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Beta adrenergic receptor mediation of stress-induced reinstatement of extinguished cocaine-induced conditioned place preference in mice: roles for beta-1 and beta-2 adrenergic receptors

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BNST: bed nucleus of the stria terminalis; CRF: corticotropin releasing factor; VTA: ventral tegmental area

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ABSTRACT

Stress can trigger relapse of drug use in recovering cocaine addicts and reinstatement in rodent models through mechanisms that appear to involve norepinephrine release and beta adrenergic receptor activation. The present study examined the role of beta adrenergic receptor subtypes in the stressor-induced reinstatement of extinguished cocaine-induced (15 mg/kg, ip) conditioned place preference in mice. Forced swim (6 min at 22ºC) stress or activation of central noradrenergic neurotransmission by administration the selective alpha-2 adrenergic receptor antagonist, BRL-44,408 (10 mg/kg, ip) induced reinstatement in wild-type but not beta adrenergic receptor-deficient *Adrb1*/*Adrb2* double-knockout mice. By contrast, cocaine administration (15 mg/kg, ip) resulted in reinstatement in both wild-type and beta adrenergic receptor knockout mice. Stress-induced reinstatement likely involved beta-2 adrenergic receptors. The beta-2 adrenergic receptor antagonist ICI-118,551 (1 or 2 mg/kg, ip) blocked reinstatement by forced swim or BRL-44,408, while administration of the non-selective beta adrenergic receptor agonist, isoproterenol (2 or 4 mg/kg, ip), or the beta-2 adrenergic receptor-selective agonist, clenbuterol (2 or 4 mg/kg, ip), induced reinstatement. Forced swim, but not BRL-44,408, -induced reinstatement was also blocked by a high (20 mg/kg) but not low (10 mg/kg) dose of the beta-1 adrenergic receptor antagonist betaxolol and isoproterenol-induced reinstatement was blocked by pretreatment with either ICI-118,551 or betaxolol, suggesting a potential cooperative role for beta-1 and beta-2 adrenergic receptors in stress-induced reinstatement. Overall, these findings suggest that targeting beta adrenergic receptors may represent a promising pharmacotherapeutic strategy for preventing drug relapse, particularly in cocaine addicts whose drug use is stress-related.

INTRODUCTION

In many cocaine addicts, stress appears to serve as a potent trigger for drug use. Reports that stressful stimuli can precipitate craving for cocaine in human subjects in a laboratory setting (Sinha et al., 2000) are paralleled by findings that stressors can induce reinstatement in rodent models of drug relapse (Shaham et al., 2000a). In abstinent cocaine addicts, the ability of stress to promote drug use is particularly problematic because, unlike many triggers for relapse (e.g., drug use and exposure to many drug-associated contexts and cues), the occurrence of stress is usually unavoidable. For this reason, understanding the receptor mechanisms through which stressors evoke drug-seeking behavior is likely important for the identification of better pharmacotherapeutic strategies for relapse prevention.

Noradrenergic signaling in the brain has been implicated in stress-induced relapse (Koob, 1999; Weinshenker et al., 2007). In rodent and non-human primate models, central delivery of norepinephrine (Brown et al., 2009) or activation of central noradrenergic neurotransmission via antagonism of alpha-2 adrenergic receptors (Lee et al., 2004; Feltenstein and See, 2006) reinstates extinguished cocaine seeking. Similarly, functional antagonism of noradrenergic neurotransmission through administration of alpha-2 adrenergic receptor agonists blocks stress-induced reinstatement in preclinical models (Erb et al., 2000) and attenuates stress-induced craving in human cocaine addicts (Fox et al., 2012; Jobes et al., 2011). The apparent involvement of noradrenergic systems in stress-induced drug use poses adrenergic receptors as potential medicinal targets for relapse prevention.

One method for the preclinical investigation of drug relapse is the conditioned place preference/reinstatement approach, in which the ability of stimuli to re-establish cocaine-induced conditioned place preference following extinction is used to model drug relapse (Mueller and Stewart, 2000). An advantage of this method is that it can be used to evaluate reinstatement in mice, thus permitting assessment in available genetically modified rodent models. We and others have reported that exposing mice to a variety of stressors, including forced swim in 22ºC water (Kreibich and Blendy, 2004; Mantsch et al., 2010), social stress (Ribeiro Do Couo et al., 2006), and electric footshock (Redila and Chavkin, 2008), reinstates extinguishing drug-induced conditioned place preference, suggesting that this procedure can be used to examine the neurobiological processes that contribute to stress-induced relapse.

Using this the conditioned place preference/reinstatement approach, we previously reported that reinstatement by a stressor, forced swim, or by activation of central noradrenergic neurotransmission via administration of the alpha-2 adrenergic receptor antagonist, yohimbine, is blocked by pretreatment with the non-selective beta adrenergic receptor antagonist, propranolol, but not by the alpha 1 adrenergic receptor antagonist, prazosin, implicating beta, but not alpha-1, adrenergic receptors in stress-induced relapse (Mantsch et al., 2010). A preliminary experiment suggested the involvement of beta-2 adrenergic receptors in reinstatement in response to forced swim (Mantsch et al., 2010). Notably, while these findings are indicative of a selective role for beta adrenergic receptors in stress-induced reinstatement, it has been reported that prazosin can block yohimbine- or footshock stress-induced reinstatement of extinguished food- or alcohol-seeking in rats (Lê et al., 2011). In the present study we further investigate the role of beta adrenergic receptors in stress-induced relapse by testing for reinstatement of extinguished

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cocaine-induced conditioned place preference in beta adrenergic receptor-deficient mice. Additionally, we examine the role of beta adrenergic receptor subtypes in stress-induced reinstatement by testing for the abilities of beta adrenergic receptor agonists to induce reinstatement and of beta adrenergic receptor antagonists to block reinstatement following forced swim in wild-type mice. Our data support previous findings demonstrating beta adrenergic receptor involvement in reinstatement in response to stress but not cocaine administration and suggest that activation of beta-2 adrenergic receptors is both necessary for stress-induced cocaine seeking and sufficient for reinstatement, while the precise role of beta-1 adrenergic receptors remains unclear.

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MATERIALS AND METHODS

Subjects

A total of 103 male mice were used. All mice were 8-9 weeks old at the start of the study. Mice were housed singly in a temperature- and humidity-controlled, AAALAC-accredited animal facility under a 12 h/12 h light/dark cycle (lights on at 7:00 AM) and had access to food and water at all times, except when in the experimental chambers. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH.

The role of beta adrenergic receptors in place conditioning, extinction and reinstatement was initially examined using beta adrenergic receptor-deficient *Adrb1*/*Adrb2* double-knockout mice purchased from Jackson Laboratories (Bar Harbor, ME). These mice were created by Rohrer et al. (1999) by mating *Adrb1* homozygous knockout mice with *Adrb2* homozygous knockout mice to generate compound heterozygotes, the offspring of which were then mated to obtain compound homozygotes. Backcrossed mice (seven generations) were maintained by Jackson Laboratories by mating double homozygote null mice (eight generations). These mice have no gross physical or behavioral abnormalities and show no deficits in basal metabolic rate but display attenuated effects of exercise and lack the tachycardia and hypotensive responses to the beta adrenergic receptor agonist isoproterenol (Rohrer et al., 1999). A number of strains have contributed to the background (C57BL/6J, DBA/2, 129, FVB/N, CD-1) of these mice. Due to the lack of availability of hybrid controls, C57BL/6J mice (Harlan Laboratories) were used as wild-type control mice in the experiments conducted using the double-knockout mice. C57BL/6J mice were also used for the remainder of the experiments in the study.

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Drugs

Cocaine HCl was acquired from the National Institute on Drug Abuse (NIDA) through the NIDA Drug Supply Program. The selective beta-1 adrenergic receptor antagonist betaxolol HCl, the selective beta-2 adrenergic receptor antagonist ICI-118551 HCl, and the selective beta-2 adrenergic receptor agonist clenbuterol HCl were purchased from Sigma-Aldrich. The non-selective beta adrenergic receptor agonist isoproterenol hydrochloride and the highly selective (relative to yohimbine) alpha-2 adrenergic receptor antagonist BRL-44,408 maleate were acquired from Tocris Bioscience. All drugs were dissolved in saline (0.9% NaCl solution) and were administered IP in a volume of 0.1 ml/20 g body mass.

Equipment

Behavioral testing was conducted using six ENV-3013 mouse place preference chambers from Med-Associates, Inc. The stainless steel and polyvinyl chloride chambers consisted of three distinct compartments separated by 5 cm w x 5.9 cm high manual guillotine doors. The two 46.5 x 12.7 x 12.7 cm side compartments consisted of a white compartment with a 6.35 x 6.35 mm stainless steel mesh floor and a black compartment with a stainless steel grid rod floor consisting of 3.2 mm rods spaced 7.9 mm apart. The side compartments were attached via a gray-colored 7.2 cm long center compartment with a smooth floor. The clear tops of the compartments were hinged to permit placement and removal of the mice. Ceiling lights were attached to each top. To balance unconditioned side preference, only the light in the black compartment was illuminated during training/testing. Automated data collection was accomplished using photobeams (6 beams for the white and black test areas and 2 beams for the center gray area) which were evenly spaced across the length of the chamber and interfaced with a computer

containing Med-PC software. Using this automated photobeam system, entry into a side compartment was defined as consecutive breaks of the first two photocell beams in that compartment located adjacent to the door separating that compartment from the center compartment. Exiting of a side compartment (and entry into the center compartment) was indicated by occlusion of the beams in the center compartment.

