly as when no cocaine was present.

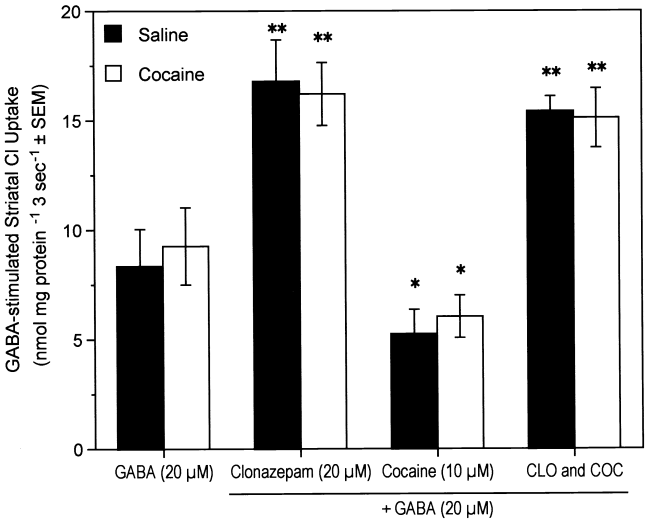

Fig. 4. Changes in striatal GABA-stimulated $^{36}Cl^-$ uptake after cocaine sensitization with the addition of in vitro clonazepam or in vitro cocaine. In vitro clonazepam significantly enhanced [$F(1,68)=72.64$, $P<.0001$] and in vitro cocaine significantly decreased striatal $^{36}Cl^-$ uptake [$F(1,68)=4.68$, $P<.05$]. There were no differences in $^{36}Cl^-$ uptake between repeated in vivo saline and cocaine treatment. Data are expressed as the mean ± S.E.M. for $n=10-11$. * $P<.05$ compared to GABA (20 μM) alone; ** $P<.0001$ compared to GABA (20 μM) alone.

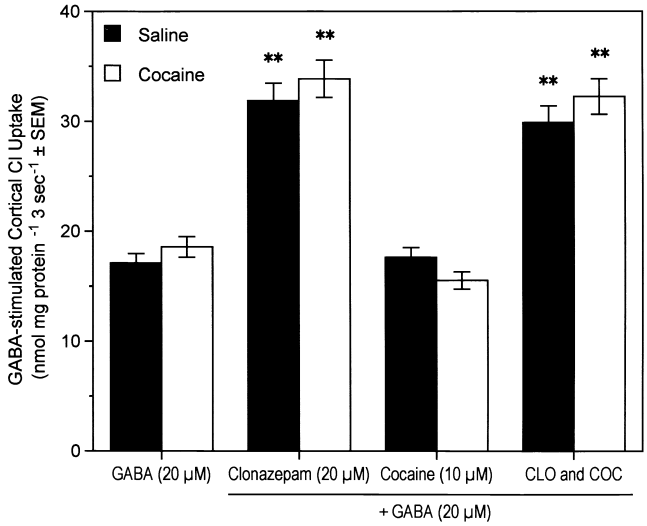

Fig. 5. Changes in cortical GABA-stimulated $^{36}Cl^-$ uptake after cocaine sensitization with the addition of in vitro clonazepam or in vitro cocaine. In vitro clonazepam significantly enhanced cortical $^{36}Cl^-$ uptake [$F(1,76)=63.53$, $P<.0001$] in both treatment groups. There were no differences in $^{36}Cl^-$ uptake between repeated in vivo saline and cocaine treatment. Data are expressed as the mean ± S.E.M. for $n=10-11$. ** $P<.0001$ compared to GABA (20 μM) alone.

4. Discussion

Previous reports characterizing changes in striatal $GABA_A$ receptor number after repeated cocaine have been equivocal (Goeders, 1991; Pecins-Thompson and Peris, 1993). A major difference was the ligand used to label the $GABA_A$ receptor: [^{3}H]-Ro 15-1788, a BZD antagonist (Goeders, 1991), and [^{35}S]-TBPS, which binds to the convulsive site of the receptor (Pecins-Thompson and Peris, 1993). Consequently, the changes measured did not occur at the neurotransmitter binding site, but rather at different noncompetitive binding sites. In addition, the previous studies did not address either allosterically modulated $GABA_A$ receptor binding or allosterically modulated GABA-stimulated $^{36}Cl^-$ uptake after repeated cocaine, which may have accounted for changes seen after repeated cocaine. Thus, it was important to determine three features about the striatal $GABA_A$ receptor after repeated cocaine: (1) changes in muscimol binding, (2) changes in allosterically modulated receptor binding, and (3) changes in allosterically modulated $GABA_A$ receptor $^{36}Cl^-$ uptake.

