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Brain-derived neurotrophic factor signaling modulates cocaine induction of reward-associated ultrasonic vocalization in rats

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RUNNING TITLE

BDNF Modulates Cocaine-induced USV Behavior

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Abbreviations: BDNF, brain-derived neurotrophic factor; USV, 50-KHz ultrasonic vocalization; ELISA, enzyme-linked immunosorbent assay

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ABSTRACT

Cocaine exhibits high liability for inducing addictive behaviors, but the mechanisms of neuroplasticity underlying the behavioral effects remain unclear. As a crucial mediator of neuroplasticity in diverse functional models, brain-derived neurotrophic factor (BDNF) could contribute to the mechanisms of addiction-related neuroplasticity. Here, we addressed the hypothesis that cocaine increases synaptic dopamine which induces BDNF protein expression to initiate addiction-related behavior in the rat. Enzyme-linked immunosorbent assay was used to measure BDNF protein expression in rat striatal tissues. For behavioral readout, we used the Metris Sonotrack system to measure the emission of 50-KHz ultrasonic vocalization (USV), a response that correlates with electrical brain stimulation and conditioned place-preference behavior in rodents. A single injection of cocaine significantly increased BDNF protein expression, but this effect was not further augmented by repeated cocaine administration. A single administration of cocaine elicited significant and dose-related USV responses, and the magnitude of the behavior increased with repeated drug administration. SCH23390, but not raclopride, significantly attenuated cocaine-induced BDNF protein expression, whereas either the D_1-like or D_2-like receptor antagonist blocked cocaine-induced USV behavior. Furthermore, significant USV behavior was elicited by the nonselective dopamine agonist, apomorphine, but not by agonists that are selective for D_1-like or D_2-like receptors. Intracerebroventricular injection of the neurotrophin TrkB receptor inhibitor, K252a, blocked cocaine-induced USV behavior, but not locomotor activity. These results suggest that neurotrophin signaling downstream of dopamine receptor function probably constitutes a crucial link in cocaine induction of USV behavior, and may contribute to the mechanisms underlying the development of addiction-related behaviors.

INTRODUCTION

Cocaine is a psychostimulant agent that alters behavior primarily by preventing the re-uptake of dopamine from neural synapses into pre-synaptic neurons. This action results in elevated synaptic concentrations of dopamine thereby leading to exaggerated activation of postsynaptic dopamine receptors. Although much is known about the acute actions of cocaine, the mechanisms that underlie the transition from acute drug use to chronic drug abuse are still unclear. Substantial evidence suggests that the acute actions of cocaine initiate a process of neuroplasticity that is thought to ultimately lead to the development of drug-related behaviors (Graybiel et al., 1990; Nestler, 2001a; Nestler, 2001b; Kalivas and Volkow, 2005). Neuroplasticity generally involves the establishment of a new steady state in structure or function within cells or tissues previously exposed to abnormal levels of stimulation. While various intracellular mediators have been implicated in this phenomenon, one signaling mediator that is consistently implicated is brain-derived neurotrophic factor (BDNF). BDNF is a neurotrophin that is involved in different forms of plasticity including long-term potentiation (LTP) and long-term depression (LTD) (Thoenen, 1995).

Psychostimulant agents such as cocaine induce the expression of BDNF mRNA and protein in various brain regions (Meredith et al., 2002; Le Foll et al., 2005; Filip et al., 2006; Liu et al., 2006). Furthermore, BDNF infusion into the mesolimbic dopamine system dramatically enhances the rewarding effects of cocaine as measured by the conditioned place preference paradigm (Horger et al., 1999), while heterozygous BDNF knockout mice are significantly less responsive to cocaine-induced sensitization and motor activation (Hall et al., 2003). Hence, BDNF could play a crucial role in cocaine-related addictive behaviors. Additionally, cocaine-induced BDNF expression is attenuated in dopamine D_1 receptor knockout mice (Zhang et al.,

2002). Furthermore, dopamine and the selective D_1-like receptor agonist SKF38393 can increase BDNF mRNA and protein expression in brain tissues and this effect is blocked by the selective D_1-like receptor antagonist SCH23390 (Okazawa et al., 1992; Inoue et al., 1997; Kuppers and Beyer, 2001; Williams and Undieh, 2009). These findings suggest a potential relationship between cocaine-induced rise in synaptic dopamine levels, increased BDNF expression, and the induction and/or maintenance of addiction-related behaviors. Thus, we proposed in this study to examine the hypothesis that cocaine increases synaptic dopamine which induces BDNF expression to initiate addiction-related behavior in the rat. Given that signaling activity is dependent on BDNF protein rather than mRNA, and that mRNA levels may not consistently translate to protein expression, we chose in the present study to directly measure BDNF protein using a sensitive and highly specific enzyme-linked immunosorbent assay (ELISA) procedure.

