Zyklophin, a systemically active selective kappa opioid receptor peptide antagonist with short duration of action

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The cyclic peptide zyklophin {[*N*-benzylTyr^1,*cyclo*(D-Asp^5,Dap^8)-dynorphin A-(1–11)NH_2, Patkar KA, et al. (2005) *J Med Chem* 48: 4500–4503} is a selective peptide kappa opioid receptor (KOR) antagonist that shows activity following systemic administration. Systemic (1–3 mg/kg s.c.) as well as central (0.3–3 nmol intracerebroventricular, i.c.v.) administration of this peptide dose-dependently antagonizes the antinociception induced by the selective KOR agonist U50,488 in C57BL/6J mice tested in the 55 °C warm water tail withdrawal assay. Zyklophin administration had no effect on morphine- or SNC-80-mediated antinociception, suggesting that zyklophin selectively antagonizes KOR in vivo. Additionally, the antagonism of antinociception induced by centrally (i.c.v.) administered U50,488 following peripheral administration of zyklophin strongly suggests that the peptide crosses the blood-brain barrier to antagonize KOR in the CNS. Most importantly, the antagonist activity of zyklophin (3 mg/kg s.c.) lasts less than 12 h, which contrasts sharply with the exceptionally long duration of antagonism reported for the established small-molecule selective KOR antagonists such as norbinaltorphimine (nor-BNI) that last weeks after a single administration. Systemically administered zyklophin (3 mg/kg s.c.) also prevented stress-induced reinstatement of cocaine-seeking behavior in a conditioned place preference assay. In conclusion, the peptide zyklophin is a KOR-selective antagonist that exhibits the desired shorter duration of action, and represents a significant advance in the development of KOR-selective antagonists.

cocaine abuse | dynorphin A analog | kappa opioid receptor antagonist | opioid peptide | stress

Interest in kappa opioid receptor (KOR) ligands has focused on agonists that have potential therapeutic applications, such as anti-inflammatory activity (1) and the acute suppression of the rewarding effects of cocaine (2) [see (3) for a review], in addition to their analgesic properties. Until recently, KOR-selective antagonists have only been used as pharmacological tools. However, recent reports describing antidepressant-like effects (4–6), anxiolytic-like effects (7, 8), efficacy against opiate dependence (9), and the ability to prevent stress-induced reinstatement of cocaine-seeking behavior (5, 10) indicate that KOR antagonists could be promising therapeutic agents [see (3) for a review].

Several selective non-peptide antagonists, in particular norbinaltorphimine (nor-BNI) (11), 5′-guanidinonaltrindole (GNTI) (12), and the phenylpiperidine JDTic (13), have been studied extensively in animal models (4–8, 14–16). All of these compounds, however, exhibit exceptionally long activity in vivo, lasting weeks to more than a month after a single dose [see (14) for a detailed review], an effect that could limit their therapeutic use. While the mechanism producing this prolonged activity is poorly understood, to date there have been no reports of small molecule-selective KOR antagonists with activity lasting less than 1 day.

Peptide ligands are metabolized by proteases, and therefore peptide KOR ligands are expected to exhibit shorter durations of action than the non-peptide KOR-selective antagonists, thereby avoiding the prolonged activity associated with the latter compounds. This prompted us to examine peptide KOR antagonists in vivo. Previous in vivo studies with the selective peptide KOR antagonist arodyn ([AcPhe^{1,2,3},Arg^4,D-Ala^8]dynorphin A-(1–11) amide) (17) developed in our laboratory demonstrated that a peptide KOR antagonist not only antagonized the KOR-selective agonist U50,488 in vivo, but also prevented stress-induced reinstatement of cocaine-induced conditioned place preference (CPP) (10). These findings are consistent with the results produced by the nonpeptide KOR antagonist JDTic (5), but as expected, the peptide arodyn exhibits a substantially shorter duration of action than the non-peptide antagonists nor-BNI and JDTic. However, arodyn is subject to rapid metabolism by proteases in blood (18), and therefore was administered centrally [by intracerebroventricular (i.c.v.) injection] to be effective in these studies.