The repeated treatment with cocaine (15 mg kg^{-1}) led to the higher behavioral scores on Day 14 compared to Day 1

in 80% of the Group C15 rats (Fig. 1A). The variability of behaviors of Group C15 rats on Days 1 and 14 was within the range of behaviors seen in the previous study. However, only 8 of 11 Group C15 rats showed an increase in behavioral score on Day 14 when compared to Day 1 (data not shown). Group C20 rats had significantly higher behavior on Day 14 compared to Day 1, but not on Day 7 compared to Day 1 ($P > .07$) (Fig. 1B). Increasing the dose of cocaine (20 mg kg^{-1}) improved both the number of rats showing higher behavioral score on Day 14 compared to Day 1 and the range in the behavioral scores on all days measured (data not shown). However, Group C20 behavior was not a ceiling effect because the maximal score was only obtained at a few points.

Binding to $GABA_A$ receptors was determined with two different ligands: muscimol and flunitrazepam. Muscimol binds to the GABA recognition site on the $GABA_A$ receptor and represents changes in $GABA_A$ receptor number, while flunitrazepam binds to the BZD site (Sieghart, 1992). After 14 days of repeated cocaine, there were no changes either in [^{3}H]-muscimol binding, or in [^{3}H]-flunitrazepam binding in the regions studied (CTX, STR, and NAc). The possibility exists that separate and distinct subpopulations of the $GABA_A$ receptor were measured in these experiments. However, these results indicate that the number of $GABA_A$ receptors in each population did not change after withdrawal from repeated cocaine. This could suggest that the previous changes in $GABA_A$ receptor number may represent alternative changes, e.g., receptor uncoupling, changes in endogenous BZDs, or changes in receptor subunit composition.

[^{3}H]-Muscimol binding has been reported to occur at two affinity sites with K_d values of 2.2 and 60 nM (Beumont et al., 1978). In this study, quantitative autoradiography binding was performed with 30 nM [^{3}H]-muscimol. Hence, the high affinity site should have been saturated, while the low affinity site would not have been saturated. However, specific muscimol binding was lower when compared to previous reports (Beumont et al., 1978; Palacios et al., 1981). The low specificity seemed to affect the variability in the modulation of [^{3}H]-muscimol binding by flunitrazepam such that significant enhancement did not occur. [^{3}H]-Muscimol binding to the GABA site may have been affected by endogenous GABA. If endogenous GABA interfered with [^{3}H]-muscimol binding, then it is most likely that the low-affinity [^{3}H]-muscimol binding site was only partially occupied in these experiments, which would obscure differences in [^{3}H]-muscimol binding between treatment groups. It may be necessary to both improve [^{3}H]-muscimol quantitative autoradiography and also measure changes in binding affinity to determine if there are real differences between treatment groups. [^{3}H]-Flunitrazepam binding both specific and allosterically modulated binding are consistent with previous reports (Ruano et al., 1992; Slaney et al., 1995). Thus, the results from both [^{3}H]-flunitrazepam binding and [^{3}H]-flunitrazepam modulated binding may provide better support for no differences in binding and receptor coupling after repeated cocaine.

The ability of allosteric ligands to modulate $GABA_A$ receptor binding is an important measure of receptor function. Chronic BZD treatment results in uncoupling of the allosteric BZD binding site from the GABA site of the $GABA_A$ receptor (Gallager et al., 1984; Mele et al., 1984; Tietz et al., 1989). In the previous report, BZD binding was increased 20 min after withdrawal, but not 2 and 14 days after withdrawal (Goeders, 1991). In the present experiments with 1 day withdrawal, repeated cocaine did not change allosteric binding when measured either at the GABA site (Fig. 2) or the BZD site (Fig. 3). Consequently, the previously observed increase in BZD binding does not appear to be caused by uncoupling of the $GABA_A$ receptor from repeated cocaine. The lack of uncoupling and no difference in [^{3}H]-flunitrazepam binding after 1 day withdrawal could also indicate that the $GABA_A$ receptor has reverted back to the nonsensitized form, which would be consistent with the previous report (Goeders, 1991). Thus, although repeated cocaine did not affect allosteric binding by uncoupling the GABA site from the BZD site, the affinity of either the GABA or BZD site could still change binding, which was not determined in these experiments.