While several previous studies have examined the relationship between BDNF and reward-associated behaviors such as place preference or intracranial self-administration (Horger et al., 1999; Hall et al., 2003; Graham et al., 2007; Bahi et al., 2008), the behaviors analyzed required prior exposure to the psychostimulant drug. This could confound the subsequent neurochemical analyses if the goal was to trace changes from initial drug exposure to the point of acquiring drug-seeking behaviors. Here, for the first time, we elected to test the emission of 50-KHz ultrasonic vocalization (USV). Fifty-kilohertz USV's have been consistently linked to positive affective or appetitive behavior. For example, rats emit increased rates of 50-KHz USVs before delivery of electrical brain stimulation to several reward-associated brain regions (Burgdorf et al., 2000; Burgdorf et al., 2007; Ciucci et al., 2007). Furthermore, rats vocalize more in an experimental chamber that is associated with the rewarding effects of previously administered amphetamine than in the control area (Knutson et al., 1999), while intra-accumbens

amphetamine injections produce robust and dose-dependent increases in 50-KHz USVs (Burgdorf et al., 2001; Thompson et al., 2006). Thus, there appears to be a high anatomical, pharmacological, and functional correlation between the generation of 50-KHz USVs and other rewarding behaviors in rats. In the present work, 50-KHz USV behavior was selected as a read-out of positive affective responses to cocaine, with the goal to test the relationship between this behavior and the production and signaling of BDNF protein.

METHODS

Animals: Male Sprague–Dawley rats weighing 175-200g were obtained from Zivic Laboratories (Pittsburgh, PA). The animals were caged in groups of three and housed in climate-controlled facilities with a 12-h light/dark cycle and free access to food and water. Protocols for the care and use of the experimental animals were approved by the Institutional Animal Care and Use Committee and conformed to the NIH *Guide for the Care and Use of Laboratory Animals.*

Drug Treatment: Cocaine hydrochloride (obtained from the National Institute on Drug Abuse, Bethesda, MD) was dissolved in sterile 0.9% sodium chloride and administered intraperitoneally (i.p.); (+/-)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol (SKF38393) was obtained through the National Institute of Mental Health Chemical Synthesis Program, while (R-) Apomorphine HCl), SCH23390, raclopride, and quinpirole (all were purchased from Sigma Aldrich, (St. Louis, MO). Cocaine was dissolved in sterile saline and administered intraperitoneally (i.p.), while the other drugs were dissolved in saline and administered subcutaneously (s.c). The trkB receptor antagonist K252a (Sigma Aldrich, St. Louis, MO) was dissolved in 25% DMSO/saline and diluted to a final concentration of 25 µg/µl. K252a was injected intracerebroventricularly (i.c.v.) into the lateral ventricle. Where indicated, SCH23390, raclopride, and K252a were injected 20 min prior to cocaine administration. Sterile saline (0.9% sodium chloride) was used for vehicle control injections.

Measurement of in vivo BDNF protein expression: Animals were rapidly decapitated 24 h after the last injection of drug. The whole brain was removed and the anatomically intact striatum was quickly dissected out, transferred to a polypropylene tube containing ice-cold

homogenization buffer [137 mM NaCl, 20 mM Tris (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mM protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO)] and homogenized for 30 seconds pulsing using an ultrasonic homogenizer. Tubes containing the homogenates were incubated for 20 min at 4 ºC on a rocking platform and then centrifuged at 17000 g and 4 ºC for 15 min. The supernatant was transferred to another tube and aliquots of the protein extract were assayed for BDNF protein using the Promega BDNF ELISA immunoassay kit (Promega, Madison WI) according to the manufacturer's protocol. Briefly, 96-well plates pre-coated with anti-BDNF monoclonal antibody were incubated with blocking buffer to saturate nonspecific binding. The immobilized anti-BDNF monoclonal antibody was incubated with BDNF standards or test samples followed by incubation with anti-human BDNF polyclonal antibody. After incubation with anti-IgY horse radish peroxidase conjugate, 3,3´,5,5´-tetramethylbenzidine (TMB) One solution was added and color detection was accomplished at 450 nm using a Spectramax Pro plate reader (Molecular Devices, Sunnyvale CA).

Intracranial drug administration: Animals were surgically implanted with a stainless steel bilateral guide cannula (Plastics One, Roanoke VA) having an external diameter of 0.5 mm in order to facilitate the subsequent acute administration of drugs. Cannulas were aseptically implanted under ketamine/xylanine (80%/12%) anesthesia. The intracerebroventricular (i.c.v.) coordinates used were AP -0.8 mm, ML ±1.5 mm, and DV -2.5 mm based on the atlas of Paxinos and Watson. Following the surgical procedure, animals were allowed at least one week to recover before being used in experiments. The drug to be administered was dissolved and diluted to appropriate concentration as described above and loaded into a Hamilton syringe connected through a 21 gauge silane tubing to the injection cannula; care was taken to expel all

air from the system. Prior to each injection, rats were briefly anesthetized in a chamber of isoflurane delivered from a controlled vaporizer. The injection cannula was carefully inserted into the guide cannula and 1 µL of drug material was slowly infused into each of the lateral ventricles at the rate of 2 µl/min. The injection cannula was left in place for an extra minute and then was slowly withdrawn and replaced with a dummy cannula to prevent loss of the drug or cerebrospinal fluid.