Cocaine-Induced Conditioned Place Preference

Cocaine-induced conditioned place preference was established using a design in which one of the side compartments was randomly designated as the cocaine compartment and the other as the saline compartment, as previously reported (Mantsch et al., 2010). On the first day of the procedure, mice were placed into the center compartment of the chamber and provided free access to both side compartments for 30 minutes in the absence of saline or cocaine pretreatment to determine pre-conditioning preference. During the 8-day conditioning phase of the experiment, mice received cocaine (15 mg/kg, ip) and saline injections on alternating days after which time they were confined to the randomly designated treatment-appropriate compartment for 30 minutes. After the final conditioning session, mice were tested for the expression of cocaine-induced conditioned place preference by once again placing them into the center compartment of the chamber and providing them with free access to the side compartments for 30 minutes. Conditioned place preference was defined as the change in time spent (sec) within the cocaine-paired compartment after conditioning when compared to the initial pre-conditioning session.

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Extinction

Following conditioning, daily extinction training was conducted. During the extinction sessions, mice were placed into the center compartment and once again provided with free access to the side compartments for 30 min. Mice underwent daily extinction training until the preference for the cocaine-paired compartment during the session (i.e., change in time spent in the cocaine side relative to pre-conditioning values) was reduced by at least 50% compared to the post-conditioning test session, at which time reinstatement testing was conducted.

Reinstatement Testing

The reinstatement sessions were identical to the extinction sessions except that mice underwent forced swim and/or received drug injections prior to the session. Reinstatement was defined according to the time spent in the compartment previously paired with cocaine. In most cases, mice were tested more than once for reinstatement. In these cases, the sequence of reinstatement test conditions were counterbalanced in order to address concerns related to altered responsiveness with repeated testing. Reinstatement sessions were separated by additional extinction sessions. Mice were required to reach the extinction criterion once again before the next reinstatement test was conducted. To confirm that alterations in locomotor activity secondary to receptor deficiency or drug administration did not contribute to the observed effects by increasing or decreasing exploration of the paired compartments in the 3-chamber apparatus, the total combined amount of time spent in the compartments previously paired with cocaine or saline was also examined during reinstatement.

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EXPERIMENT #1: Stress and cocaine-induced reinstatement in beta-adrenergic receptor-deficient and wild-type control mice.

We previously reported that pharmacological blockade of beta-adrenergic receptors prevents stress-, but not cocaine-, induced reinstatement of extinguished conditioned place preference in mice (Mantsch et al., 2010). To further investigate the role of beta adrenergic receptors in stress-induced relapse, reinstatement following stress (forced swim), administration of a priming injection of cocaine, or activation of central adrenergic neurotransmission via delivery of the selective alpha-2 adrenergic receptor antagonist BRL-44,408 was tested in beta adrenergic receptor-deficient *Adrb1*/*Adrb2* double-knockout mice (n=11) and C57BL/6J wild-type control mice (n=9). These mice underwent cocaine-induced place conditioning and extinction prior to testing for reinstatement. In most cases, mice were tested for reinstatement by all three stimuli in counterbalanced sequence.

Stress-induced reinstatement

Stress-induced reinstatement was tested using a forced swim protocol as previously reported (Kreibich and Blendy, 2004; Mantsch et al., 2010). Mice were placed into a 30 cm h x 20 cm d cylindrical polypropylene container filled with water (20-25° C) for six min. Following forced swim, mice were placed back into their home cages for 3-4 min prior to introduction into the center compartment of the place conditioning chamber with free access to both of the side compartments for reinstatement testing, as described above. In cases where reinstatement was measured, swimming behavior was video recorded and the amount of time spent immobile (vs. actively swimming) was determined.

Cocaine- and BRL-44,408- induced reinstatement

Cocaine-induced reinstatement was tested by injecting mice with 15 mg/kg cocaine (ip) and then immediately placing them into the chamber for reinstatement testing. Reinstatement by the alpha-2 adrenergic receptor antagonist BRL-44,408 (10 mg/kg, ip) was tested 30 minutes after administration. BRL-44,408 is more selective for alpha-2 adrenergic receptors than other commonly used antagonists (e.g., yohimbine) and has been reported to increase central norepinephrine levels in rats (Dwyer et al., 2010). We have previously reported that this dose of BRL-44,408 is optimal for reinstatement (Mantsch et al., 2010).

EXPERIMENT #2: Reinstatement of conditioned place preference by isoproterenol

To determine if activation of beta adrenergic receptors is sufficient for reinstatement of extinguished cocaine-induced conditioned place preference, mice (n=11) were tested for reinstatement following administration of the non-selective beta adrenergic receptor agonist, isoproterenol. Mice received injections of isoproterenol (1, 2, or 4 mg/kg, ip) or vehicle 30 minutes prior to placement into the chambers and testing for reinstatement. Each mouse was tested with each dose of isoproterenol in counterbalanced sequence.

EXPERIMENT #3: Effects of beta adrenergic receptor antagonists on reinstatement

In order to determine the role of beta-1 and beta-2 adrenergic receptors in stress-induced reinstatement, mice were tested for reinstatement of extinguished place preference in response to forced swim or BRL-44,408 (10 mg/kg, ip), as described in Exp 1, following pretreatment with the selective beta-1 adrenergic receptor antagonist, betaxolol, or the selective beta-2 adrenergic receptor antagonist, ICI-118,551. Seven total mice were tested for the effects of betaxolol (10

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or 20 mg/kg, ip; 30 minute pretreatment) and vehicle on reinstatement following forced swim in counterbalanced sequence. All seven mice were tested for the effects 10 mg/kg betaxolol and vehicle. Six of the mice were also tested for the effects of 20 mg/kg betaxolol. Eight total mice were tested for the effects of ICI-118,551 (1 or 2 mg/kg, ip; 30 minute pretreatment) on swim-induced reinstatement. All eight mice were tested for the effects 1 mg/kg ICI-118,551 and vehicle. Seven of the mice were also tested for the effects of 2 mg/kg ICI-118,551. Seven mice were tested for the effects of betaxolol (10 mg/kg) or ICI-118,551 (1 mg/kg) and vehicle on reinstatement following administration of BRL-44,408 in counterbalanced sequence. Five of these mice were tested for effects of betaxolol, ICI-118,551 and vehicle. One mouse was tested only for the effects of betaxolol and vehicle, and one mouse was tested only for the effects of ICI-118,551 and vehicle. Additionally, six mice were tested for the effects of 20 mg/kg betaxolol, 2 mg/kg ICI-118,551 and vehicle on BRL-44,408-induced reinstatement in counterbalaneced sequence. Each of these mice was tested under each pretreatment condition.

Finally, eleven mice were tested for the effects of betaxolol (10 mg/kg only) or ICI-118,551 (1 mg/kg only) and vehicle on reinstatement following administration of isoproterenol (4 mg/kg, ip). Ten of these mice were tested for effects of betaxolol, ICI-118,551 and vehicle. One mouse was tested only for the effects of betaxolol on isoproterenol-induced reinstatement.

EXPERIMENT #4: Reinstatement of conditioned place preference by clenbuterol

Since reinstatement by stress and BRL-44,408 was blocked by the beta-2 adrenergic receptor antagonist, ICI-188,551, mice (n=7) were tested for reinstatement following administration of the selective beta-2 adrenergic receptor agonist, clenbuterol, to determine if activation of beta-2 adrenergic receptors is sufficient for reinstatement. These mice received injections of

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clenbuterol (1, 2, or 4 mg/kg, ip) or vehicle 30 minutes prior to placement into the chambers and testing for reinstatement. Each mouse was tested with each dose of clenbuterol in counterbalanced sequence. Additionally, eleven beta adrenergic receptor knockout and five C57BL/6J wild-type control mice were tested for reinstatement by 4 mg/kg clenbuterol to confirm that clenbuterol was producing reinstatement through actions at beta adrenergic receptors. The knockout mice were also used to test reinstatement in response to stress, cocaine, and BRL-44,408 in Exp. 1.

Locomotor Testing

To assist with dose selection, the effects of various doses of betaxolol, ICI-118,551, clenbuterol, and isoproterenol on locomotor activity were tested in 26 total mice using an automated AccuScan activity system (AccuScan Instruments, Inc., Columbus, OH) consisting of a frame containing photocells (eight per cage) in which clear Plexiglas 29.5 cm l x 19 cm w x 12.7 cm h cages were placed. Activity was measured as total photobeam breaks. During the week prior to locomotor testing, mice were habituated to the test environment during two 2-h sessions. On the test day, mice were placed into the chambers for two hrs prior to drug administration after which activity was measured over a 2-h period. The effects of betaxolol (0, 5, 10 and 20 mg/kg, ip), clenbuterol (0, 1, 2, and 4 mg/kg, ip), ICI-118,551 (0, 0.5, 1, and 2 mg/kg, ip) and isoproterenol (0, 1, 2, and 4 mg/kg, ip) were tested in separate groups of mice.

Statistical Analyses

Place preference during testing, extinction and reinstatement was defined as the time spent (sec) in the designated cocaine compartment. In the experiments comparing conditioning, extinction

and reinstatement between wild-type and beta adrenergic-deficient mice, the significance of differences was determined using 2-way ANOVA with genotype as a between subjects factor and time spent in the cocaine side during conditioning (pre- vs. post-conditioning), extinction (day one vs. day two), or reinstatement (extinction vs. reinstatement session) as a repeated measure followed by post-hoc testing using 2-tailed student's t-tests. The significance of the effects of drug pretreatments (betaxolol or ICI-118,551) on reinstatement by each of the stimuli was determined using 2-way repeated measures reinstatement x drug pretreatment ANOVA followed by post-hoc testing using 2-tailed student's t-tests. The abilities of various doses of isoproterenol or clenbuterol to induce reinstatement and the dose-dependent effects of various drugs on locomotor activity were determined using one-way repeated measures ANOVA followed by post-hoc testing using the Dunnett's test. For all analyses, significance was defined as $p<0.05$.