Kappa opioid peptide ligands designed to be to metabolically stable have been shown to penetrate the CNS, and may prove to be useful therapeutic agents. Earlier studies demonstrated that E2078 {[NMeTyr^1,NMeArg^7,D-Leu^8]dynorphin A-(1–8) N-ethyl amide}, an analog of the endogenous opioid peptide dynorphin A (Dyn A) stabilized to slow metabolic degradation, can cross the blood-brain barrier (BBB) (19, 20) and produces analgesia in humans following systemic (intramuscular) administration (21). In more recent preliminary studies, we found that several Dyn A-(1–11) amide analogs could cross a model of the BBB (18). Therefore, we expected that a peptide KOR antagonist derived from dynorphin A and stabilized to metabolism would exhibit activity in the CNS after systemic administration.

Based on metabolism studies of arodyn (18) we anticipated that a Dyn A derivative cyclized in the middle of the sequence would exhibit substantially improved metabolic stability and remain active for a significant but finite time (i.e., for hours, not days) after systemic administration. We had reported that the cyclic peptide [*N*-benzylTyr^1,*cyclo*(D-Asp^5,Dap^8)]Dyn A-(1–11) amide (now named zyklophin) exhibits reasonable affinity and high selectivity for KOR [K_i (KOR) = 30 nM and K_i ratios (KOR/MOR/DOR) = 1/194/>330 in radioligand binding assays], and nanomolar antagonist potency against KOR in vitro (22). Previously, modifications to Dyn A that have resulted in antagonist activity have been located in the N-terminal tetrapeptide "message" sequence. Zyklophin, however, is a Dyn A-based antagonist with modifications in the C-terminal "address" domain that alter efficacy. In initial testing, zyklophin antagonized KOR in vivo (18). These promising preliminary results prompted us to undertake additional studies of this peptide.

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Conflict of interest statement: The authors have filed a U.S. patent application on using zyklophin entitled "Method for Treating and/or Preventing Drug Seeking Behavior."

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Here we report the detailed in vivo characterization of zyklophin, including evaluation of the dose dependency following both central and peripheral administration, the duration, and KOR selectivity of zyklophin-mediated antagonism. We also examined the ability of this peptide to prevent stress-induced reinstatement of cocaine-seeking behavior to evaluate the potential therapeutic application of this lead compound in the treatment of cocaine abuse. Most importantly, we found that this peptide is not only centrally active following systemic (s.c.) administration in vivo, but that the duration of zyklophin-mediated antagonist activity is less than 12 h. Thus, zyklophin is a selective KOR antagonist that exhibits the desired finite duration of action and represents a significant advance in the development of selective KOR antagonists.

Results

Antagonist Activity of Zyklophin in Analgesic Assays. The in vivo effects of zyklophin on opioid-mediated antinociception were examined in C57BL/6J mice using the 55 °C warm-water tail-withdrawal test. Initial tests demonstrated that zyklophin lacks antinociceptive effects after either central or peripheral administration. Intracerebroventricular administration of zyklophin (3 nmol) did not significantly change the tail-withdrawal latency from baseline up to 60 min later (1.84 ± 0.41 s latency versus 1.42 ± 0.14 s baseline latency, $P = 0.11$, n.s.). Likewise, s.c. administration of zyklophin (3 mg/kg) did not alter the tail-withdrawal response (1.40 ± 0.08 s latency after the peptide, $P = 0.87$, n.s.), similar to the results of s.c. administration of vehicle alone (1.58 ± 0.18 s latency versus 1.53 ± 0.16 s baseline latency, $P = 0.85$, n.s.). In contrast, administration of the KOR agonist U50,488 (10 mg/kg i.p.) produced significant antinociception (Fig. 1). Intracerebroventricular pretreatment with zyklophin (0.3, 1, or 3 nmol) 1 h before testing significantly antagonized the antinociceptive effect of the KOR-selective agonist U50,488 (Fig. 1*A*). Moreover, peripheral pretreatment with zyklophin (1 or 3 mg/kg s.c.) 1 h before testing also significantly antagonized the antinociceptive effect of U50,488 (Fig. 1*B*). Importantly, peripheral administration of zyklophin (3 mg/kg s.c.) 1 h before testing also antagonized the antinociceptive effect of U50,488 administered centrally (40 nmol i.c.v. 20 min before testing, Fig. 1*C*), which is strong evidence that the peptide antagonist crossed the BBB in vivo to act on KOR in the brain.