Measurement of locomotor activity: As a general behavioral index of dopaminergic drug action, locomotor activity was recorded for each animal using a Med-Associates Open Field Activity Monitoring cages (Med-Associates, St. Albans, VT). Rats were carefully lifted out of the cages and held for drug injection and afterwards placed back into the cages and locomotor activity was recorded continuously for 90 min.

Measurement of ultrasonic vocalization (USV) behavior: Animals were tested following an acute injection of saline or cocaine to naïve or previously drug-treated subjects. For each experiment, matching sets of animals were administered saline to serve as controls. On the day of testing, animals were first acclimated to the test apparatus which consisted of modified Med-Associates Activity Chambers each over-fitted with a pair of high-sensitivity SONOTRACK ultrasonic microphones (Metris B.V., KA Hoofddorp, The Netherlands). The main Sonotrack unit was connected to a Dell Computer workstation running the Sonotrack software in a Windows XP environment. After 15 min in the chamber, the apparatus was activated for 10 min in order to record any baseline USV. The animals were then carefully injected with saline or drug and placed back into the chamber. Both the Med-Associates activity monitoring system (see

above) and the Sonotrack ultrasonic system were then simultaneously activated and monitored concurrently. Drug-induced USV behavior was continuously monitored for up to 90 min. The Sonotrack system captures multiple parameters for each emission, including the time (from start of experiment), the actual frequency of the emission, and its amplitude and duration. The combination of vocalization frequency and duration characterized two modes of emissions – a long-duration (>500 ms) 22 (±4)-KHz mode that is associated with aversive responses, and a short-duration (<300 ms) 50 (±6)-KHz mode that is reported to associate with reward or appetitive behaviors in rodents (Burgdorf et al., 2001; Portfors, 2007). Ultrasonic vocalizations were first identified and counted by the Sonograph system based on the established spectral characteristics for 22 KHz and 50-KHz emissions. The raw data were exported into Microsoft Excel and the data re-examined and validated using an in-house algorithm. There was consistently strong correlation ($r>0.99$) between the machine counts and the manual determinations. We report here on the 50-KHz responses that characteristically lasted less than 300 ms and showing a strong dose-dependence for cocaine as previously reported for other rewarding drugs (Burgdorf et al., 2001; Thompson et al., 2006), and as confirmed in our preliminary experiments. No other form of vocalization between 15-100 KHz and lasting from 1-1000 ms was emitted to any appreciable degree by the animals throughout the course of these experiments.

Data Analysis: Each experiment was performed on multiple occasions for the molecular studies or in multiple animals for the behavioral studies so that sample sizes of 6-9 were accumulated. In general, the data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software, Inc, San Diego, CA), followed post-hoc either by the

Dunnett test to determine which of the tested treatments differed significantly from the respective control group, or by the Tukey test to determine which among the various pairs of treatment or control groups were significantly different. Statistical comparisons were considered significant at $p<0.05$ or better.

RESULTS

Single or repeated injections of cocaine alter BDNF protein expression: Rats injected with a single cocaine dose of 3 mg/kg or 30 mg/kg and examined 24 h afterwards showed significant increases in BDNF protein expression in the striatum (Figure 1). Repeated once daily injection of 30 mg/kg cocaine for 3 days also resulted in an increase in BDNF protein expression; however, this increase was not significantly different from the effect of the single injection of 30 mg/kg cocaine or 3 mg/kg treatment ($p>0.05$, Tukey posthoc test).

D_1-like dopamine receptors mediate cocaine-induced BDNF protein expression: While animals administered 20 mg/kg cocaine showed a significant increase in BDNF protein expression, pretreatment with the selective D_1-like dopamine receptor antagonist SCH23390 (0.1 mg/kg) blocked the effect of cocaine on BDNF expression. The selective D_2-like receptor antagonist, raclopride at 0.1 mg/kg, failed to modulate the effect of cocaine on BDNF expression (Figure 2). SCH23390 or raclopride alone did not exert any significant effect on BDNF protein expression.

D_1-like and D_2-like dopamine receptors mediate cocaine-induced 50-KHz USV behavior: Rats administered cocaine at a selected dose of 20 mg/kg produced markedly increased USV responses as compared to saline-treated animals (Figure 3). This effect of cocaine was significantly blocked by either 0.1 mg/kg SCH23390 or 0.1 mg/kg raclopride, whereas neither antagonist alone elicited any significant effect on USV responses.

Apomorphine (2.0 mg/kg) significantly increased USV emissions whereas SKF38393 (up to 3.0 mg/kg) or quinpirole (up to 1.0 mg/kg) had no such effect (Figure 4).