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RESULTS

EXPERIMENT #1: Stress and cocaine-induced reinstatement in beta-adrenergic receptor-deficient and wild-type control mice

Place conditioning and extinction in beta adrenergic receptor deficient mice

In order to examine the role of beta adrenergic receptors in stress-induced relapse, a total of eleven beta adrenergic receptor-deficient *Adrb1*/*Adrb2* double-knockout mice and nine wild-type mice underwent place conditioning and extinction and were tested for reinstatement. Both wild-type and double-knockout mice developed conditioned place preference and no difference in the magnitude of cocaine-induced place preference was observed between genotypes (Figure 1A). A 2-way place conditioning (time spent in coc side pre vs. post conditioning; repeated measure) x genotype ANOVA showed that, overall, conditioned place preference was observed (significant main effect of conditioning; $F_{1,18}$=14.812, p=0.001). However no effect of genotype or conditioning x genotype interaction was found.

Extinction of conditioned place preference in beta adrenergic receptor-deficient and wild-type mice is shown in Figures 1B and 1C. Beta adrenergic receptor knockout mice extinguished more quickly than wild-type controls (Figure 1B). Wild-type mice reached the extinction criteria in 5.0 ± 0.68 sessions, compared to 3.1 ± 0.23 for knockout mice (t_{14}=2.167; p<0.01). This observation is consistent with reports that pharmacological inhibition of beta adrenergic receptors produces post-retrieval disruption of conditioned place preference, thereby reducing subsequent time spent in the cocaine-paired environment (Bernardi et al. 2009; Otis and Mueller, 2011). To further examine this possibility, we compared time spent in the cocaine compartment between genotypes on the two days after testing for the expression of place preference (i.e., on post-conditioning days two and three). A 2-way ANOVA examining differences in time spent

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on the cocaine paired side on these days between genotypes showed significant main effects of genotype ($F_{1,18}$=5.665; p<0.05) and session/day (repeated measure $F_{1,18}$=4.772; p<0.05) and a non-significant trend towards a genotype x session/day interaction (p=0.065). Despite the lack of a significant interaction, planned comparisons examining the amount of time spent in the cocaine-paired compartment on days two and three post-conditioning in each genotype were performed. A significant reduction in the time spent in the cocaine-paired compartment on day three vs. day two only observed in knockout mice (t_{10}=2.697; p<0.05) and, on day three post-conditioning, knockout mice spent less time in the cocaine-paired compartment compared to wild-type controls (t_{18}=3.149; p<0.01).

Stress- and cocaine-induced reinstatement in beta adrenergic receptor-deficient mice

The ability of various stimuli to reinstate extinguished cocaine-induced conditioned place preference in beta adrenergic receptor-deficient and wild-type mice is shown in Figure 2. Reinstatement of extinguished cocaine-induced place preference by a stressor, forced swim, was tested in ten beta adrenergic receptor knockout mice and five wild-type mice (Figure 2A). Swim-induced reinstatement was observed in wild-type but not knockout mice. A 2-way ANOVA showed a significant interaction between genotype and reinstatement condition ($F_{1,13}$=10.815; p<0.01). Post-hoc testing showed that swim stress increased the time spent in the cocaine compartment relative to extinction in wild-type mice (paired t_4=4.722, p<0.01) but not in knockout mice. Additionally, the amount of time spent in the cocaine compartment following forced swim was significantly increased in wild-type vs. knockout mice (unpaired t_{13}=3.431, p<0.01). There was no significant difference between genotypes during the preceding extinction session. Active immobility time during the 6-min swim sessions did not significantly differ between wild-type and beta adrenergic receptor-deficient mice (Table 1).

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Reinstatement in response to administration of cocaine (15 mg/kg, ip) was determined in eleven knockout and six wild-type mice (Figure 2B). In contrast to swim stress, cocaine administration reinstated extinguished place preference in both knockout and wild-type mice. Two-way ANOVA showed a significant overall reinstatement effect (reinstatement vs. extinction; $F_{1,15}$=13.184, p<0.01) but no main effect of genotype or reinstatement x genotype interaction.

Since we previously reported that activation of central noradrenergic neurotransmission through administration of the alpha-2 adrenergic receptor antagonist, BRL-44,408 reinstates extinguished place preference (Mantsch et al., 2010), we determined the ability of 10 mg/kg BRL-44,408 (ip) to induce reinstatement in eight wild-type and ten beta adrenergic receptor knockout mice (Figure 2C). Similar to stress, reinstatement following BRL-44,408 administration was observed in wild-type but not knockout mice. A 2-way ANOVA showed a significant interaction between genotype and reinstatement condition ($F_{1,16}$=5.409; p<0.05). Post-hoc testing revealed that BRL-44,408 increased the amount of time spent in the cocaine-paired compartment in wild-type but not knockout mice (paired t_7=2.768, p<0.05) during reinstatement testing when compared to the preceding extinction session. Additionally, time spent in the cocaine compartment following BRL 44,408 was significantly increased in wild-type vs. knockout mice (unpaired t_{16}=3.050, p<0.01). Notably, post-hoc testing also showed that the time spent in the cocaine compartment during the extinction sessions prior to testing for BRL 44,408 induced reinstatement was reduced in knockout mice (p<0.05).

To confirm that differences in locomotor activity between genotypes under extinction and reinstatement conditions did not significantly contribute to the observed effects of beta adrenergic receptor deficiency on reinstatement, the total combined amount of time spent in the

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cocaine and saline compartments of the 3-chamber apparatus was also measured. In all cases (swim, cocaine and BRL-44,408), 2-way ANOVA failed to show significant overall effects of genotype or reinstatement condition or significant genotype x reinstatement condition interactions (data not shown), suggesting that the observed effects on reinstatement were not associated with differences in the amount of time that the mice spent outside of the center compartment in which they were placed at the start of the test session.

EXPERIMENT #2: Reinstatement of conditioned place preference by isoproterenol

To determine if activation of beta adrenergic receptors is sufficient to reinstate extinguished place preference, nine mice were tested with the non-selective beta adrenergic agonist, isoproterenol. These mice showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 698.70 ± 45.801 sec, post-conditioning time spent = 1012.99 ± 52.59 sec; paired test; t_8=4.581; p<0.01). Isoproterenol dose-dependently reinstated extinguished place preference (Figure 3). Two-way repeated measures ANOVA showed a significant interaction ($F_{3,24}$=4.922; p<0.01) between isoproterenol dose (0, 1, 2, and 4 mg/kg) and test condition (extinction vs. reinstatement). Post-hoc analysis showed that significant reinstatement was observed at the 2 mg/kg (t_8=2.382; p<0.05 vs. extinction) and 4 mg/kg (t_8=3.983; p<0.01 vs. extinction) but not following administration of vehicle or the lowest (i.e., 1 mg/kg) isoproterenol dose tested. Isoproterenol did not increase the total amount of time spent in the cocaine and saline compartments combined during reinstatement testing, suggesting the reinstatement was not an artifact of increased locomotor activity (data not shown).

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EXPERIMENT #3: Effects of beta adrenergic receptor antagonists on reinstatement

Role of Beta-1 and Beta-2 Adrenergic Receptors in Stress-Induced Reinstatement

In order to determine which beta adrenergic receptor subtype mediates stress-induced reinstatement of extinguished cocaine-induced conditioned place preference, the effects of the selective beta-1 receptor antagonist, betaxolol (10 or 20 mg/kg), or the selective beta-2 antagonist, ICI-118,551 (1 or 2 mg/kg), on reinstatement following forced swim were tested (Figure 4). For this experiment, mice were tested for stress-induced reinstatement multiple times in counter-balanced sequence. Seven mice were tested for the effects of betaxolol on reinstatement. All of these mice were tested for the effects of 10 mg/kg betaxolol and six of them were tested for the effects of the 20 mg/kg betaxolol dose. These mice showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 531.66 ± 95.89 sec, post-conditioning time spent = 828.36 ± 92.03 sec; paired test; t_6=5.196; p<0.01). Reinstatement was not blocked by the 10 mg/kg betaxolol dose (Figure 4A). A 2-way repeated measures ANOVA showed that forced swim produced reinstatement (significant overall main effect of swim vs. extinction; $F_{1,5}$=10.729; p<0.05). However, no main effect of 10 mg/kg betaxolol and no significant betaxolol x reinstatement interaction were observed. By contrast, a 2-way ANOVA showed a significant interaction between betaxolol pretreatment and reinstatement condition in mice that received 20 mg/kg betaxolol ($F_{1,6}$=8.210; p<0.05; Figure 4B). In this group, post-hoc testing revealed significant reinstatement in mice pretreated with vehicle (paired t_6=2.512; p<0.05) but not 20 mg/kg betaxolol.

In contrast to betaxolol, both doses of ICI-118,551 that were tested blocked swim-induced reinstatement. Eight total mice were tested for the effects of ICI-118.551 on reinstatement. All eight of these mice were tested for the effects of the 1 mg/kg ICI-118,551

dose (Figure 4C), while only seven were tested for the effects of the 2 mg/kg dose (Figure 4D). These mice showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 535.22 ± 83.12 sec, post-conditioning time spent = 847.94 ± 82.07 sec; paired test; t_7=6.016; p=0.001). For both doses of ICI-118,551, significant interactions between ICI-118,551 treatment (drug vs. vehicle) and reinstatement conditions (swim vs. extinction) were observed ($F_{1,7}$=5.659 for the 1 mg/kg dose; $F_{1,6}$=7.065 for the 2 mg/kg dose; p<0.05 for each). In both cases, post-hoc testing revealed that forced swim produced reinstatement in mice pretreated with vehicle (paired t_7=2.350, p=0.05 for the 1 mg/kg dose vehicle; paired t_6=2.925, p<0.05 for the 2 mg/kg dose vehicle), but not ICI-118,551.

Despite a tendency to reduce immobility during forced swim testing at the lower doses, neither betaxolol (10 or 20 mg/kg) nor ICI-118,551 (1 or 2 mg/kg) significantly altered active immobility time during the 6-min sessions relative to vehicle treatment (Table 1). Additionally, neither betaxolol (10 or 20 mg/kg) nor ICI-118,551 (1 or 2 mg/kg) reduced the total amount of time spent in the cocaine and saline compartments combined during reinstatement testing, suggesting that the inhibition of reinstatement was not attributable to a general reduction of locomotor activity and decreased movement out of the center start compartment (data not shown).

