Published in final edited form as:

Eur J Pharmacol. 2007 August 13; 569(1-2): 84–89.

Reinstatement of cocaine place-conditioning prevented by the peptide kappa-opioid receptor antagonist, Arodyn

A.N. Carey^a, K. Borozny^a, J.V. Aldrich^b, and J.P. McLaughlin^{a,*}

^aDepartment of Psychology; Northeastern University, Boston, MA, USA 02115

^bDepartment of Medicinal Chemistry; University of Kansas, Lawrence, KS, USA 66047

Abstract

Stress contributes to the reinstatement of cocaine-seeking behavior in abstinent subjects. Kappa-opioid receptor antagonists attenuate the behavioral effects of stress, potentially providing therapeutic value in treating cocaine abuse. Presently, the peptide arodyn produced long-lasting kappa-opioid receptor antagonism, suppressing kappa-opioid receptor agonist-induced antinociception at least 3 days after intracerebroventricular administration of 0.3 nmol. C57Bl/6J mice demonstrated cocaine-conditioned place preference, extinction over 3 weeks, and a subsequent reinstatement of place preference. Arodyn pretreatment suppressed stress-induced, but not cocaine-exposed, reinstatement of cocaine place preference. These results verify that arodyn and other kappa-opioid receptor antagonists may be useful therapeutics for cocaine abuse.

Keywords

Cocaine; Reward; Reinstatement; Arodyn; Kappa Opioid Antagonist

1. Introduction

Despite the dangers and complications of cocaine abuse and addiction, cocaine remains an illicit drug of abuse with nearly 2 million American cocaine users (National Institute on Drug Abuse (NIDA), 2005). Unfortunately, no Food and Drug Administration-approved medication is currently available for the treatment of cocaine abuse, even with our understanding of the neurobiological actions of cocaine. Cocaine is known to inhibit dopamine transporter activity, thus enhancing extracellular dopamine signaling (Heikkila et al., 1975). This cocaine-induced increase of synaptic dopamine levels in the A10 mesolimbic dopamine pathway is associated with the increased perception of cocaine's rewarding and addictive effects (Kuhar et al., 1991). From this, it is theorized that medications that suppress cocaine-induced increases in mesolimbic dopamine might prove to be effective treatments for cocaine abuse (Carroll et al., 1999).

Medications activating the kappa-opioid system reduce mesolimbic dopaminergic signaling (Spanagel et al., 1992), and may therefore prove useful in the treatment of cocaine abuse. However, the use of kappa-opioid receptor agonists can have therapeutic limitations. For example, while kappa-opioid receptor agonists given acutely are noted to suppress cocaine

*Corresponding author: Jay P. McLaughlin Northeastern University Department of Psychology 125 Nightingale Hall 360 Huntington Avenue Boston, MA, USA 02115 Tel: 617-373-2361 Fax: 617-373-8714 E-mail: j.mclaughlin@neu.edu

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self-administration (Kreek et al., 1987), recent reports demonstrate that repeated administration paradoxically potentiates cocaine reward in a manner prevented by kappa-opioid receptor antagonists (Negus et al., 2004; McLaughlin et al., 2006). These data suggest that the long-term maintenance of cocaine-abstinent patients may be facilitated by preventing the activation of kappa-opioid receptors with receptor-specific antagonists. Supporting this, kappa-opioid receptor antagonists were shown to prevent stress-induced potentiation of the rewarding effects of cocaine (McLaughlin et al., 2003). Moreover, the kappa-opioid receptor antagonist (3R)-7-hydroxy-N-{(1S)-1-[[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]}-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide (JDTic) was previously reported to suppress stress-induced reinstatement of cocaine self-administration, while cocaine-primed reinstatement of cocaine self-administration was unaffected (Beardsley et al., 2005). These findings suggest that kappa-opioid receptor antagonists may be valuable in the treatment of stress-induced relapse to drug-seeking behavior.

Arodyn, (Ac[Phe^(1,2,3),Arg⁽⁴⁾,D-Ala⁽⁸⁾]dynorphin A-(1–11) amide), is a novel peptide ligand based on the structure of dynorphin A (Bennett et al., 2002,2005), an endogenous ligand for the kappa opioid receptor. Arodyn was previously characterized as a potent, highly selective kappa-opioid receptor antagonist (Bennett et al., 2002). As such, we hypothesized that arodyn pretreatment would prevent stress-induced reinstatement of cocaine-conditioned place preference.