BDNF signaling mediates cocaine-induced 50-KHz USV behavior: This experiment tested the role of intact BDNF signaling via the TrkB receptor in the acute or sensitizing effects of cocaine. Groups of rats pretreated with either saline or 25 µg of K252a per brain hemisphere were administered a fixed dose (20 mg/kg) of cocaine on a once daily schedule for 5 days; on each occasion, USV behavior was immediately assessed for up to 90 min.. Cocaine-treated animals showed a significant ($p<0.001$) trend of increasing USV responses with each test session (Figure 5). Rats pretreated with K252a prior to administration of cocaine showed decreased responses to cocaine-induced USVs as compared to animals receiving cocaine alone (Figure 5). K252a alone had no effect on USV emissions. The inhibitory effect of K252a on cocaine action was evident throughout the course of daily cocaine treatment up to the 5^{th} and last day of testing.

Locomotor activity behavior also increased with each exposure to cocaine (Figure 5). However, in contrast with the USV measures, pretreatment with K252a did not alter the development of cocaine-induced locomotor sensitization (Figure 5).

DISCUSSION

The present results show a significant role of the dopaminergic system in modulation of BDNF protein expression and further suggest that BDNF signaling mediates cocaine-induced USV behavior. BDNF protein expression was significantly increased in the striatum after a single injection of cocaine, while pretreatment with SCH23390 significantly attenuated cocaine-induced BDNF protein expression. Cocaine significantly increased USV emission, and this response was blocked by SCH23390 or raclopride. While the dopamine receptor subtype-selective agonists SKF38393 and quinpirole did not induce USV behavior, the nonselective agonist apomorphine significantly induced USV behavior. Further, the data implicate a role for BDNF in the mediation of USV behavior, seeing that the TrkB receptor inhibitor, K252a, significantly attenuated cocaine-induced USV behavior but did not block concurrent locomotor activity.

This study measured the protein expression of BDNF, a neurotrophic factor that mediates the survival and function of brain dopamine neurons (Hyman et al., 1991). Our results show that a single injection of cocaine produced significant increases in BDNF protein expression in the rat striatum which, as dissected, probably included the nucleus accumbens (Graham et al., 2007). Repeated daily injections of cocaine, however, did not produce additional increases in BDNF protein beyond the levels observed following the first injection. While this may suggest that the levels observed with the first drug exposure may be the maximum the system can yield, it is also possible that drug treatments subsequent to the first exposure produced increases in pro-BDNF that was not being converted to mature BDNF – the form that is detected by the ELISA assay (Fumagalli et al., 2007).

Among dopamine receptors, both D_1-like and D_2-like subtypes play critical roles in the behavioral, cellular, and molecular responses to the catecholamine. Additionally, dopamine receptors are coupled to multiple intracellular signaling pathways (Undie and Friedman, 1990; Brami-Cherrier et al., 2002; Anderson and Pierce, 2005; Sahu et al., 2009) that activate protein kinases capable of phosphorylating CREB, a major transcriptional regulator of BDNF expression. For this purpose, additional experiments were done to determine the involvement of dopamine receptors in cocaine-induced BDNF protein expression. The D_1-like dopamine receptor antagonist SCH23390 blocked cocaine-induced BDNF expression, whereas the D_2-like dopamine receptor antagonist raclopride had no effect. These results suggest that cocaine-induced BDNF expression is mediated through dopamine D_1-like receptor activation.

Previous studies have shown a direct relationship between increased 50-KHz USVs and the expression of other behaviors traditionally measured to index the activity of brain reward systems, such as conditioned place preference (Knutson et al., 1999). Furthermore, while other commonly used behavioral paradigms such as drug self-administration or conditioned place preference require prior and multiple drug exposures before the subject can be tested, USV endpoints are obtainable both in naïve subjects and in subjects previously or chronically exposed to drug, as demonstrated in our preliminary experiments (data not shown). Our present results have shown that cocaine increased 50-KHz USV calls, while selective antagonism of D_1-like receptors or D_2-like receptors significantly blocked the vocalization response. These observations are consistent with previous findings among dopaminergic systems wherein the coactivity of both subfamilies of dopamine receptors (D_1-like and D_2-like) is necessary to induce an appetitive state (Thompson et al., 2006). The observation that apomorphine induced USV behavior, while SKF38393 and quinpirole produced no significant effect further strengthens the

notion that D_1-like and D_2-like receptor activation coordinately mediates USV behavior; an inference that is consistent with receptor interactions in the mediation of various other dopaminergic behaviors (Minematsu et al., 1994; Geter-Douglass and Riley, 1996; Filip and Przegalinski, 1997; Thompson et al., 2006).