Role of Beta-1 and Beta-2 Adrenergic Receptors in Reinstatement by BRL-44,408

The effects of betaxolol (10 and 20 mg/kg) and ICI-118,551 (1 and 2 mg/kg) on reinstatement by the selective alpha-2 adrenergic receptor antagonist, BRL-44,408 are shown in Figure 5. Mice tested for reinstatement in response to BRL-44,408 (n=13 total), showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 650.66 ± 59.13 sec, post-conditioning time spent = 981.67 ± 645.118.00 sec; paired test; t_{12}=5.170; p<0.01). Five of

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these mice were tested for effects of 10 mg/kg betaxolol and 1 mg/kg ICI-118,551. One mouse was tested only for the effects of 10 mg/kg betaxolol and one mouse only for the effects of 1 mg/kg ICI-118,551. An additional six mice were tested for the effects of both 20 mg/kg betaxolol and 2 mg/kg ICI-118,551 on reinstatement by BRL-44,408.

Neither 10 mg/kg (Figure 5A) nor 20 mg/kg (Figure 5B) betaxolol (ip) blocked BRL-44,408-induced reinstatement. Two-way repeated measures ANOVA showed a significant reinstating effects of BRL-44,408 ($F_{1,5}$=11.196; p<0.05; 10 mg/kg betaxolol group or $F_{1,5}$=22.117; p=0.005; 20 mg/kg betaxolol group) but no significant main effects of betaxolol (10 or 20 mg/kg) and no betaxolol x reinstatement condition interactions. By contrast both 1 mg/kg (Figure 5C) and 2 mg/kg (Figure 5D) ICI-118,551 (ip) blocked reinstatement by BRL-44,408. At the 1 mg/kg ICI-118,551 dose, a 2-way repeated measures ANOVA showed a significant interaction between ICI-118,551 pretreatment and BRL-44,408 induced reinstatement ($F_{1,5}$=25.175; p<0.01). Post-hoc testing revealed that BRL-44,408 reinstated place preference in mice following vehicle, but not ICI-118,551, pretreatment (BRL 44,408 vs. extinction; paired t_5=3.589, p<0.05). At the 2 mg/kg ICI-118,551 dose, a 2-way repeated measures ANOVA showed a near-significant interaction between ICI-118,551 pretreatment and BRL-44,408 induced reinstatement ($F_{1,5}$=6.115; p=0.056). A planned comparison t-test revealed that BRL-44,408 reinstated place preference in mice following vehicle, but not ICI-118,551 pretreatment (BRL 44,408 vs. extinction; paired t_5=2.553, p=0.05). As was the case with forced swim, neither betaxolol nor ICI-118,551 reduced the total amount of time spent in the cocaine and saline compartments combined during reinstatement testing, suggesting that general reductions in locomotor activity and decreased movement out of the center start compartment did not contribute to the effects on reinstatement (data not shown).

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Role of Beta-1 and Beta-2 Adrenergic Receptors in Isoproterenol-Induced Reinstatement

To examine the role of beta-1 and beta-2 adrenergic receptor subtypes in reinstatement in response to the non-selective beta receptor agonist, isoproterenol, mice were tested for reinstatement in response to 4 mg/kg isoproterenol following pretreatment with the beta-1 adrenergic receptor selective antagonist, betaxolol (10 mg/kg, ip; Figure 6A) or the beta-2 receptor selective antagonist, ICI-118,551 (1 mg/kg, ip; Figure 6B). These mice (n=11 total) showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 579.75 ± 59.87 sec, post-conditioning time spent = 944.08 ± 39.28 sec; paired test; t_{10}=6.704; p<0.001). Surprisingly, isoproterenol-induced reinstatement was blocked by either betaxolol (n=11) or ICI-118,551 (n=10) pretreatment. A 2-way repeated measures ANOVA examining the effects of betaxolol on isoproterenol-induced reinstatement showed a significant interaction between betaxolol pretreatment (10 mg/kg betaxolol vs. vehicle) and reinstatement ($F_{1,10}$=10.981; p<0.01). Likewise, a 2-way repeated measures ANOVA examining the effects of ICI-118,551 on reinstatement showed a significant interaction between ICI-118,551 pretreatment (1 mg/kg ICI-118,551 vs. vehicle) and reinstatement ($F_{1,9}$=5.420; p<0.05). In both cases, significant reinstatement of place preference was observed following pretreatment with vehicle (t_{10}=3.338, p<0.01 for betaxolol experiment; t_9=2.890, p<0.05 for ICI-118,551 experiment) but not following administration of either beta adrenergic receptor antagonist. Effects on the total amount of time spent in the cocaine and saline compartments combined during reinstatement testing were not observed (data not shown).

EXPERIMENT #4: Reinstatement of conditioned place preference by clenbuterol

To determine if beta-2 adrenergic receptor activation alone is sufficient to reinstate extinguished place preference, seven mice were tested for reinstatement following administration of the

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selective beta-2 receptor agonist, clenbuterol. These mice showed significant cocaine-induced place preference (pre-conditioning time spent in the cocaine side = 603.03 ± 53.88 sec, post-conditioning time spent = 987.06 ± 37.52 sec; paired test; t_6=5.675; p<0.01). Clenbuterol dose-dependently reinstated extinguished place preference (Figure 7A). Two-way repeated measures ANOVA showed a significant interaction ($F_{3,18}$=5.206; p<0.01) between clenbuterol dose (0, 1, 2, and 4 mg/kg) and test condition (extinction vs. reinstatement). Post-hoc analysis showed that significant reinstatement was observed at the 2 mg/kg (t_6=3.18; p<0.05 vs. extinction) and 4 mg/kg (t_6=4.667; p<0.01 vs. extinction) but not following administration of vehicle or the lowest (i.e., 1 mg/kg) clenbuterol dose. As was the case with isoproterenol, clenbuterol did not increase the total amount of time spent in the cocaine and saline compartments combined during reinstatement testing, suggesting the reinstatement was not an artifact of increased locomotor activity (data not shown). As expected, clenbuterol-induced reinstatement was not observed in beta adrenergic receptor-deficient mice (Figure 7B). A 2-way reinstatement x genotype ANOVA showed a significant interaction between genotype (knockout vs. wildtype) and clenbuterol-induced reinstatement ($F_{1,14}$=15.930; p<0.01). Clenbuterol (4 mg/kg, ip) reinstated place preference in wild-type (t_4=6.474; p<0.05; n=5) but not *Adrb1*/*Adrb2* double-knockout mice (n=11).

Effects of Drugs on Locomotor Activity

To aid in data interpretation, the unconditioned locomotor (ambulatory) responses to isoproterenol, betaxolol, ICI-118,551, and clenbuterol were examined in separate groups of mice (n=5 per group) and are shown in Table 2. Each of the drugs dose-dependently reduced ambulatory activity compared to their respective vehicle controls (repeated measures ANOVA; $F_{3,12}$=18.406, p<0.001 for betaxolol; $F_{3,12}$=16.315, P<0.001 for ICI-118,551; $F_{3,12}$=4.264, p<0.05

for isoproterenol; $F_{3,12}$=5.291, p<0.05 for clenbuterol). Post-hoc testing using a Dunnett's test showed that betaxolol significantly reduced ambulatory activity compared to vehicle at all doses tested (5, 10 and 20 mg/kg; p<0.05) and ICI-118,551 significantly reduced activity at the two higher doses that were tested (1 and 2 mg/kg; p<0.05) while isoproterenol and clenbuterol only reduced activity at the highest tested dose (4 mg/kg; p<0.05).

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DISCUSSION

We previously reported that disinhibition of central noradrenergic neurotransmission via alpha-2 adrenergic receptor antagonism using yohimbine or the more selective antagonist, BRL-44,408, is sufficient to reinstate extinguished cocaine-induced conditioned place preference, while functional antagonism of noradrenergic activity via administration of the alpha-2 adrenergic receptor agonist, clonidine, prevents reinstatement by a stressor, forced swim, in mice (Mantsch et al., 2010). Further, we reported that stress- and yohimbine-induced reinstatement is prevented by propranolol, but not the alpha-1 adrenergic receptor antagonist, prazosin (Mantsch et al., 2010). In this study, we confirm the role of beta adrenergic receptors in stress-induced reinstatement by demonstrating that reinstatement by forced swim or BRL-44,408 is not observed in beta receptor-deficient *Adrb1*/*Adrb2* double-knockout mice and that beta receptor activation using isoproterenol, a non-selective agonist, is sufficient to induce reinstatement in wild-type mice. Although suggestive of a role for beta adrenergic receptors, the absence of reinstatement in knockout mice should be interpreted with caution due to the lack of hybrid background strain controls. However, it should be noted that stress-induced reinstatement of cocaine-induced place preference has been observed in a number of mouse strains, including other hybrid background strains (Kreibich and Blendy, 2004; Redila and Chavkin, 2008; Land et al., 2009; Mantsch et al., 2010; Briand et al., 2010; Bruchas et al., 2011) .

Our findings suggest that beta-2 adrenergic receptor activation is both necessary for stress-induced reinstatement and sufficient to reinstate and that stress-induced reinstatement may also involve beta-1 receptors. The selective beta-2 receptor antagonist, ICI-118,551 blocked reinstatement by forced swim or BRL-44,408 at all doses tested, while the selective beta-2 receptor agonist, clenbuterol, dose-dependently reinstated. Since the beta-1 receptor antagonist,

betaxolol, also interfered with swim- (but not BRL-44,408) induced reinstatement at a high dose, we can't rule out a contribution of beta-1 receptors to stress-induced reinstatement. In contrast to ICI-118,551 which has a 300-fold higher affinity for beta-2 relative to beta-1 receptors, the selectivity for betaxolol for beta-1 versus beta-2 receptors is much lower, approximately a 35-fold affinity difference (Wellstein et al., 1986). Thus, it is possible that, at the higher betaxolol dose, there was some beta-2 receptor antagonism. However, considering that it has been reported that the same betaxolol dose can alter other stress-induced behaviors in mice on which ICI-118,551 has no effect (Stone et al., 1996), a coordinated roles for both receptors is possible. Accordingly, reinstatement by isoproterenol was blocked by either betaxolol or ICI-188,551. Since activation of beta-2 receptors alone is sufficient for reinstatement (clenbuterol reinstates), the requirement for beta-1 receptors for reinstatement is intriguing, and may imply that tonic levels of beta-1 receptor activation, possibly by basal levels of norepinephrine, is necessary for the activation of beta-2 receptors and/or their effects on cocaine seeking.