This hypothesis was tested after first confirming the *in vivo* activity and duration of kappa-opioid receptor antagonism induced by arodyn. The antagonizing effect of a single administration of arodyn on kappa-opioid receptor agonist-induced antinociception was tested in C57Bl/6J mice using the 55°C warm-water tail-withdrawal test. Once determined, the effect of arodyn pretreatment on mice exposed to stress or cocaine to induce reinstatement of cocaine-conditioned place preference was measured. Vehicle-pretreated mice demonstrated both stress- and cocaine-induced reinstatement of cocaine-conditioned place preference, whereas arodyn pretreatment prevented stress-, but not cocaine-induced, reinstatement. The results support the hypothesis that kappa-opioid receptor antagonists may prevent stress-induced reinstatement of cocaine reward, and suggest they may have therapeutic value in the treatment of relapse to psychostimulant abuse.

2. Materials and Methods

2.1 Subjects and compounds

Arodyn was synthesized as described previously (Bennett et al., 2002,2005). The kappa-opioid receptor agonist ($\pm$)-*trans*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U50,488) was provided by the NIDA Intramural Drug Program (Bethesda, Maryland, USA). All other compounds were from Sigma (St. Louis, Missouri, USA). Adult male C57Bl/6J mice weighing 19–27 grams were obtained from commercial vendors (Jackson Labs, Bar Harbor, Maine, USA), and were housed and cared for in accordance with the 1996 National Institute of Health Guide for the Care and Use of Laboratory Animals and as approved by the Institutional Animal Care Committee. C57Bl/6J mice were selected for this study because of their established responses to stress and cocaine place-conditioning (Szumlinski, et al, 2002;McLaughlin et al., 2003).

2.2 Antinociceptive testing and intracerebroventricular injection technique

The 55°C warm-water tail-withdrawal assay was used as described earlier (McLaughlin et al., 1999), with the latency of the mouse to withdraw its tail taken as an endpoint. After determining baseline tail-withdrawal latencies, mice received a single intracerebroventricular (i.c.v.) dose of vehicle (artificial cerebrospinal fluid, 146 mM NaCl, 2.7 mM KCl, 1.2 mM, CaCl₂, 1.0 mM

MgCl₂) or a graded dose of aroclor (0.3 or 1 nmol, i.c.v.) and the tail-withdrawal latency again tested 40 min later. For i.c.v. injections, each mouse was lightly anesthetized with isoflurane, an incision was made in the scalp, and the injection was made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm directly into the lateral ventricle, as detailed previously (McLaughlin et al., 1999). The volume of these injections was 5 µl, using a 10-µl Hamilton microliter syringe. The initial doses of aroclor examined were selected based on the previous *in vitro* characterization of aroclor (Bennett et al., 2002) and the *in vivo* activity of the parent compound, dynorphin A. Similar results were obtained for both doses of aroclor; further studies used the lower dose (0.3 nmol) of aroclor. Additional mice pretreated with aroclor were returned to their home cages and allowed to recover 80 min, 23.3 h, 71.3 h or 167.3 h to determine the duration of the kappa-opioid receptor antagonist effects produced by aroclor. After recovery, a single dose of the kappa-opioid receptor agonist, U50,488 (10 mg/kg, i.p.) was administered. The dose of U50,488 was selected based on previous demonstration of significant kappa-opioid mediated antinociception in C57Bl/6J mice (McLaughlin et al., 2006). Mice administered U50,488 were subsequently tested 40 min later for their tail-withdrawal latencies to determine the duration of kappa-opioid receptor antagonism produced by aroclor.