The duration of KOR antagonism produced by a single s.c. dose of zyklophin was then determined. Mice were pretreated with zyklophin (3 mg/kg) 20 min to 23.3 h before administration of U50,488 (10 mg/kg i.p.), and antinociception measured in the 55 °C warm-water tail-withdrawal test (Fig. 2). Zyklophin pretreatment significantly antagonized U50,488-induced antinociception for at least 8 h, but for less than 12 h. In contrast, as reported previously [see, for example, (23)], mice pretreated with a single dose of the prototypical KOR antagonist nor-BNI showed long lasting antagonism of KOR agonist-induced antinociception; mice pretreated 7 days prior with nor-BNI (10 mg/kg i.p.) still demonstrated significant antagonism of U50,488-induced antinociception (13.2 ± 5.58% antinociception, $P = 0.005$, compared to 3.16 ± 1.31% when administered 2 h before testing). These findings demonstrate a reversible, relatively short duration of KOR antagonism produced by zyklophin, unlike established KOR-selective non-peptide antagonists that exhibit exceptionally long activity after a single dose (14).

The receptor selectivity of zyklophin was determined in the mouse 55 °C warm-water tail-withdrawal assay using selective opioid receptor agonists. Pretreatment of mice with zyklophin blocked the antinociceptive effect of U50,488, but not of morphine, which preferentially activates MOR, or the DOR-selective agonist SNC-80 (Fig. 3). These results demonstrate zyklophin-mediated antagonism in vivo is selective for KOR.

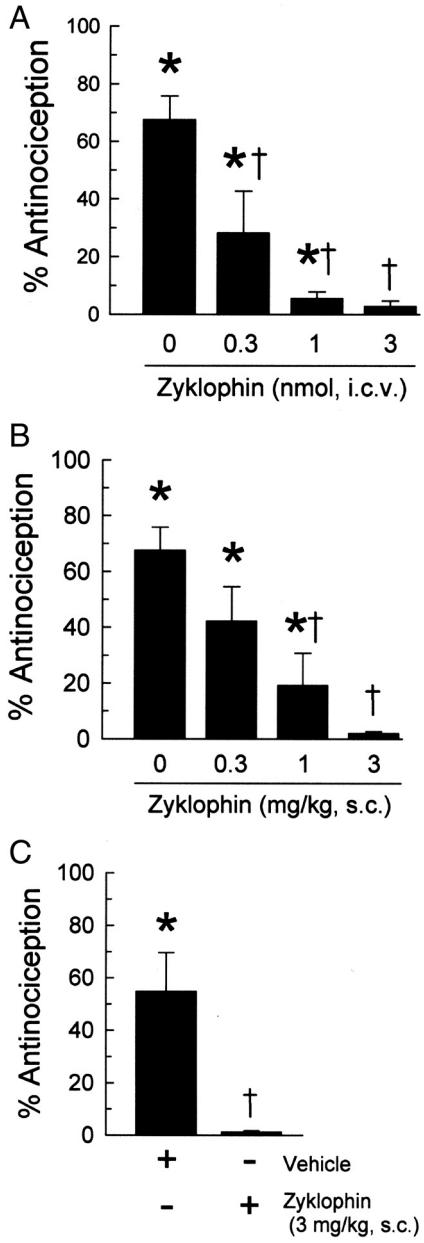

Fig. 1. Zyklophin crosses the blood brain barrier to antagonize U50,488-induced antinociception in the mouse 55 °C warm-water tail-withdrawal test. The antinociceptive effects of U50,488 (10 mg/kg i.p.) were determined 40 min after administration in mice pretreated 1 h with vehicle or zyklophin through the (*A*) i.c.v. (0.3–3 nmol) or (*B*) s.c. route (0.3–3 mg/kg) of administration. (*C*) The antinociceptive effect of centrally administered U50,488 (40 nmol i.c.v.) was also antagonized in mice peripherally pretreated 60 min with zyklophin (3 mg/kg s.c.). Tail-withdrawal latencies in *C* were measured 20 min after injection of U50,488. Plus and minus signs under bars denote whether the listed agent was administered or not. Data are presented as the mean percent antinociception ± SEM from 6–8 mice. *, significantly different from baseline tail-withdrawal latency; †, significantly different from U50,488-induced antinociception after vehicle pretreatment, $P < 0.05$; Student's *t*-test.