Having observed a relationship between cocaine-induced increases in dopamine receptor activation, BDNF expression and USV behavior, we next addressed whether enhanced BDNF expression simply correlated with behavior or if in fact BDNF signaling was involved in mediating drug-induced USV behavior. We found that both USV behavior and locomotor activity showed sensitization followed repeated cocaine injection and these observations are consistent with previous findings (Mu et al., 2009). Additionally, using intracranial delivery of K252a to inhibit BDNF signaling via TrkB receptors, we observed a marked attenuation of cocaine-induced 50-KHz USV behavior. Thus, BDNF signaling is critical in the mechanism of cocaine-induced USV behavior, an inference that is consistent with previous findings on reward or addiction-related behaviors (Horger et al., 1999; Hall et al., 2003; Graham et al., 2007; Bahi et al., 2008). TrkB receptor signaling appears to be the likely underlying mechanism for these results as previous findings have shown that cocaine modulates several signaling cascades downstream of TrkB receptors. For instance, cocaine self-administration in rats induces activation of TrkB-mediated Phospholipase Cγ signaling in the nucleus accumbens (NAc) (Graham et al., 2007). Cocaine also dramatically increases ERK phosphorylation in the NAc, ventral tegmental area, and prefrontal cortex, following acute or chronic drug administration (Valjent et al., 2004; Jenab et al., 2005; Valjent et al., 2005; Sun et al., 2007). Furthermore, chronic cocaine increases phosphatidylinositol-3-kinase activity in the NAc shell and decreases the activity in the NAc core (Zhang et al., 2006). Thus, cocaine administration is associated with

changes at multiple sites of the BDNF/TrkB signaling cascade. Interestingly, although Horger and colleagues (1999) have reported that TrkB inhibition modulated locomotor activity, the effects of the TrkB receptor inhibitor k252a in the present study were evident in cocaine-induced affective behavior but not in the induction or sensitization of locomotor behavior. Perhaps, differences in cocaine dosages used between the two studies could account for the apparent inconsistencies. Nevertheless, the present results imply that cocaine-mediated affective and motor behaviors could be differentiated at the level of BDNF/trkB receptor signaling possibly as a result of localization of modulated BDNF expression in reward-related regions of the brain.

In conclusion, the observations demonstrate that BDNF signaling could provide a critical link between dopamine receptor activation and cocaine-induced USV behavior. Thus, increased BDNF protein expression following cocaine administration could lead to neuroadaptations germane to the induction of sustained enhancement of cocaine-induced behavior. Clarifying the link between neurotrophin signaling and psychostimulant action should contribute to a greater understanding of the molecular mechanisms underlying addiction-related neuroadaptations.

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FOOTNOTES

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LEGENDS FOR FIGURES

FIGURE 1: Effects of single or repeated injections of cocaine on BDNF protein expression. Male Sprague Dawley rats were given ip injections of cocaine 3 mg/kg, 30 mg/kg, or 30 mg/kg repeated once daily for three days (30X3). Animals were killed 24 h after drug treatment and BDNF levels in dissected striatal tissues measured by ELISA method. BDNF levels were computed as percentages relative to basal BDNF levels. Each bar is the mean ± SEM (n=6-9 animals). **p<0.01, ***p<0.001, compared to the control group.

FIGURE 2: Effects of dopamine receptor antagonists on cocaine-induced BDNF protein expression *in vivo*. Male Sprague Dawley rats were pretreated with SCH23390 (SCH) or raclopride (RAC) 20 minutes prior to injection of 30 mg/kg cocaine (COC). Animals were killed 24 h later and BDNF protein levels assayed in striatal tissues. The data were calculated as percentages relative to saline-treated controls. Each bar is the mean ± SEM (n=8). ***p<.001 compared to saline group; # p<.05 compared to cocaine group as determined by one-way ANOVA followed with Tukey's post hoc analysis. N.S. means "not significant".

Figure 3: Effects of dopamine receptor antagonists on cocaine-induced 50-KHz USV behavior. Groups of male Sprague Dawley rats pretreated with either saline, SCH23390 (SCH, 0.1 mg/kg) or raclopride (RAC, 0.1 mg/kg) were each divided into two subgroups and administered either saline (1 ml/kg) or cocaine (COC, 20 mg/ml/kg). USV behavior was monitored for 90 min. Each bar is the mean ± SEM (n=6 rats). Data were analyzed by ANOVA followed with Tukey's posthoc test. ***p<.001 compared to the cocaine group.

Figure 4: Effect of dopamine receptor agonists on 50-KHz USV behavior. Male Sprague Dawley rats received a single injection of saline (SAL), apomorphine (APO), SKF38393 (SKF) or quinpirole (QUIN) and USV behavior was monitored for 90 min. Each bar represents the mean ± SEM (n=7-9 animals). *** p<.001 compared to saline group by Tukey's post hoc analysis.

Figure 5: Effects of TrkB receptor inhibition on cocaine-induced USV and locomotor behaviors. Groups of male Sprague Dawley rats were injected once daily for five days with either 20 mg/kg cocaine alone or the same dose of cocaine after pretreatment with an i.c.v. injection of the TrkB receptor antagonist K252a (50µg). Following each drug treatment, USV behavior (Top panel) and locomotor activity (Bottom panel) were concurrently measured for 90 min. Each bar is the mean +/- SEM (N=6 animals). *p<0.05, **p<0.01, ***p<0.001, compared to the control group, ###p<.001 compared to cocaine group as determined by ANOVA.