2.3 Cocaine-conditioned place preference, extinction and reinstatement

Conditioned place preference. C57Bl/6J mice were conditioned using a protocol similar to the previously established biased cocaine-conditioned place preference paradigm (Szumlini et al., 2002;McLaughlin et al., 2003 and 2006). The apparatus was a compartmentalized box divided into two equal-sized outer compartments (25 cm × 25 cm × 25 cm) with distinct cues, each joined to a small central section (8.5 cm × 25 cm × 25 cm) accessed through a single doorway (3 cm high). The entire unit was fitted with infrared beams, the breaking of which allows an automated measure of the time animals spend in each chamber (San Diego Instruments, San Diego, California, USA). The compartments differ in wall striping (vertical vs. horizontal alternating black and white lines, 1.5 cm in width) and floor texture (lightly mottled vs. smooth). Note that the biased place-conditioning protocol involves administration of cocaine to mice in the outer compartment opposite of their preference response in an initial preconditioning preference test. The biased conditioned place preference protocol produces a sensitive indicator of conditioned drug reward that is consistent across studies (Shimosato and Ohkuma, 2000;Szumlini et al., 2002,McLaughlin et al., 2003 and 2006), equivalent to alternative methods (e.g., the counterbalanced design; see Bardo et al., 1995 for a review). Moreover, the biased conditioned place preference design has the advantage of controlling for the individual animal's bias in the apparatus, allowing for the more efficient use of the animals available. It has also been demonstrated as an effective protocol for the study of extinction and reinstatement (Szumlini et al., 2002).

Time spent in each compartment was measured by allowing individual mice to move freely between all three compartments over a 30-min testing period. The apparatus is balanced, with animals on average demonstrating an equivalent amount of time in each of the three compartments (593±16 seconds on the left compartment, 595±18 seconds on the right compartment, and 612±18 seconds in the central compartment) that did not statistically differ (one-way ANOVA, $F_{(2,38)}=0.36$, $p = 0.70$). Place-conditioning began immediately following cocaine administration (10 mg/kg, s.c.), whereupon mice were consistently confined for 30 min in the appropriate outer compartment. A dose of 10 mg/kg s.c. cocaine was selected for this study as it has been shown previously to produce a reliable conditioned-place preference response in C57Bl/6J mice (Zhang et al., 2002;Kreibich and Blendy, 2004;Brabant et al., 2005). Conditioning with assay vehicle (0.9% saline, 0.3 ml/30 g body weight, s.c.) followed 4 h later in a similar manner, but paired to the opposite chamber. This conditioning cycle was repeated once on each of four days, which has been demonstrated to be effective in maintaining the place preference response for approximately 3 weeks (Brabant et al., 2005). Data are plotted

as the difference in time spent in the eventual cocaine- and vehicle-paired compartments, such that by convention the initial bias generates a negative value, and a positive value reflects a conditioned preference for the cocaine-paired side. Note that conditioned place aversion, where animals avoid the drug-paired compartment, is also detectable under this method when animals spend significantly more time in the initially preferred side. However, this was not demonstrated in this study under any conditions.

Extinction.—Place preference for the cocaine-paired compartment was re-examined weekly to confirm extinction. Placing animals repeatedly into the apparatus with free access to all compartments for 30 min produced extinction, defined as a statistically significant decrease in the time spent in the cocaine-paired compartment during the extinction trial as compared to the immediate postconditioning response. As expected for the C57Bl/6J strain of mice, conditioned place preference responses subsided with repeated testing over the three week period (Szumlinski et al., 2002; Kreibich and Blendy, 2004; Brabant et al., 2005).

Reinstatement.—Reinstatement of drug preference was examined after either exposure to forced swim stress (see section 2.4, below) or an additional cycle of cocaine place-conditioning. Note that a single cycle of cocaine place-conditioning was found to be insufficient to produce conditioned place preference alone in C57Bl/6J mice (Brabant et al., 2005). Mice were pretreated i.c.v. with vehicle or aroclor one hour prior to either cocaine conditioning or forced swimming on the first day. The day after completion of stress exposure or cocaine conditioning, animals were tested for place preference.

2.4 Forced swim stress

A two-day forced swim stress protocol was used as previously detailed (McLaughlin et al., 2003) to produce stress-induced reinstatement of cocaine-conditioned place preference. Mice were pretreated on the first day with vehicle or aroclor one hour prior to forced swimming. A one hour delay prior to exposure to forced swim stress was used both to be consistent with previous methodology (McLaughlin et al., 2003), and to ensure the animals recovered fully from the effects of anesthesia and the i.c.v. injection itself. Tail-withdrawal latencies were collected prior to pretreatment and again immediately after the conclusion of each day's swimming as described previously (McLaughlin et al., 2003) to assess the stress-induced activation of the endogenous kappa-opioid system and the effect of aroclor pretreatment as a kappa-opioid receptor antagonist. One hour after the final exposure to forced swim stress, the place preference responses of mice were tested as described above to determine possible reinstatement of extinct conditioned place preference.