Activity of Zyklophin in a CPP Model of Stress-Induced Reinstatement of Cocaine-Seeking Behavior. We previously demonstrated that the peptide KOR antagonist arodyn can suppress stress-induced reinstatement of cocaine-seeking behavior (10). Therefore, we expected that the KOR antagonist zyklophin would similarly prevent this stress-induced reinstatement. To test this hypothesis, C57BL/6J mice were first place-conditioned over 4 days with

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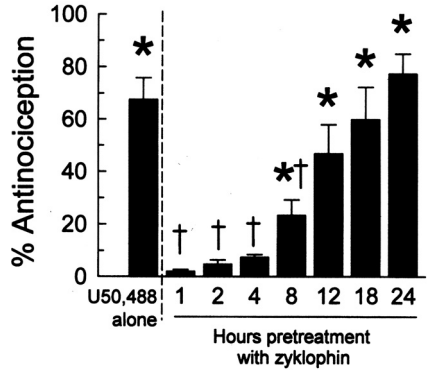

Fig. 2. Time course of zyklophin-mediated antagonism of U50,488-induced antinociception in the mouse 55 °C warm-water tail-withdrawal test. Antinociceptive effect of U50,488 (10 mg/kg i.p.) in mice pretreated for 1–24 h with zyklophin (3 mg/kg s.c.). Tail withdrawal latencies were determined 40 min after agonist administration. Data are presented as the mean percent antinociception ± SEM from 8–12 mice. *, significantly different from the baseline tail-withdrawal latency; †, significantly different from U50,488-induced antinociception without pretreatment, $P < 0.05$; Student's *t*-test.

cocaine (see Fig. 4*A*). Mice demonstrated a cocaine-conditioned place preference that was significantly greater than that of the initial preference [Fig. 4*B*, left bars; $F_{(3, 320)} = 45.5$, $P < 0.0001$; one-way ANOVA with Tukey HSD post-hoc test]. This place preference lasted more than 2 weeks (Fig. 4*B*, dark gray bar). After 3 weeks, mice demonstrated extinction with a place preference response similar to the initial preference response and which was statistically significantly less than the initial preference immediately after place-conditioning (Fig. 4*B*, light gray bar, $P < 0.01$).

Following extinction of cocaine CPP, mice were administered vehicle (0.9% saline) or zyklophin (1 or 3 mg/kg s.c.) daily for 2 days (see Fig. 4*A*) and exposed 20 min later to repeated forced swim stress. The day following the completion of exposure to stress (i.e., day 30, see Fig. 4*A*), mice were tested for place preference to examine possible reinstatement of drug-seeking behavior. As expected, stress-exposed vehicle-pretreated mice subsequently demonstrated reinstatement of CPP [Fig. 4*B*, striped center-left bar, $F_{(6, 284)} = 22.8$, $P < 0.001$; one-way ANOVA with Tukey HSD post-hoc test]. Importantly, zyklophin pretreatment prevented stress-induced reinstatement. While pretreatment with 1 mg/kg s.c. zyklophin did not prevent stress-induced reinstatement of cocaine-conditioned place preference (Fig. 4*B*, fine-thatched center bar, $P = 0.99$ as compared to vehicle-treated stress-induced control animals), mice pretreated daily with 3 mg/kg s.c. zyklophin before exposure to forced swimming demonstrated place preference responses that did not differ significantly from preconditioning or extinction responses (Fig. 4*B*, coarsely-thatched center-right bar, $P = 0.73$ and 0.70, respectively). However, treatment with zyklophin