Both ICI-118,551 and betaxolol inhibited locomotor activity at doses tested for effects on reinstatement. Since reduced locomotor activity could produce effects that resemble decreased reinstatement in the 3-compartment apparatus due to potential reductions in the overall time spent outside of the start chamber, we also examined the total time spent in the saline and cocaine compartments combined during reinstatement testing. In all cases, antagonist-induced reductions in the time spent in the cocaine compartment were accompanied by increases in time spent in the saline compartment such that the total time spent outside of the start chamber was no different from vehicle control conditions. Thus, we consider it unlikely that locomotor-suppressing effects contributed to the reductions in reinstatement.

Based on the findings by Leri et al. (2002) that combined antagonism of beta-1 and beta-2 adrenergic receptors in the bed nucleus of the stria terminalis (BNST) or amygdala using a cocktail of betaxolol and ICI-118,551 blocks reinstatement of extinguished cocaine self-administration by footshock stress in rats, we assume that our findings are attributable to the blockade or loss of receptors in the brain. However, a role for peripheral receptors cannot be ruled out, especially considering evidence that epinephrine released from the adrenal medulla can regulate a number of behavioral processes, despite its inability to cross the blood brain barrier (see e.g., McGaugh and Roozendaal, 2002). Although our earlier finding that surgical adrenalectomy fails to prevent acute stress-induced reinstatement in rats argues against a role for peripheral epinephrine in stress-induced cocaine seeking (Graf et al., 2011), the role of peripheral beta-2 receptors remains to be determined.

Beta adrenergic receptor-mediated cocaine seeking likely involves the BNST. The ventral noradrenergic bundle, comprised of projections from medullary cell groups, heavily innervates the BNST and is critical for stress-induced reinstatement (Shaham et al., 2000b). Both beta-1 and beta-2 receptors are highly expressed in the mouse BNST (Lorton and Davis, 1987) and intra-BNST delivery of a cocktail of ICI-118,551 and betaxolol blocks stress-induced reinstatement in rats (Leri et al., 2002). In the BNST, norepinephrine enhances excitatory neurotransmission through actions at beta receptors (Egli et al., 2005) via a mechanism that may involve CRF (corticotropin releasing factor; Nobis et al., 2011), consistent with the finding that intra-BNST CRF delivery induces cocaine seeking while CRF receptor antagonism in the BNST prevents stress-induced reinstatement (Erb and Stewart, 1999) and with reports that CRF is downstream from norepinephrine in the pathway that mediates stress-induced reinstatement (Brown et al., 2009). Adrenergic receptor-regulated GABAergic inputs to the ventral tegmental

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area (VTA) have been characterized (Dumont and Williams, 2000). Alternatively, beta adrenergic receptors may regulate efferents from the BNST that release CRF into the VTA (Rodaros et al., 2007). We and others have reported that delivery of CRF into the VTA can reinstate cocaine seeking in rats and that VTA CRF receptor activation is necessary for stress-induced reinstatement (Wang et al., 2005; Blacktop et al., 2011). Determination of the ability of BNST beta adrenergic receptors to regulate CRF release into the VTA awaits further investigation.

Beta adrenergic receptors have also been implicated in anxiety-related responses associated with acute cocaine delivery (Schank et al., 2008) and cocaine withdrawal (Harris and Aston-Jones, 2003). In the case of withdrawal, these effects appear to be mediated by beta-1 receptors, likely in the amygdala (Rudoy and Van Bockstaele, 2007). The relationship between withdrawal symptoms and stress-induced relapse is unclear. However, a study involving cocaine-dependent individuals undergoing outpatient treatment found that propranolol only improved treatment outcomes in subjects who displayed severe withdrawal symptoms (Kampman et al., 2001), suggesting that medications targeting beta adrenergic receptors may be more effective for relapse prevention in subpopulations of addicts whose use is stress-related.

Despite preventing stress-induced reinstatement, beta adrenergic receptor deficiency and ICI-118,551 did not significantly alter immobility time during the forced swimming bouts. This finding was unexpected and may imply that there is a disconnection between stress-induced cocaine seeking and other stress-related behaviors. Surprisingly, it has been reported that clenbuterol decreases immobility during forced swim (Finnegan et al., 1987) while ICI-118,551 and propranolol increase it (Stone et al., 1995), suggesting that reduced drug seeking could be counter-intuitively related to depression phenotype.

Beta-adrenergic receptor-deficient mice developed conditioned place preference and did not show reductions in its expression relative to wild-type controls, suggesting that beta-adrenergic receptor activation is not necessary for cocaine's rewarding effects or for the retrieval of cocaine-associated memories. Cocaine-induced reinstatement was also unaffected in knockout mice, consistent with our earlier finding that pharmacological antagonism failed to alter reinstatement in response to cocaine (Mantsch et al., 2010). These findings are inconsistent with one report that beta receptor antagonism prevented the expression/retrieval of cocaine-induced conditioned place preference in rats (Otis and Mueller, 2011). Although conditioned place preference was not altered in knockout mice, differences relative to wild-type controls were observed during extinction, with no difference on the first test day after conditioning and decreased time spent in the cocaine compartment thereafter. Several groups have reported that antagonism of beta receptors either prior to (Otis and Mueller, 2011) or after (Fricks-Gleason and Marshall, 2008; Bernardi et al., 2009) testing for place preference reduces subsequent preference, potentially due to interference with memory reconsolidation processes. Disruption of memory reconsolidation could explain the reductions in place preference with repeated testing during extinction in knockout mice. To the extent that reconsolidation of cocaine-associated memories was impaired, it did not affect subsequent cocaine-induced reinstatement, in contrast to a report by Fricks-Gleason and Marshall (2008) that pharmacological blockade of these receptors after retrieval produced deficits in later reinstatement by cocaine. Thus, it is unlikely that impaired memory reconsolidation contributed to loss of stress-induced reinstatement in knockout mice. In support, acute pretreatment with ICI-118,551 selectively inhibited reinstatement by forced swim or BRL-44,408, an effect that can't be attributed to earlier disruption of reconsolidation-related processes.

To summarize, these data indicate that beta adrenergic receptor activation is both necessary for stress-induced reinstatement of cocaine seeking and sufficient to reinstate. While our findings demonstrate a role for beta-2 receptors in this effect, they do not rule out a role for beta-1 receptors and, in fact, suggest that stress-induced cocaine seeking may involve coordination between the two receptor subtypes. Future studies will further examine the mechanism through which this potential coordination occurs as well as the brain region(s) and neurocircuitry through which these receptors contribute to stress-induced relapse.

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AUTHOR CONTRIBUTIONS

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FOOTNOTES

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FIGURE LEGENDS

Figure 1: Induction and extinction of cocaine-induced conditioned place preference in beta adrenergic receptor-deficient *Adrb1/Adrb2* double-knockout (KO) and wild-type C57BL/6J control (WT) mice. Data represent the time spent (sec ±S.E.) in the cocaine-paired compartment prior to (Pre-Cond) and after (Post-Cond) conditioning (A) and during the two extinction sessions following testing for the expression of conditioning (post-conditioning days 2 and 3; B) as well as the mean number of extinction sessions prior to reaching the extinction criterion (C). Both wild-type and knockout mice developed conditioned place preference (Figure 1A; *$p<0.05$ vs. Pre-Cond). Knockout mice showed reduced preference on post-conditioning day 3 (Figure 1B; **$p<0.05$ vs. WT) and extinguished more rapidly (Figure 1C; ***$p<0.05$ vs. WT) than wild-type mice.

Figure 2: Reinstatement following forced swim (2A), cocaine (15 mg/kg, ip) administration (2B) or delivery of the selective alpha-2 adrenergic receptor antagonist BRL-44,408 (10 mg/kg, ip; 2C) in beta adrenergic receptor-deficient *Adrb1/Adrb2* double-knockout (KO) and wild-type C57BL/6J control (WT) mice. Data represent the time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing or the preceding extinction session (Ext). Forced swim (Fig 2A) and BRL-44,408 (Fig 2C) induced reinstatement in WT but not KO mice. By contrast cocaine induced reinstatement in both WT and KO mice (*$p<0.05$ vs. Ext; **$p<0.05$ KO vs. WT; Fig 2B).

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Figure 3: Dose-dependent reinstatement by the beta adrenergic receptor agonist, isoproterenol in wild-type C57BL/6J mice. Data represent time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing following pretreatment with isoproterenol (0, 1, 2 or 4 mg/kg, ip) or during the preceding extinction session (Ext). Isoproterenol induced reinstatement at the 2 and 4 mg/kg doses (*p<0.05 vs. Extinction).

Figure 4: Effects of the selective beta-1 adrenergic receptor antagonist betaxolol (10 mg/kg, ip; Fig 4A or 20 mg/kg, ip; Fig 4B) or the selective beta-2 adrenergic receptor antagonist ICI-118,551 (1 mg/kg, ip; Fig 4C or 2 mg/kg, ip; Fig 4D) on reinstatement following forced swim in wild-type C57Bl/6J mice. Data represent the amount of time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing following six minutes of forced swim after drug or vehicle pretreatment (Veh) or during the preceding extinction session (Ext). Betaxolol blocked swim-induced reinstatement at the 20 mg/kg but not 10 mg/kg dose, while ICI-118,551 blocked swim-induced reinstatement at both doses that were tested (*Significant reinstatement; p<0.05 vs. Extinction).