2.5 Statistical analysis

Student's t-tests comparing baseline and post-treatment tail-withdrawal latencies were used to determine statistical significance for all tail-withdrawal data. Data for conditioned place preference experiments were analyzed with 2-way ANOVA using the SPSS 14.0 statistical package (Chicago, Illinois, USA). Analyses examined the main effect of conditioned place preference phase (e.g., post-conditioning, week of preference test, reinstatement) and the interaction of drug pretreatment (arodyn or vehicle) X reinstatement condition (stress or cocaine exposure). Significant effects were further analyzed using Fisher's LSD post hoc testing. All data are presented as mean $\pm$ S.E.M., with significance set at $P < 0.05$.

3. Results

3.1 Pretreatment with the kappa-opioid receptor antagonist arodyn prevents U50,488-induced antinociception for at least 3 days.

Previous studies utilized *in vitro* cellular assays to demonstrate the ability of arodyn to act as a kappa-opioid receptor antagonist (Bennett et al., 2002). We confirmed the *in vivo* kappa-opioid receptor antagonist effects of arodyn in C57Bl/6J mice using the 55°C warm-water tail-withdrawal test. Initial tests confirmed that arodyn lacked antinociceptive effect, as expected of a kappa-opioid receptor antagonist. As expected, intraperitoneal administration of the kappa-opioid receptor agonist U50,488 (10 mg/kg) produced significant antinociception 40 min after administration (12.1 ± 1.76 sec, $P < 0.05$), whereas intracerebroventricular pretreatment for 40 min with arodyn alone (1 nmol) did not significantly change the baseline tail-withdrawal latency (1.24 ± 0.05 sec baseline latency versus 1.60 ± 0.25 sec latency after arodyn, $P > 0.05$). However, consistent with the previous characterization of arodyn (Bennett et al., 2002), intracerebroventricular pretreatment with arodyn (0.3 or 1 nmol) 2 h prior to testing significantly antagonized the antinociceptive effect of U50,488 (1.52 ± 0.13 and 2.36 ± 0.69 sec, respectively, both $P < 0.05$ as compared to U50,488 alone).

A number of kappa-opioid receptor-selective antagonists, such as norbinaltorphimine, demonstrate a prolonged duration of action (Horan et al., 1992). We next determined the duration of kappa-opioid receptor antagonism produced by a single dose of arodyn. Mice were pretreated through the intracerebroventricular route with vehicle (artificial cerebrospinal fluid; Fig.1, circles) or arodyn (0.3 nmol; Fig.1, triangles) 80 min to 167.3 (7 days) in advance of an intraperitoneal administration of U50,488 (10 mg/kg), and antinociception measured in the 55°C warm-water tail-withdrawal test. Mice that were administered artificial cerebrospinal fluid prior to U50,488 showed significant increases in tail-withdrawal latencies each day of testing (Fig.1). In contrast, arodyn pretreatment antagonized U50,488-induced antinociception for at least 3 days, but less than 7 days. These findings demonstrate a long duration of kappa-opioid receptor antagonism produced by arodyn, analogous to established kappa-opioid receptor antagonists (Horan et al., 1992; Carroll et al., 2004).

3.2 Pretreatment with the kappa-opioid receptor antagonist arodyn prevents stress-induced analgesia and reinstatement of cocaine-conditioned place preference.

The kappa-opioid receptor antagonist JD1c was previously reported to suppress stress-induced reinstatement of cocaine self-administration (Beardsley et al., 2005). Since the peptide arodyn acts as a kappa-opioid receptor antagonist, it would be expected to also prevent stress-induced reinstatement of cocaine seeking behavior. To examine this hypothesis, C57Bl/6J mice were first place conditioned over four days with cocaine. Mice demonstrated a cocaine-conditioned place preference that was significantly greater than that of the initial preference response (Fig. 2, left bars; $F_{(4,176)} = 10.2$, $P < 0.01$). This place preference lasted over 2 weeks (Fig. 2, center left bar). After 3 weeks, mice demonstrated extinction with a place preference response that was statistically less than the initial postconditioning preference (Fig. 2, thatched left bar, $P < 0.05$).