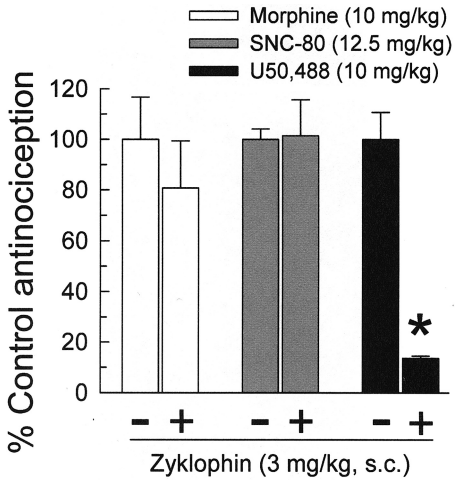

Fig. 3. Kappa-opioid receptor-selective antagonism by zyklophin. Antinociceptive effects of morphine (10 mg/kg i.p., left pair of white bars) or SNC-80 (12.5 mg/kg i.p., center pair of gray bars) were not reduced by a 1 h pretreatment with zyklophin (3 mg/kg s.c.), whereas the effect of U50,488 (10 mg/kg i.p., right pair of black bars) was significantly antagonized. Tail-withdrawal latencies were measured in the mouse 55 °C warm-water tail-withdrawal test 40 min after selective agonist administration. Data are presented as the mean percent antinociception ± SEM of the control animals treated only with the matching agonist (100%). n = 8–10 mice. *, Significantly different from matching agonist effect, $P < 0.01$; Student's *t*-test.

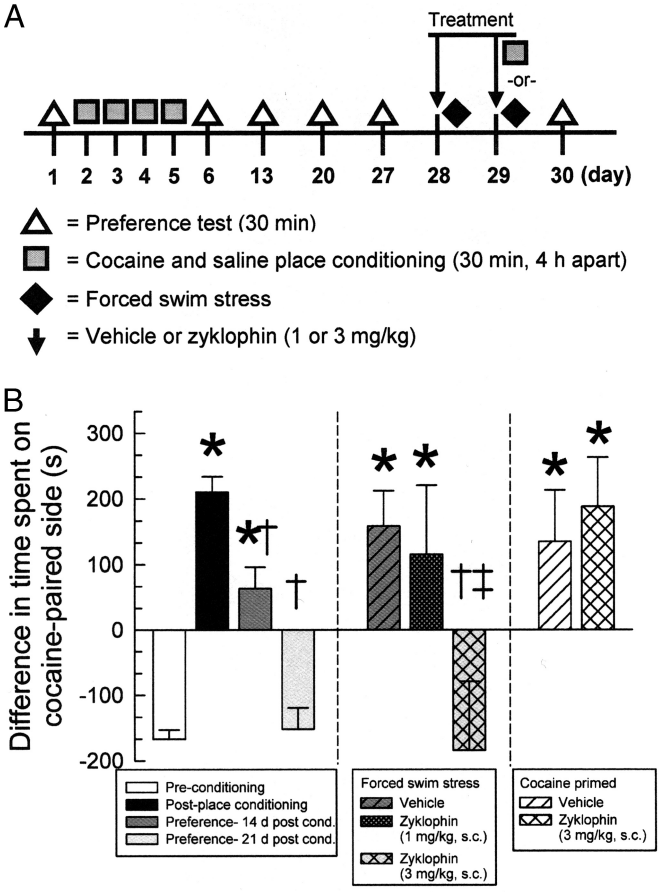

Fig. 4. Stress-induced reinstatement of cocaine CPP prevented by zyklophin pretreatment. (*A*) Schematic of reinstatement and testing protocol. Vehicle (0.9% saline) or zyklophin were administered on days 28 and 29, 20 min before initial exposure to forced swim stress (diamonds) or cocaine place-conditioning (square, day 29). (*B*) 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. Zyklophin pretreatment (3, but not 1, mg/kg s.c.) prevented stress-induced reinstatement of place preference (center bars). In contrast, zyklophin pretreatment (3 mg/kg s.c.) was ineffective at preventing cocaine-induced reinstatement of CPP (rightmost bar). n = 8–17 mice; cocaine place-conditioning data on left represents combined responses of 81 mice. *, Significantly different from preconditioning place preference response (leftmost bar); †, significantly different from post-conditioning place preference response (second bar on left); ‡, significantly different from stress-induced reinstatement of place preference response (striped gray bar, center), Fisher's LSD post-hoc test.