FIGURE 1

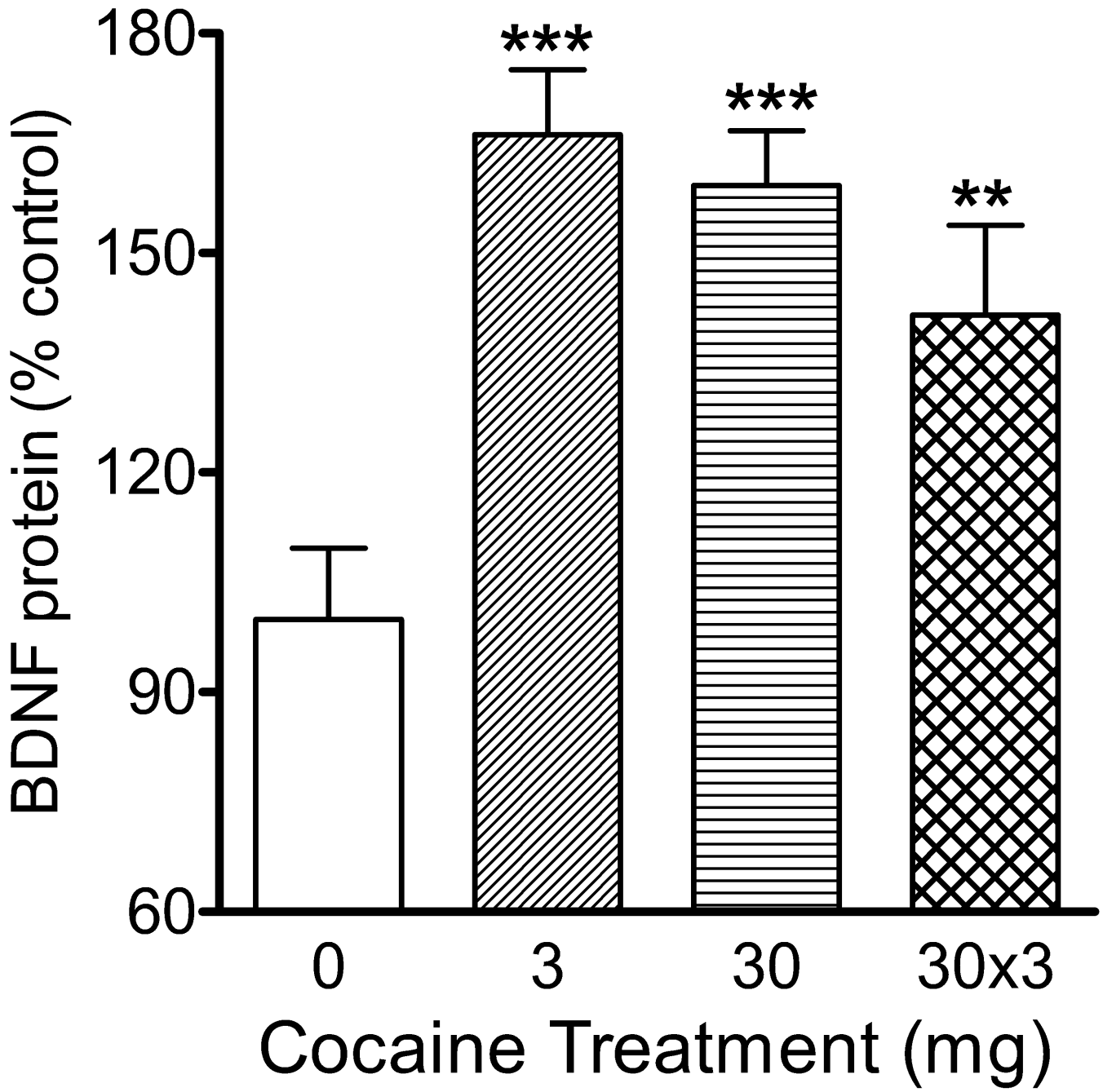

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FIGURE 2