Figure 5: Effects of the selective beta-1 adrenergic receptor antagonist betaxolol (10 mg/kg, ip; Fig 5A or 20 mg/kg, ip; Fig 5B) or the selective beta-2 adrenergic receptor antagonist ICI-118,551 (1 mg/kg, ip; Fig 5C or 2 mg/kg, ip; Fig 5D) on reinstatement following administration of BRL-44,408 (BRL; 10 mg/kg, ip) in wild-type C57Bl/6J mice. Data represent the amount of time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing following BRL administration after antagonist or vehicle pretreatment (Veh) or during the preceding extinction

session (Ext). ICI-118,551 but not betaxolol blocked BRL-induced reinstatement (*Significant reinstatement; $p<0.05$ vs. Extinction; **$p<0.05$ ICI-118,551 vs. Veh).

Figure 6: Effects of the selective beta-1 adrenergic receptor antagonist betaxolol (10 mg/kg, ip; Fig 6A) or the selective beta-2 adrenergic receptor antagonist ICI-118,551 (1 mg/kg, ip; Fig 6B) on reinstatement following administration of isoproterenol (4 mg/kg, ip) in wild-type C57Bl/6J mice. Data represent the amount of time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing following isoproterenol administration after antagonist or vehicle pretreatment (Veh) or during the preceding extinction session (Ext). Both ICI-118,551 and betaxolol blocked isoproterenol-induced reinstatement (*Significant reinstatement; $p<0.05$ vs. Extinction).

Figure 7: Dose-dependent reinstatement by the beta-2 adrenergic receptor agonist, clenbuterol in wild-type (WT) C57BL/6J mice (7A) and reinstatement following administration of 4 mg/kg clenbuterol in WT and beta-adrenergic receptor knockout (KO) mice (7B). Data represent time spent in the cocaine-paired side (sec ± S.E.) during reinstatement testing following pretreatment with clenbuterol or during the preceding extinction session (Ext). Clenbuterol produced reinstatement at the 2 and 4 mg/kg doses but failed to produce reinstatement in KO mice (*$p<0.05$ vs. Extinction; **$p<0.05$ KO vs. WT).

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TABLES

Table 1: Immobility time (sec ± S.E.) during a 6-min period of forced swim in beta adrenergic receptor-deficient *Adrb1*/*Adrb2* double-knockout mice and wild-type C57BL/6J mice pretreated with vehicle, the beta 1 adrenergic receptor antagonist, betaxolol (10 or 20 mg/kg, ip) or the beta 2 adrenergic receptor antagonist, ICI-118,551 (1 or 2 mg/kg).

	WT/Vehicle	**Beta AR KO**	**Betaxolol**		**ICI-118,551**	
Immobility Time (sec)	231.08 ± 22.28	195.25± 14.72	*10 mg/kg* *20 mg/kg*	176.55 ± 26.73 218.92 ± 16.26	*1 mg/kg* *2 mg/kg*	163.15 ± 20.41 218.00 ± 18.65

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Table 2: Effects of adrenergic drugs on locomotor activity. Data represent the mean number of ambulatory counts (± S.E.) recorded over a 2-h period following i.p. administration of various doses of betaxolol, ICI-118,551, isoproterenol, or clenbuterol.

Betaxolol		**ICI-118,551**		**Isoproterenol**		**Clenbuterol**	
Dose (mg/kg)	*Ambulatory (Cts ± S.E.)*	*Dose (mg/kg)*	*Ambulatory (Cts ± S.E.)*	*Dose (mg/kg)*	*Ambulatory (Cts ± S.E.)*	*Dose (mg/kg)*	*Ambulatory (Cts ± S.E.)*
0	3501.60 ± 385.63	*0*	2455.76 ± 248.19	*0*	2047.50 ± 466.84	*0*	2433.20 ± 633.59
5.0	1927.60 ± 368.37*	*0.5*	2408.40 ± 346.86	*1.0*	1437.8 ± 583.28	*1.0*	1685.40 ± 442.49
10.0	1519.40 ± 304.82*	*1.0*	1316.20 ± 152.39*	*2.0*	959.75 ± 107.66	*2.0*	1234.80 ± 556.03
20.0	279.80 ± 84.53*	*2.0*	806.60 ± 110.01*	*4.0*	527.75 ± 90.15*	*4.0*	537.40 ± 186.78*