(3 mg/kg s.c.) daily for 2 days immediately after exposure to forced swimming did not prevent stress-induced reinstatement (118 ± 187 s; $P = 0.79$, n.s., as compared to vehicle-treated, stress-exposed response).

In addition, mice demonstrating extinction of CPP were subsequently exposed instead to a single cycle of cocaine conditioning before place preference testing (see Fig. 4*A*). Cocaine-exposed mice pretreated with vehicle exhibited reinstatement of place preference [Fig. 4*B*, striped white right bar, $F_{(4, 262)} = 40.7$, $P < 0.01$; one-way ANOVA with Tukey HSD post-hoc test]. Mice treated daily for 2 days with zyklophin before exposure to this additional cocaine conditioning cycle also showed a significantly greater preference for the cocaine-paired compartment as compared to preconditioning and extinction preferences (Fig. 4*B*, rightmost bar, $P < 0.01$). Furthermore, the reinstated preference of zyklophin-pretreated mice was not significantly different from the response of vehicle pretreated mice (Fig. 4*B*, rightmost bars, $P = 0.63$, n.s.). Thus zyklophin pretreatment had no effect on cocaine-induced reinstatement of place preference. Overall, these results confirm a mediating role for the endogenous KOR system in stress-induced relapse of drug-seeking behavior, as pretreatment with the peptide KOR antagonist zyklophin prevented the stress-induced reinstatement.

Discussion

While non-peptide selective KOR antagonists have been studied extensively in vivo, until recent research in our laboratories (10) the evaluation of selective peptide KOR antagonists has been limited to in vitro testing. A major factor limiting in vivo studies has been the ability of the peptides to reach KOR in the CNS, which requires the ligand possess metabolic stability and the ability to cross the BBB. While the rapid metabolism of the peptide KOR antagonist arodyn precluded systemic administration, our initial studies of this peptide (10) prompted us to pursue peptides that we anticipated would be more metabolically stable, with the goal of identifying systemically active selective peptide KOR antagonists.

The present results from the 55 °C warm-water tail-withdrawal assay demonstrate that zyklophin is a systemically active, KOR selective antagonist in vivo. Whereas nor-BNI has been reported to initially exhibit modest agonist activity (24), zyklophin does not exhibit any agonist activity in the warm-water tail-withdrawal assay following either central or peripheral administration. Zyklophin administered either centrally (i.c.v., Fig. 1*A*) or systemically (s.c., Fig. 1*B*) dose-dependently antagonized the antinociception induced by the KOR-selective agonist U50,488. Because KOR agonists can produce antinociceptive effects through the activation of peripheral KOR (25) as well as through receptors in the CNS, it was important to establish whether the observed antagonism by peripherally administered zyklophin was due primarily to interaction with peripheral receptors. However, the antagonism of centrally administered U50,488 by peripherally administered zyklophin (Fig. 1*C*) strongly suggests that the peptide crosses the BBB to antagonize KOR in the CNS. The antagonism of U50,488, but not morphine or SNC-80-mediated antinociception, further demonstrates that zyklophin is a selective antagonist in vivo, consistent with the KOR selectivity demonstrated in binding assays (22).

One of the most important findings in this study was the duration of the antagonist activity of zyklophin (Fig. 2). In contrast to the non-peptide selective KOR antagonists such as nor-BNI, which exhibit exceptionally long duration of action (up to weeks after a single dose) (14), the antagonist activity of zyklophin lasts less than 12 h after systemic (s.c.) administration. The prolonged activity of the non-peptide KOR-selective antagonists could complicate their use as pharmacological tools and as potential therapeutic agents. In contrast, the time course of zyklophin-mediated KOR antagonism represents a useful duration of action for many in vivo studies, and a more typical time course for therapeutic agents.