Upon demonstration of extinction of cocaine-conditioned place preference, mice were administered vehicle (artificial cerebrospinal fluid) or the peptide kappa-opioid receptor antagonist arodyn (0.3 nmol, i.c.v.), and exposed to repeated forced swim stress. Exposure to forced swimming resulted in a stress-induced analgesia in vehicle-pretreated mice, measured as a significant increase in tail-withdrawal latencies over pre-stress baseline values (Day 1, 1.29 ± 0.1 vs. 2.32 ± 0.25 sec post stress, $P < 0.01$; Day 2, 1.22 ± 0.08 vs. 3.43 ± 0.48 sec post stress, $P < 0.01$). In contrast, a single arodyn pretreatment prevented the stress-induced increase in tail-withdrawal latency (Day 1, 1.28 ± 0.11 vs. 1.53 ± 0.16 sec post stress, $P = 0.18$; Day 2, 1.49 ± 0.18

vs. 1.66 ± 0.24 sec post stress, $P=0.58$). These results demonstrate that arodyn pretreatment prevented stress-induced analgesia through antagonism of the kappa-opioid receptor, similar to previous findings (McLaughlin et al., 2003;2006).

Following the exposure to stress, mice were tested for place preference to examine possible reinstatement of drug seeking behavior. Importantly, stress-exposed mice pretreated with vehicle subsequently demonstrated reinstatement of conditioned place preference (Fig. 2, striped center bar, $F_{(3,176)} = 7.27$, $P < 0.01$). In contrast, arodyn pretreatment prevented stress-induced reinstatement, with mice demonstrating place preference responses that did not differ significantly from preconditioning responses (Fig. 2, white center bar, $P=0.47$). Alternately, mice demonstrating extinction of place preference were subsequently exposed to a single round of cocaine conditioning prior to place preference testing. Cocaine-exposed mice pretreated with vehicle exhibited a reinstatement of place preference (Fig. 2, thatched right bar, $P < 0.05$). Notably, arodyn pretreatment had no effect on cocaine-induced reinstatement of place preference. Mice treated with arodyn before exposure to an additional cocaine conditioning cycle showed a significantly greater preference for the cocaine-paired compartment as compared to pre-conditioning preference (Fig. 2, rightmost bar, $P < 0.05$). Furthermore, the reinstated preference of arodyn pretreated mice was not significantly different than the response of vehicle pretreated mice (Fig. 2, rightmost bars, $P=0.37$). Overall, these results confirm a mediating role for the endogenous kappa-opioid system in stress-induced relapse of drug seeking behavior, as pretreatment with the novel peptide kappa-opioid receptor antagonist arodyn prevented the stress-induced reinstatement.

4. Discussion

The present study demonstrated that the peptide kappa-opioid receptor antagonist arodyn selectively blocked stress-induced, but not cocaine-exposed, reinstatement of cocaine-conditioned place preference. These results support the hypothesis that kappa-opioid receptor antagonists may prevent reinstatement of cocaine seeking behavior, and suggest that they may have therapeutic value in the treatment of relapse to psychostimulant abuse.

Stress is known to potentiate the rewarding properties of drugs of abuse (Piazza et al, 1990) and is a major contributor to the reinstatement of drug-seeking behavior in abstinent subjects (Shaham et al., 2000). This increase in drug seeking behavior may be due in part to the activity of the endogenous kappa-opioid system. Exposure to stress was demonstrated to increase levels of the endogenous kappa-opioid receptor agonist dynorphin in C57Bl/6J mouse brains (Shirayama et al., 2004). Moreover, mice exposed to forced swim stress before cocaine place-conditioning demonstrated a two-fold increase in cocaine place preference that was blocked by kappa-opioid receptor gene deletion or antagonism with nor-binaltorphimine (McLaughlin et al., 2003 and 2006). From this, it has been posited that kappa-opioid receptor antagonists may prevent stress-induced relapse. Supporting this, the kappa-opioid receptor antagonist (-)- $\alpha 5,9$ -diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan (MR-2266) prevented psychological stress-induced analgesia (Takahashi et al., 1990). Furthermore, the novel kappa-opioid receptor antagonist JD1c dose-dependently suppressed stress-induced, but not cocaine-primed, reinstatement of cocaine self-administration (Beardsley et al., 2005). The present data are consistent with these reports, and verify that exposure to stress activates the kappa-opioid receptor through release of endogenous dynorphin peptides.

Exogenous receptor agonist activation of the kappa-opioid receptor tonically inhibits dopamine signaling (Spanagel et al., 1992), and acutely suppresses the rewarding effects of cocaine (Mello and Negus, 2000;McLaughlin et al., 2006). However, repeated kappa-opioid receptor agonist treatment paradoxically enhanced dopaminergic signaling (Thompson et al., 2000), and U50,488 administration potentiated cocaine-conditioned place preference in a time-

dependent, norbinaltorphimine-sensitive manner (McLaughlin et al., 2006). As such, it would appear that the kappa-opioid receptor agonists might be better suited as acute therapeutic interventions to suppress cocaine reward and craving, whereas kappa-opioid receptor antagonists may be useful in preventing the relapse to cocaine-seeking behavior.