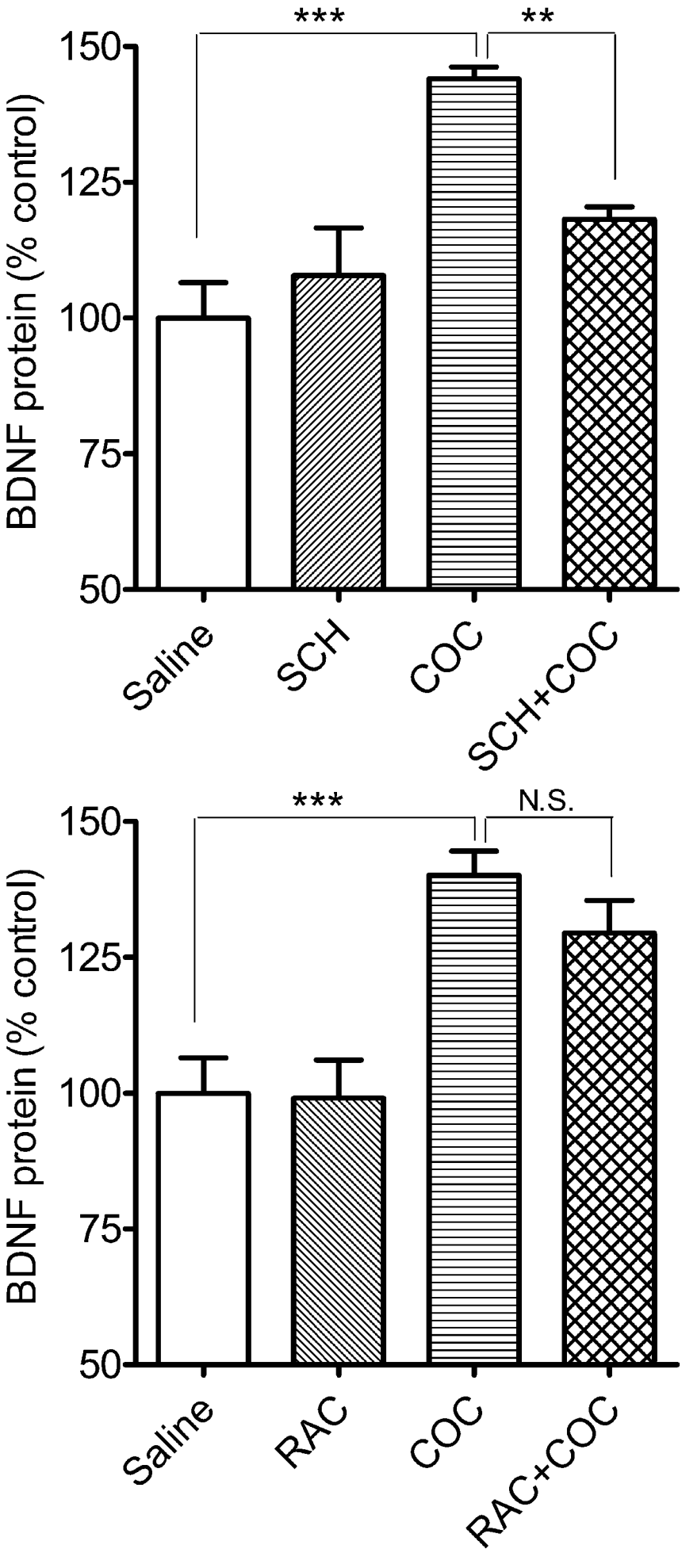

FIGURE 3

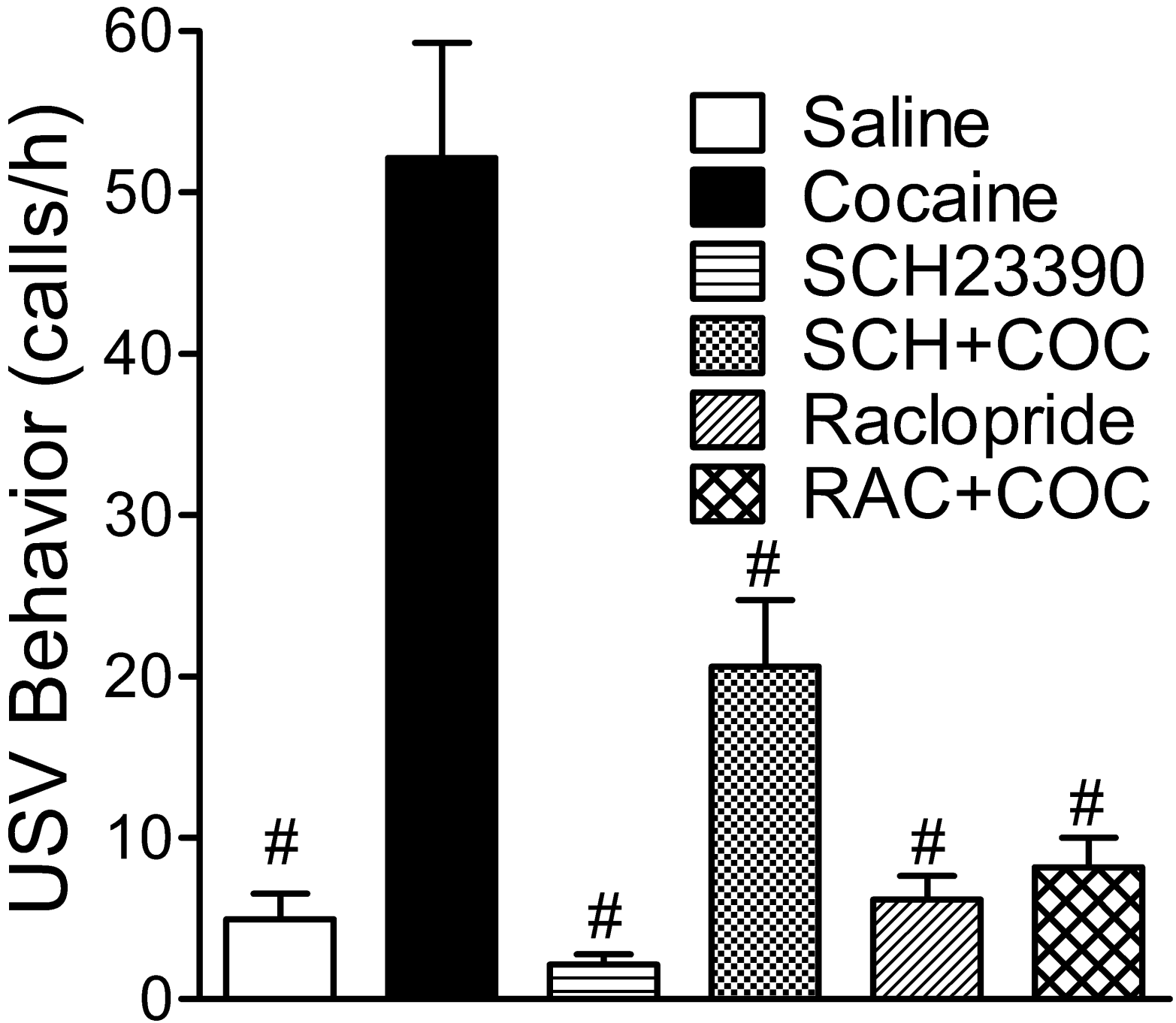

FIGURE 4