The activity of systemically administered zyklophin in preventing stress-induced reinstatement of cocaine-seeking behavior is another strong indication that this peptide crosses the BBB to reach KOR in the CNS. It is thought that increases in extracellular synaptic dopamine levels in the A10 mesolimbic dopamine pathway of the brain are associated with the increased perception of reward and the addictive effects of psychostimulants including cocaine (26–28). KOR and their endogenous ligands the dynorphins are colocalized with this reward pathway, and acute activation of KOR is well known to result in tonic inhibition of dopamine signaling [see (29) for a review]. Importantly, the endogenous kappa opioid system has also been found to play a key role in the responses to stress. Stress itself potentiates the rewarding properties of drugs of abuse (30, 31), and contributes to the likelihood of reinstatement of drug-seeking behavior in abstinent subjects (32). Exposure to stress has been reported to modulate dynorphin levels (33, 34), and repeated exposure to stress potentiates cocaine-seeking behavior through a mechanism that requires KOR activation (35, 36). Consistent with the central location of the KOR involved in this response, central administration of the peptide KOR antagonist arodyn suppressed reinstatement of stress-induced cocaine-seeking behavior in the CPP assay (10). Since the reward pathways and the KOR involved in the response to stress discussed above are restricted to the brain, the present findings that zyklophin prevents stress-induced potentiation of cocaine CPP following peripheral administration provides further evidence that zyklophin crosses the BBB to antagonize KOR in the CNS. Notably, zyklophin did not prevent reinstatement when administered after exposure to stress. These results suggest that it is the prophylactic blockade of the stress-induced activation of the endogenous KOR system that prevented stress-induced reinstatement of cocaine CPP.

The systemic activity of zyklophin not only facilitates its use as a pharmacological tool, but suggests an important development in the search for potential therapeutic agents. Peptides with appropriate pharmacokinetic properties have been used successfully as therapeutic agents [e.g., the LH-RH analog leuprolide and the HIV fusion inhibitor enfuvirtide (Fuzeon™)] and can have some advantages as drugs, including high activity, high specificity, low toxicity, and the minimization of drug-drug interactions (37). While the delivery of peptides as therapeutic agents remains a challenge, the development of alternatives to injection for systemic administration will facilitate their clinical use. The recent demonstration of the activity of Dyn A-(1–13) analogs following inhalation (38) shows that other routes of administration can be used for opioid peptides that could increase their acceptance as therapeutic agents.

In conclusion, zyklophin represents a major advance in the selective KOR antagonist area. Its relatively short duration of action makes it an ideal pharmacological tool for a variety of in vivo studies. Its systemic activity makes it a promising lead compound for further development, with possible future therapeutic value. There is currently no medication approved by the FDA for the treatment of cocaine abuse or for the prevention of relapse to cocaine use in former cocaine addicts or abusers. The findings reported here add to previous evidence (5, 10) that suggest that KOR-selective antagonists may serve as valuable therapeutics in the prevention of stress-induced reinstatement of cocaine abuse.

Methods

Subjects and Compounds. Zyklophin was synthesized as described previously (22). The KOR agonist (±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U50,488) was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, Maryland). All other com-

pounds were obtained from Sigma. Adult male C57BL/6J mice weighing 19–27 grams were obtained from Jackson Labs, 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 (10, 35, 39).

Antinociceptive Testing. The 55 °C mouse warm-water tail-withdrawal assay was used as described earlier (40), with the latency of the animal to withdraw its tail taken as the endpoint. After determining baseline tail-withdrawal latencies, mice were administered a graded dose of compound through the i.c.v., i.p., or s.c. route as noted. For i.c.v. injections, vehicle was artificial cerebrospinal fluid (146 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, and 1.0 mM $MgCl_2$), whereas all peripheral administrations were made in 0.9% saline as the vehicle except for SNC-80, which was dissolved in 35% DMSO/65% saline. Intracerebroventricular injections were made as described previously (40). The volume of these injections was 5 μL, using a 10-μL Hamilton syringe. After agonist administration, the tail-withdrawal latency was determined 40 min later unless otherwise specified. The initial doses of zyklophin examined were selected based on the previous in vitro characterization of zyklophin (22) and the in vivo activity of the peptide KOR antagonist arodyn (10). Mice pretreated with zyklophin were returned to their home cages for 20 min; for the determination of the duration of KOR antagonist activity mice were also pretreated for 1.3, 3.3, 7.3, 11.3, 17.3, or 23.3 h. Mice were then administered a single dose of the KOR selective agonist U50,488 (10 mg/kg i.p.) and subsequently tested 40 min later for their tail-withdrawal latencies to determine the duration of the KOR antagonist effects produced by zyklophin. The dose of U50,488 used (10 mg/kg i.p.) was selected based on the previous demonstration of significant, selective KOR-mediated antinociception in C57BL/6J mice at this dose (10, 36, 41). Likewise, morphine (10 mg/kg i.p.) was used at a dose reported to activate primarily MOR (42). The dose of SNC-80 (12.5 mg/kg i.p.) was chosen for both DOR selectivity (43) and to avoid negative side effects such as seizures (44); 40 min after administration no negative side effects were observed with this dose.