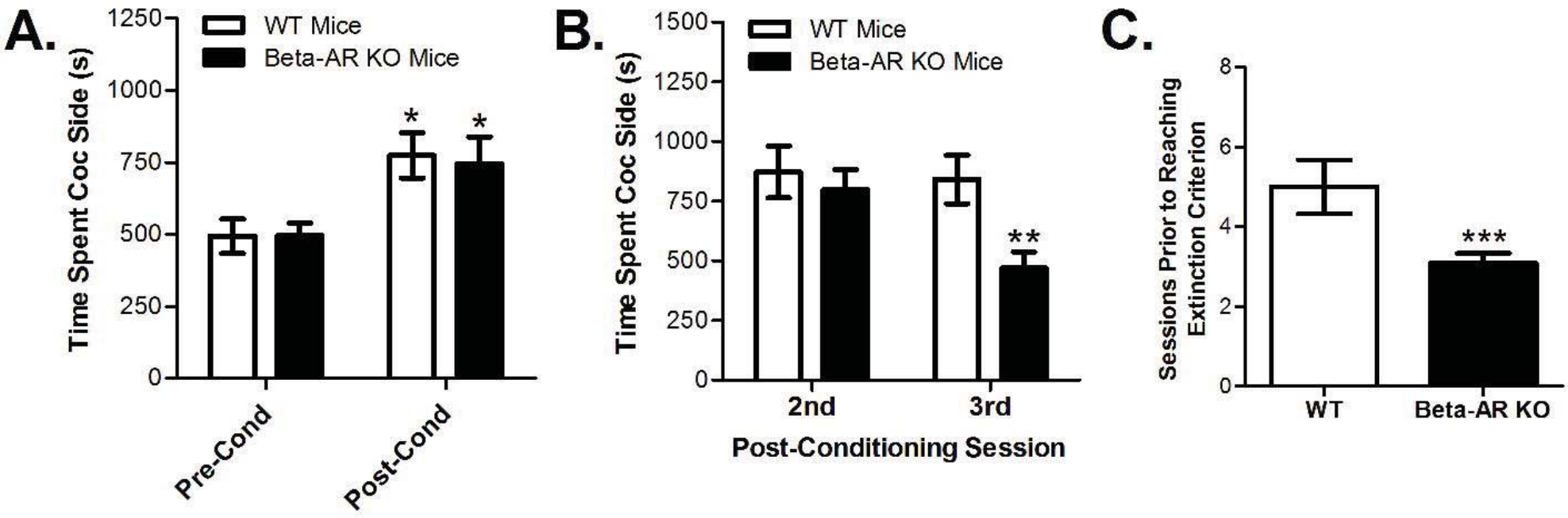

FIGURE 1

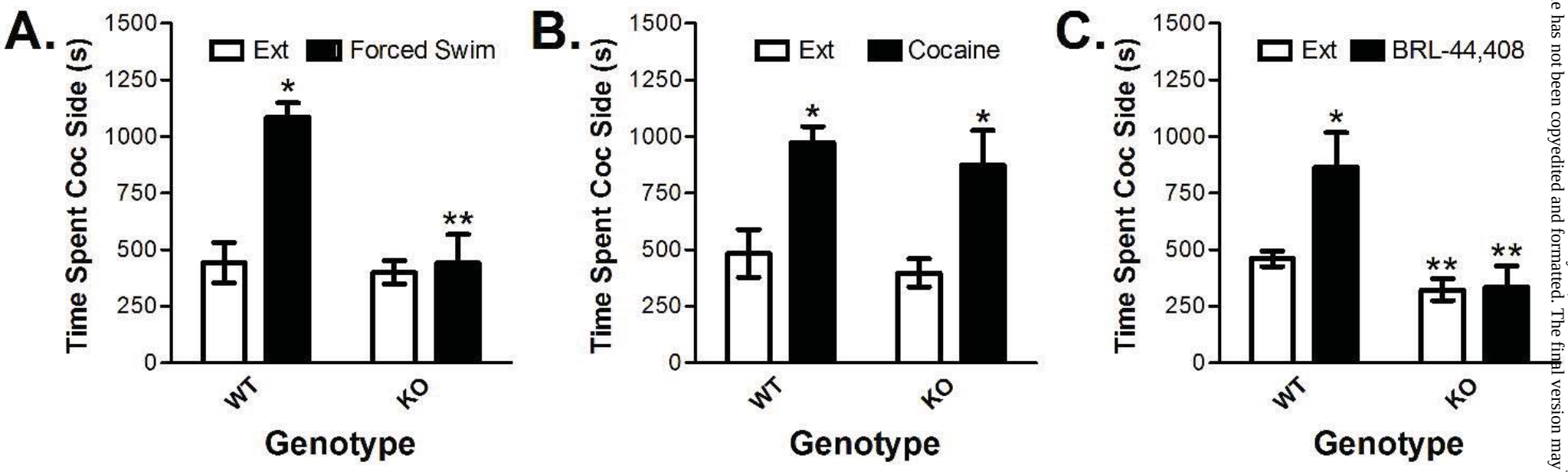

FIGURE 2

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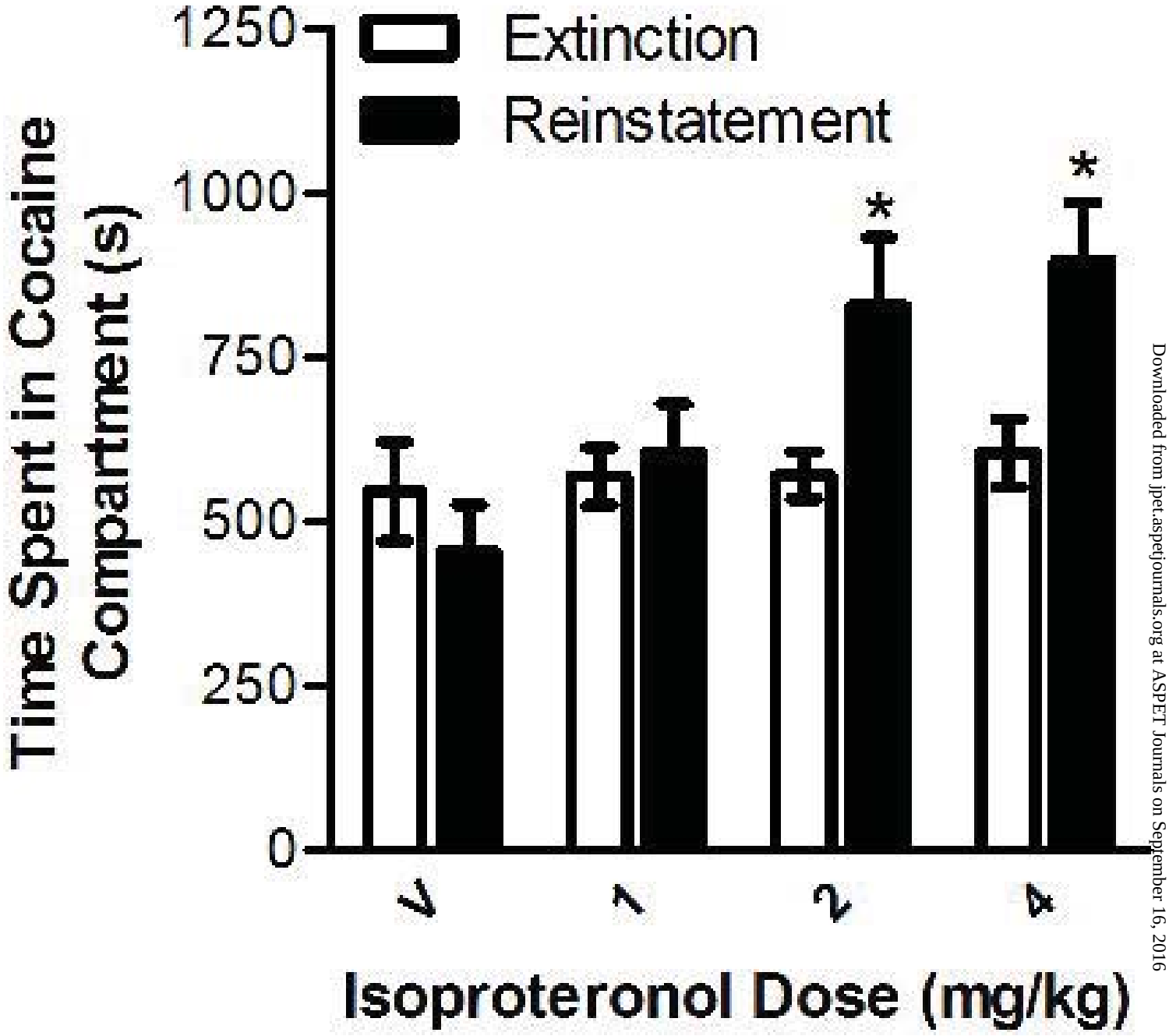

FIGURE 3

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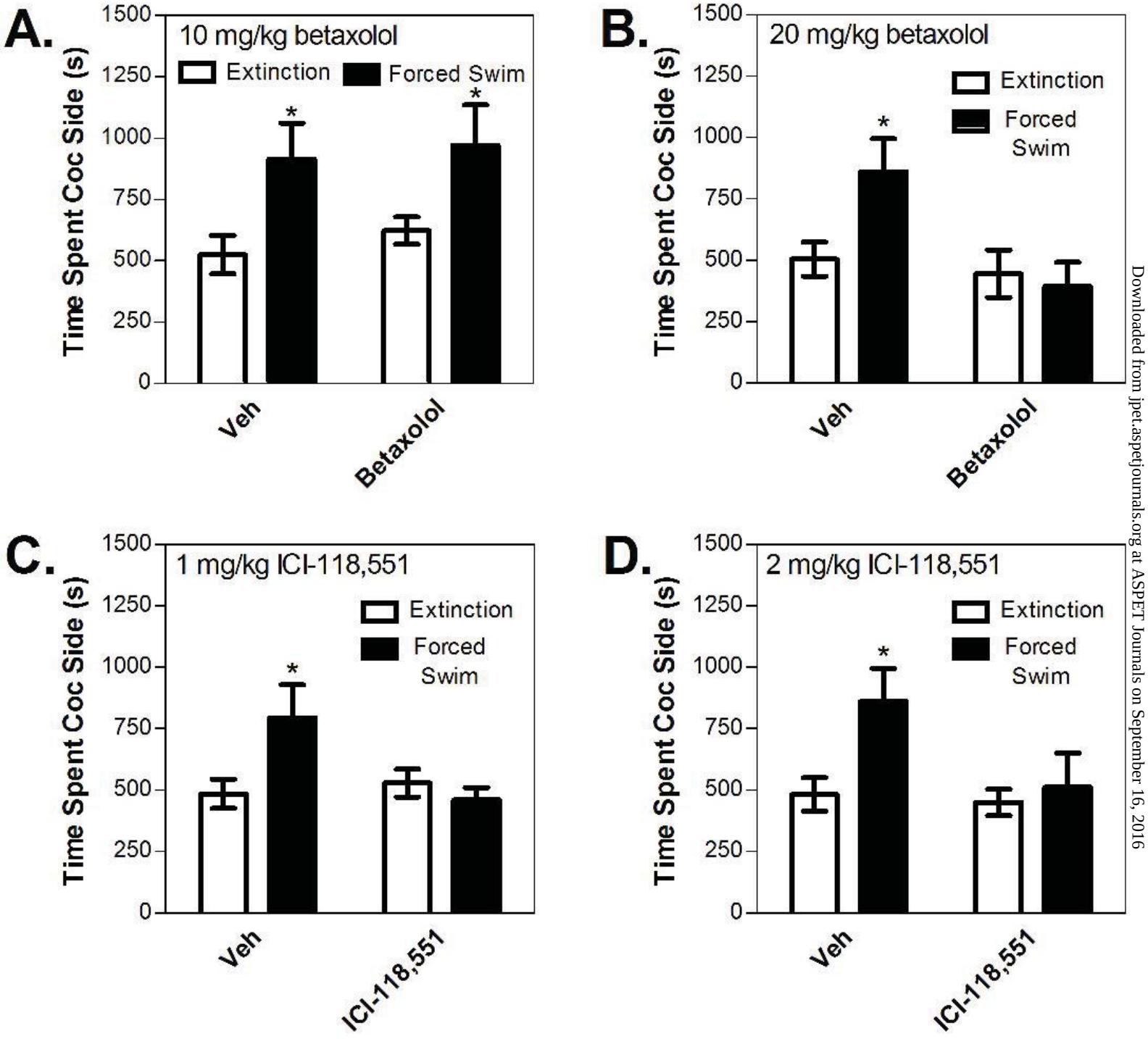

FIGURE 4

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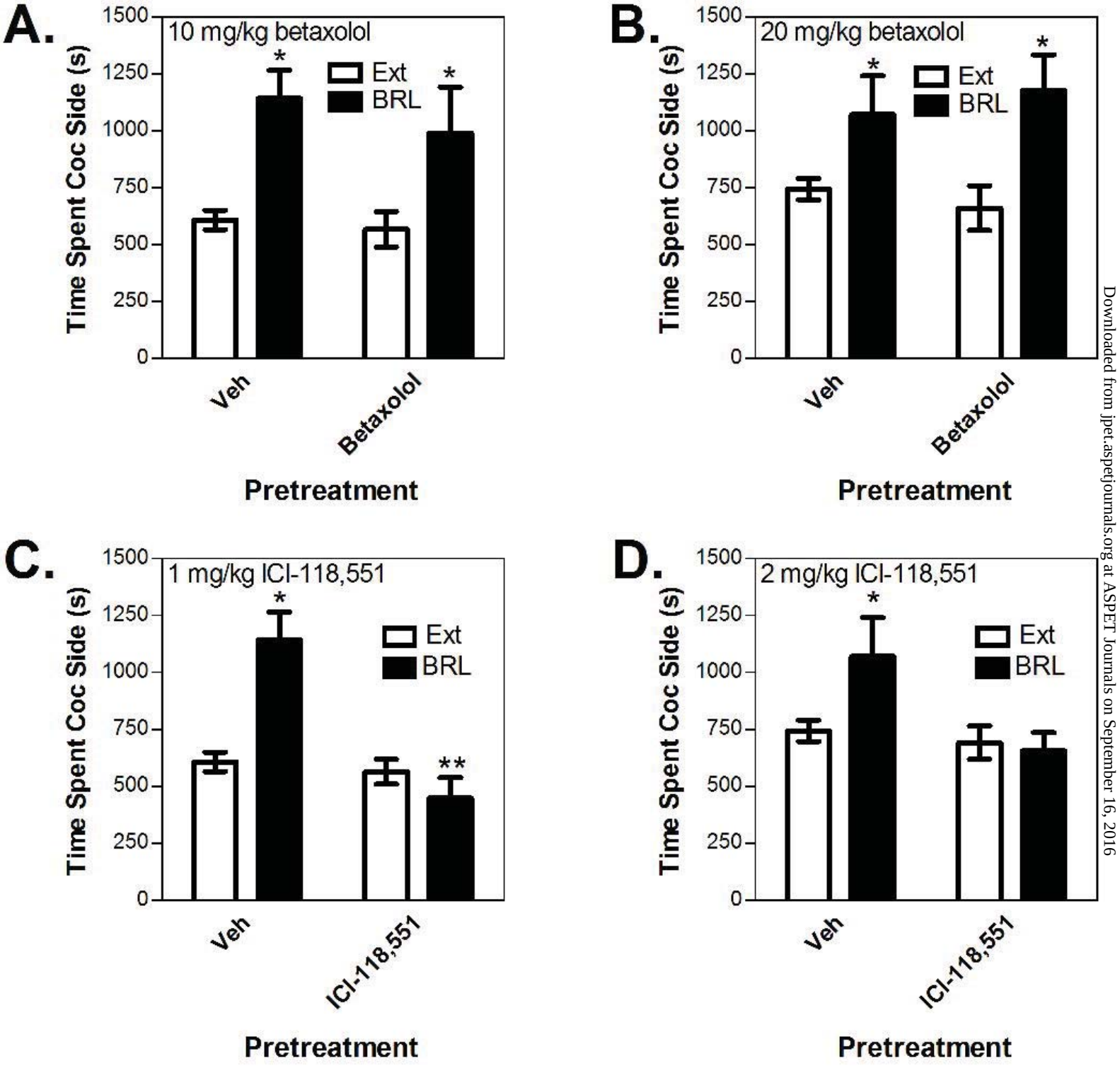