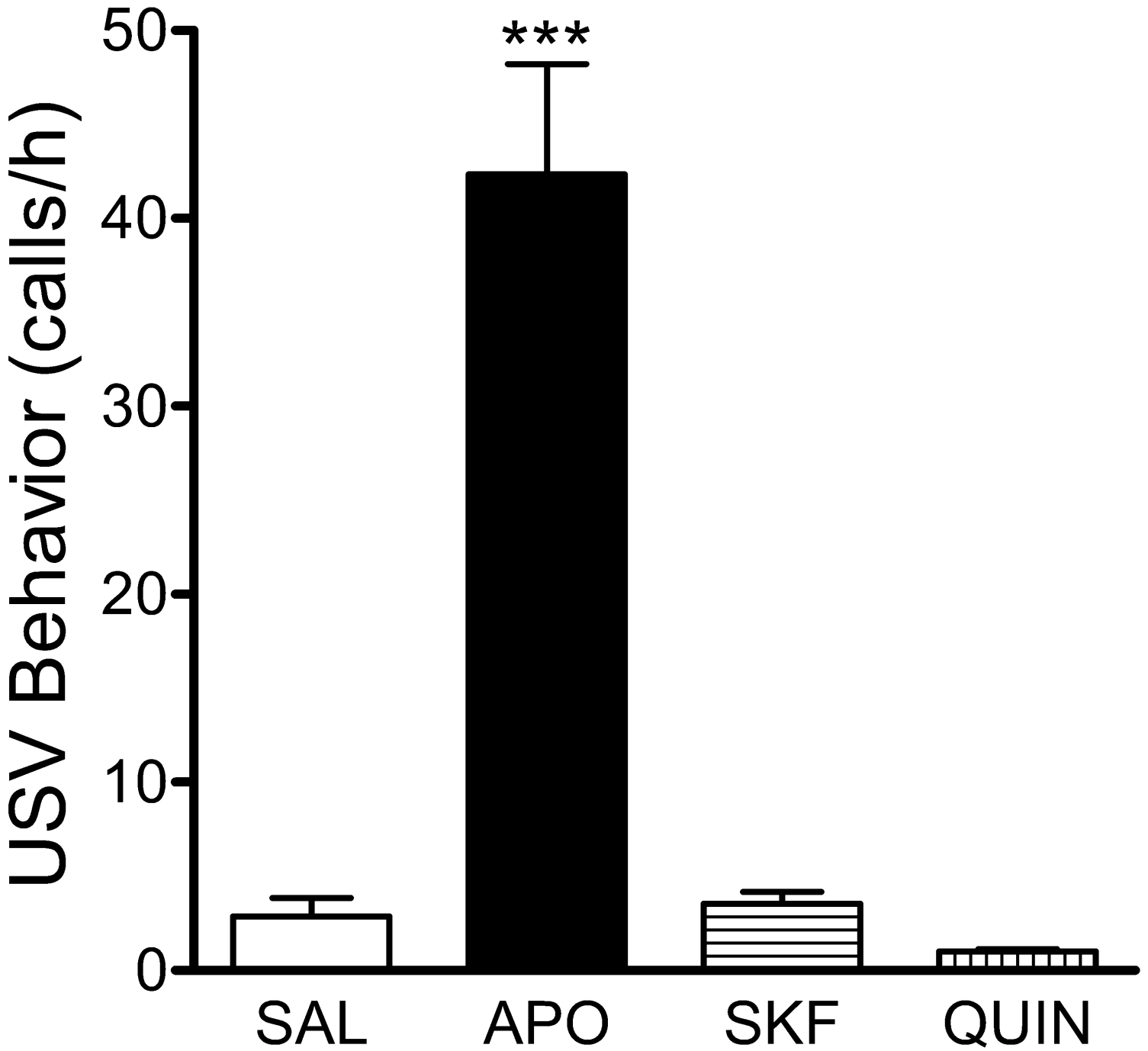

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FIGURE 5