A cut-off time of 15 s was used in this study; if the mouse failed to display a tail-withdrawal response during that time, the tail was removed from the water and the animal assigned a maximal antinociceptive score of 100%. At each time point, antinociception was calculated according to the following formula: % antinociception = 100 × (test latency − control latency)/(15 − control latency). For opioid receptor selectivity testing, the antinociceptive results after zyklophin pretreatment are expressed as a percentage of the matching control group administered agonist (morphine, SNC-80, or U50,488) alone.

Cocaine-Conditioned Place Preference, Extinction, and Reinstatement. ***Conditioned place preference.*** C57BL/6J mice were conditioned using a protocol (Fig. 4*A*) similar to the previously established biased cocaine CPP paradigm (10, 35, 36, 39). It has also been demonstrated as an effective protocol for the study of extinction and reinstatement (10, 39).

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 animals on average initially demonstrated an equivalent amount of time in each of the two outer conditioning compartments (630 ± 14 and 609 ± 14 s in the left and right compartments, respectively, $P = 0.36$, n.s., Student's *t*-test). Place-conditioning began immediately following cocaine administration (10 mg/kg s.c.) on day 2, whereupon mice were 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 CPP response in C57BL/6J mice (45, 46). 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 each day on days 3–5 (see Fig. 4*A*), and animals were then tested for conditioned place preference the day after the final cycle of conditioning (day 6). This protocol has been demonstrated to be effective in producing and maintaining a conditioned-place preference response for approximately 2–3 weeks (10, 46). Data are plotted as the difference in time spent in the cocaine- and vehicle-paired compartments. By convention the initial bias generates a negative value, and a positive value reflects a conditioned preference for the cocaine-paired side. Conditioned place aversion, where animals avoid the drug-paired compartment and spend significantly more time in the initially preferred side, was not detected in this study under any conditions.

Extinction. Place preference for the cocaine-paired compartment was re-examined once a week (see Fig. 4*A*) to determine 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 weekly testing over the 3-week period (10, 39, 46).

Reinstatement. Reinstatement of drug preference was examined after either exposure to forced swim stress (see below) or an additional cycle of cocaine place-conditioning (see Fig. 4*A*). Note that a single cycle of cocaine place-conditioning has been found to be insufficient to produce conditioned place preference alone in C57BL/6J mice (46). Mice were pretreated s.c. with vehicle or zyklophin daily for 2 days 20 min before either cocaine place-conditioning (for 1 cycle as detailed above) or forced swimming (see below). The day after completion of stress exposure or cocaine place-conditioning, animals were tested for place preference (see Fig. 4*A*).

Forced Swim Stress. A 2-day forced swim stress protocol was used as previously detailed (10, 35) to produce stress-induced reinstatement of cocaine CPP. Mice were pretreated each day with vehicle or zyklophin 20 min before exposure to forced swim stress (see Fig. 4*A*). The day after the final exposure to forced swim stress, the place preference responses of mice were tested as described above to determine possible reinstatement of extinguished CPP. An additional set of animals was administered zyklophin immediately after the completion of forced swimming each day, and the place preference responses tested as described above.

Statistical Analysis. Student's *t*-tests comparing baseline and posttreatment tail-withdrawal latencies were used to determine statistical significance for all tail-withdrawal data. Data for conditioned place preference experiments were analyzed with ANOVA using the SPSS 14.0 statistical package. Analyses examined the main effect of CPP phase (e.g., post-conditioning, week of preference test, reinstatement) and the interaction of drug pretreatment (zyklophin or vehicle) × reinstatement condition (stress or cocaine exposure). Significant effects were further analyzed using Tukey HSD post-hoc testing. All data are presented as mean ± SEM, with significance set at $P < 0.05$.

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