Peptide ligands may be advantageous as therapeutic agents because of their high activity, high specificity and minimization of drug-drug interactions (Marx, 2005). Perhaps surprisingly, little has been done with either endogenous or synthetic peptide kappa-opioid receptor antagonists, although research has illustrated the utility of peptide mu-opioid receptor antagonists. For example, unlike naltrexone and naloxone, the highly selective mu-opioid receptor antagonist CTAP (Corbett et al., 1993) produced minimal morphine withdrawal behaviors (Bilsky et al., 1996), and also dose-dependently blocked reinstatement of cocaine-self administration (Tang et al., 2005). These studies support the possible utility of highly selective peptide opioid receptor antagonists in treatment of drug withdrawal and relapse, and suggest the value of developing additional peptide kappa-opioid receptor antagonists. While the present study has utilized i.c.v. administration of arodyn, a number of studies have shown that dynorphin A analogs can cross the blood-brain barrier and remain active after systemic administration (Yu et al., 1997; Butelman et al., 1999; Hiramatsu et al., 2001; Brugos et al., 2004). In recent work, arodyn has been shown to cross a model of the blood-brain barrier (Chappa, Fang, Aldrich and Lunte, manuscript in preparation). Ongoing research is focusing on identifying analogs with enhanced pharmacokinetic properties for systemic administration.

The present study demonstrated that a single administration of arodyn prevented both U50,488- or stress-induced antinociception for at least 3 days. This long duration of arodyn-mediated kappa-opioid receptor antagonism *in vivo* is perhaps surprising. Arodyn is a peptide based on the structure of dynorphin A(1–11), which is known to have a short half-life of activity *in vivo* (Young et al., 1987). However, other kappa-opioid receptor antagonists such as norbinaltorphimine (Horan et al., 1992) and JD1c (Carroll et al., 2004) also produce persistent antagonism, consistent with the present results.

In summary, the novel peptide kappa-opioid receptor antagonist arodyn produced a long-lasting antagonism of U50,488-induced antinociception and suppressed stress-induced reinstatement of cocaine seeking behavior. These data suggest novel peptide kappa-opioid antagonists may be of therapeutic value for the treatment of relapse to cocaine abuse.

Acknowledgements

We thank Weijie Fang for the synthesis of the arodyn used in this study. This research was supported by NIDA R01 DA018832 (to JVA) and NIDA R03 DA016415 and a Northeastern University Provost's RSDF (to JPM).

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Figure 1. Aroclor antagonism of U50,488-induced antinociception lasted for at least 3 days in the 55°C warm-water tail-withdrawal assay.

Baseline tail-withdrawal responses were characterized for all mice (points left of the dashed line). Mice were then administered i.c.v. vehicle (artificial cerebrospinal fluid, circles) or aroclor (0.3 nmol, triangles), allowed to recover from 1.3 hours to 7 days, and administered a single dose of the kappa-opioid receptor agonist U50,488 (10 mg/kg). Tail-withdrawal latency was measured 40 min after receptor agonist administration. (Points each represent n=8 mice. *=significantly different from baseline tail-withdrawal response (points left of dashed bar); †=significantly different from U50,488-induced tail-withdrawal latency after vehicle-pretreatment, $P < 0.05$; Student's T-test.)

Figure 2. Aroclor prevented stress-induced reinstatement of cocaine-conditioned place preference. After 4 days of cocaine (10 mg/kg s.c. daily), mice exhibited significant preference for the cocaine paired environment, with extinction occurring by 3 weeks (left bars). Mice were exposed to forced swim stress (center bars) or an additional round of cocaine place-conditioning (right bars), reinstating place preference. Aroclor pretreatment (0.3 nmol, open bars center and right) prevented stress-, but not cocaine-induced reinstatement of place preference. (Bars represent $n=6-40$ mice. *=Significantly different from preconditioning place preference response (leftmost bar); †=significantly different from postconditioning place preference response (second bar on left); ‡=significantly different from stress-induced reinstatement of place preference response (striped bar, center), Fisher's LSD post hoc test.)