FIGURE 5

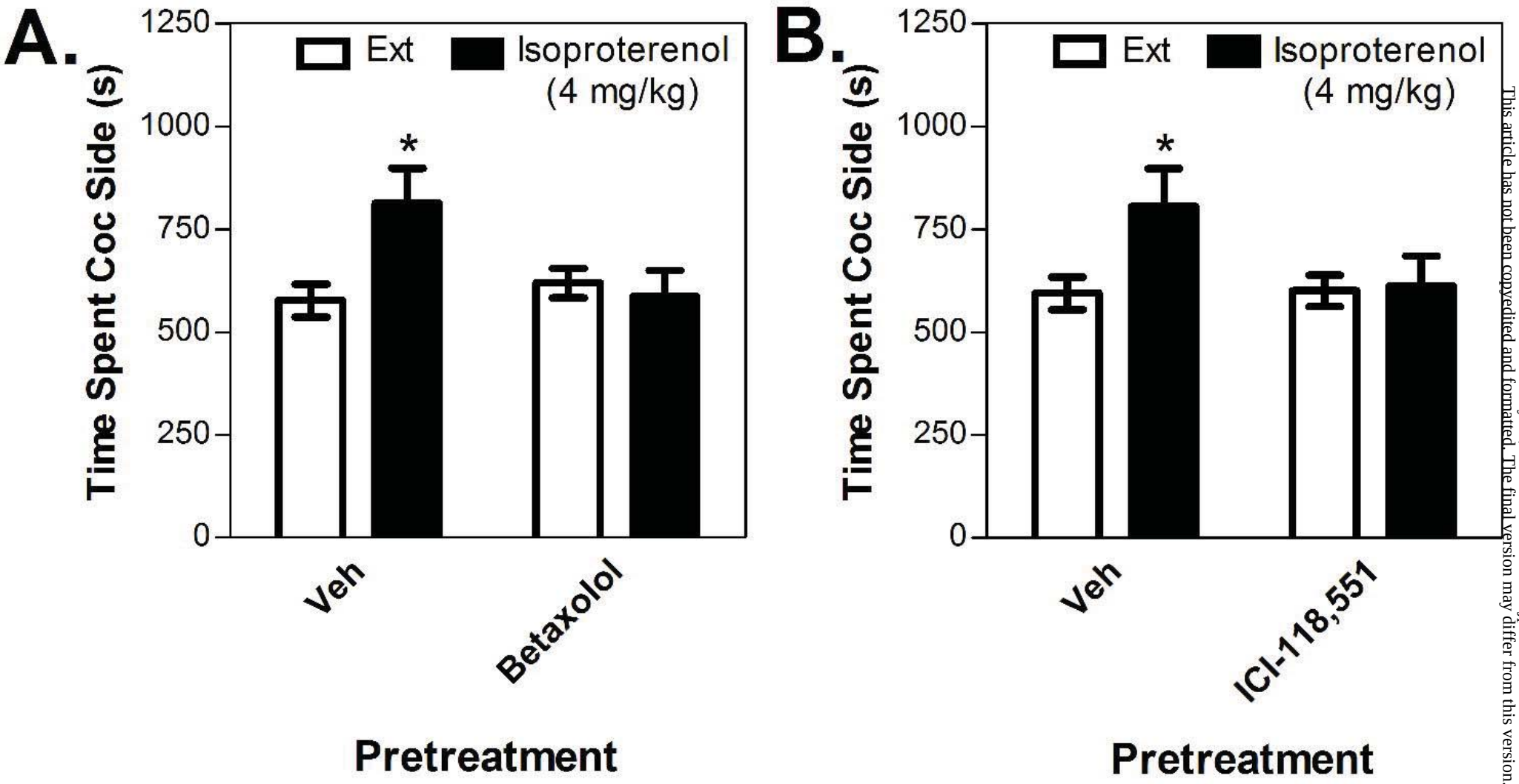
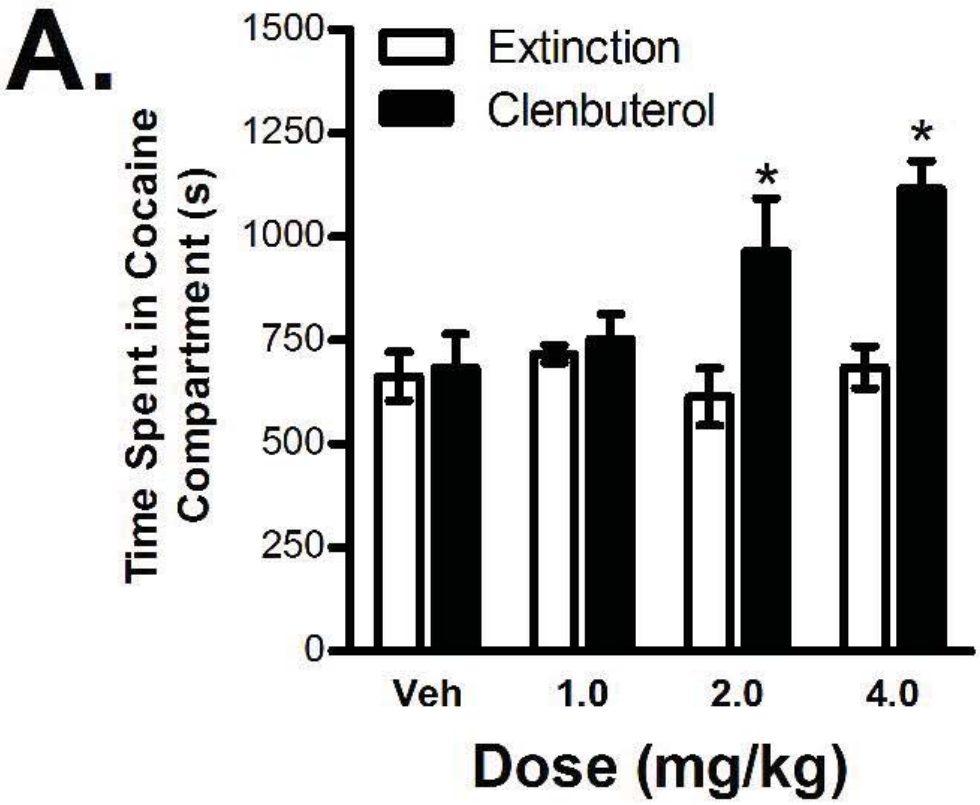


FIGURE 6



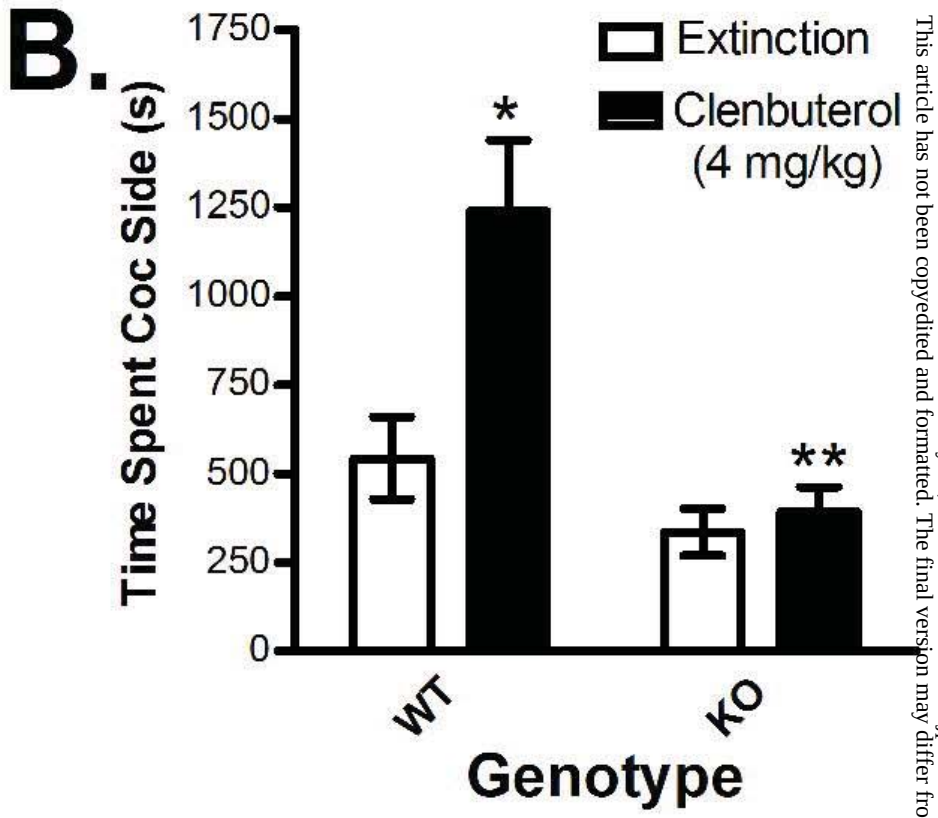


FIGURE 7