The time of withdrawal after the last drug treatment may be critical in the demonstration of receptor uncoupling. In previous reports of $GABA_A$ receptor $^{36}Cl^-$ uptake uncoupling, the receptor was uncoupled from the BZD site immediately (Li et al., 1993) and 6 h (Buck and Harris, 1990) after withdrawal, but not 1 (Buck and Harris, 1990) and 2 days (Li et al., 1993) after withdrawal. In the present study, $GABA_A$ receptor $^{36}Cl^-$ uptake was measured 1 day after the last treatment. It is possible that the receptor may initially be uncoupled, then recouples during the withdrawal period. It would be important to determine if the $GABA_A$ receptor is uncoupled at earlier periods after withdrawal because the reason for the increase in BZD binding after 20 min withdrawal from repeated cocaine is unknown. However, the possibility that the receptor recouples the GABA site and BZD site after cocaine withdrawal limits the contribution of the uncoupling mechanism to cocaine sensitization because BZD uncoupling would be much shorter in duration compared to sensitization. It is tempting to speculate that the change from increased BZD binding 20 min after withdrawal (Goeders, 1991) to a lack of difference in BZD binding 1 (here), 2, and 14 days after withdrawal (Goeders, 1991) results from the $GABA_A$ receptor returning to the drug-naive state. Thus, the contribution of the allosteric BZD site to the maintenance of cocaine sensitization may be limited.

Previously, repeated cocaine treatment decreased striatal $GABA_A$ receptor $^{36}Cl^-$ uptake (Peris, 1996; Resnick et al., 1999). In this study, there was no difference in striatal GABA-stimulated $^{36}Cl^-$ uptake between saline- and cocaine-treated rats. It is unclear as to why there was no difference in the present study because the rats were subjected to the same treatment schedule that resulted in cocaine sensitization. Although the dose of cocaine used to cause cocaine sensitization was greater, this dose increase should theoretically

increase behavior and could cause a greater decrease in $GABA_A$ receptor function. A possibility that there was no difference in striatal GABA-stimulated $^{36}Cl^-$ uptake is that the amount of GABA-stimulated $^{36}Cl^-$ uptake in this study was low compared to previous results (Peris, 1996). The only difference between the two microsac preparations was the inclusion of protease inhibitors in this study, which could have decreased the variability between the different preparations. In a future experiment, it may be critical to show both behavioral sensitization and decreased striatal $GABA_A$ receptor function after repeated cocaine, before examining the modulation of GABA-stimulated $^{36}Cl^-$ uptake.

The mechanism by which in vitro cocaine specifically decreases striatal $GABA_A$ receptor $^{36}Cl^-$ uptake is unknown. Previously, it was reported from this laboratory that there was no effect of in vitro cocaine on striatal $GABA_A$ receptor function, but in that study, the microsac preparation did not include protease inhibitors (Peris, 1996). Hence, proteins critical for the effect of in vitro cocaine may have been degraded in the previous assays. It would be important to identify these proteins in future experiments. The decrease by in vitro cocaine was not likely due to a direct effect on the $GABA_A$ receptor. At the present time, there is no reported cocaine binding site on the $GABA_A$ receptor. It has been reported that GABA current is inhibited by cocaine (Ye et al., 1997, 1999), but this occurs at much higher cocaine concentrations and could be the result of a local anesthetic effect of cocaine. More recently, specific subunits of the nicotinic acetylcholine receptor were shown to be inhibited by cocaine and contain a cocaine binding domain (Francis et al., 2000). It is tempting to speculate that the $GABA_A$ receptor subunits may also contain an analogous binding site, since it also belongs to the ligand-gated superfamily.

In summary, repeated cocaine did not alter the number of $GABA_A$ receptors as measured by [^{3}H]-muscimol binding or [^{3}H]-flunitrazepam binding. The lack of change in [^{3}H]-muscimol binding was surprising because of previously reported changes in $GABA_A$ receptor number by BZD and convulsant binding. The GABA binding site was not uncoupled from the allosteric binding site, but it is possible that repeated cocaine affects the affinity of the receptor, which was not measured, more than the number at these allosteric sites. In addition, cocaine sensitization did not change allosterically modulated striatal $GABA_A$ receptor $^{36}Cl^-$ uptake. However, in vitro cocaine decreased stimulated $GABA_A$ receptor $^{36}Cl^-$ uptake in both saline- and cocaine-treated rats. Thus, repeated cocaine does not appear to change coupling or function of the $GABA_A$ receptor after 1 day withdrawal.

Acknowledgments

This research was supported by NIDA grant DA05890 (BJJ). We thank Amanda Vasquez, Tammy McLellen, and Cindy Achat for expert technical assistance.

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