Inhibition of ERK pathway or protein synthesis during reexposure to drugs of abuse erases previously learned place preference

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Repeated association of drugs of abuse with context leads to long-lasting behavioral responses that reflect reward-controlled learning and participate in the establishment of addiction. Reactivation of consolidated memories is known to produce a reconsolidation process during which memories undergo a labile state. We investigated whether reexposure to drugs had similar effects. Cocaine administration activates extracellular signal-regulated kinase (ERK) in the striatum, and ERK activation is required for the acquisition of cocaine-induced conditioned place preference (CPP). When mice previously conditioned for cocaine-place preference were reexposed to cocaine in the drug-paired compartment after systemic administration of SL327, an inhibitor of ERK activation, CPP response was abolished 24 h later. This procedure also abolished the phosphorylation of ERK and glutamate receptor-1 observed in the ventral and dorsal striatum, 24 h later, during CPP test. Erasure of CPP by SL327 required the combination of cocaine administration and drug-paired context and did not result from enhanced extinction. Similarly, reexposure to morphine in the presence of SL327 long-lastingly abolished response of previously learned morphine-CPP. The effects of SL327 on cocaine- or morphine-CPP were reproduced by protein synthesis inhibition. In contrast, protein synthesis inhibition did not alter previously acquired locomotor sensitization to cocaine. Our findings show that an established CPP can be disrupted when reactivation associates both the conditioned context and drug administration. This process involves ERK, and systemic treatment preventing ERK activation during reexposure erases the previously learned behavioral response. These results suggest potential therapeutic strategies to explore in the context of addiction.

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Abbreviations: CPP, conditioned place preference; DA, dopamine; DStr, dorsal striatum; ERK, extracellular signal-regulated kinase; GluR1, glutamate receptor-1; NAcc, nucleus accumbens; P-ERK, phosphorylated ERK; P-GluR1, phospho-Ser845 GluR1; MEK, mitogen-activated protein kinase/ERK kinase.

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Reexposure to cocaine in drug-paired compartment in the presence of a MEK inhibitor erases CPP and its biochemical correlates. (A) Experimental design: S, saline; C, cocaine, 20 mg/kg; Veh, vehicle; SL327, 30 mg/kg. Each group included 8 mice. (B) After conditioning, mice developed a significant preference for the cocaine-paired side (test 1, \( F_{(3,28)} = 16.77, P < 0.01 \)). Inhibition of ERK activation by systemic administration of SL327 1 h before cocaine reexposure suppressed the place preference when animals were tested 24 h later (test 2, \( F_{(3,28)} = 20.79, P < 0.01 \)). Data are means ± SEM. Post hoc comparison (Bonferroni test), group I vs. group II to IV: **, \( P < 0.01 \); group III vs. group IV: **, \( P < 0.01 \). (C–E) To assess protein phosphorylation during the CPP test, P-ERK and total ERK were similarly analyzed (E and F). Results were expressed as a ratio of phosphoprotein/total protein and normalized as percentage of controls. Data are means ± SEM (seven to eight mice per group). One way ANOVA for P-ERK2 was as follows: NAcc, \( F_{(3,25)} = 11.34, P < 0.01 \); DStr, \( F_{(3,27)} = 9.41, P < 0.01 \); Pf Cx, \( F_{(3,25)} = 4.98, P < 0.01 \). One way ANOVA for P-GluR1 was as follows: NAcc, \( F_{(3,25)} = 10.46, P < 0.01 \); DStr, \( F_{(3,25)} = 7.24, P < 0.01 \); Pf Cx, \( F_{(3,25)} = 0.62, NS \). Bonferroni test was as follows: group I vs. group II to IV, *, \( P < 0.05 \); **, \( P < 0.01 \); group III vs. group IV, **, \( P < 0.01 \).

To examine the biochemical correlates of the behavioral response, we measured by immunoblotting the activatory phosphorylation of ERK (P-ERK) and GluR1. Conditioning in the presence of SL327 erases previously acquired CPP.

Thus, administration of SL327 during reexposure to cocaine in the drug-paired environment had profound consequences 24 h later during the CPP test: it prevented activation of ERK and cAMP-dependent protein kinase (PKA), as indicated by lack of GluR1 phosphorylation, as well as the behavioral response of place preference. This finding shows that biochemical and behavioral aspects of the conditioned reaction acquired during previous training were apparently erased by blockade of the ERK pathway during simultaneous reexposure to conditioned cocaine.
MEK Inhibitor Does Not Increase Extinction of Cocaine-Induced Conditioning. When mice are repeatedly injected with saline in the previously drug-paired compartment, an extinction of the conditioned response is observed (32). The effect of SL327 could be accounted for by a powerful extinction of the learned CPP. To test this hypothesis, cocaine-conditioned mice received an injection of saline in the previously drug-paired compartment in the presence of vehicle (group V) or SL327 (group VI) and were compared with four groups treated as in Fig. 1 (Fig. 2A). When these mice were tested 24 h later (test 2), they still displayed a significant CPP (groups V and VI, Fig. 2B). This result shows the absence of significant extinction in these mice and rules out a persistent effect of SL327 during the CPP test. On the other hand, a disappearance of the conditioned response was observed in group IV (Fig. 2B), as was found in the first experiment.

A characteristic of extinguished conditioned responses is that they can be reinduced by a priming injection of cocaine before testing CPP (33, 34). We tested the effects of a priming injection of cocaine just before the place preference test (test 3, Fig. 2A and B). Priming did not restore the erased CPP in mice reexposed to cocaine in the drug-paired environment in the presence of SL327 (group IV, test 3, Fig. 2B). These results further indicate that SL327 does not exert its effects by an increased extinction mechanism. It should also be noted that cocaine treatment in the presence of SL327 does not have aversive properties (9).

The Ability of SL327 to Erase CPP Requires Reexposure to Cocaine in the Drug-Paired Environment. We then tested whether SL327-induced suppression of previously acquired CPP required reexposure to cocaine in the drug-paired environment, or whether the same effect could be obtained after cocaine injection in a neutral environment. To examine this point, we repeated a protocol similar to that described in Fig. 1, except that additional groups of mice received an injection of cocaine in their home cage in the presence of vehicle (group VII) or SL327 (group VIII) (Fig. 2A and C). CPP was abolished only in animals that had been reexposed to cocaine in the presence of SL327 in the drug-paired compartment (Group IV, test 2, Fig. 2C). In contrast, mice that received cocaine in the presence or absence of SL327 in their home cage displayed a normal CPP (groups VII and VIII, test 2, Fig. 2C).

In mice that received SL327 and were placed in the drug-paired compartment in the absence of cocaine (saline injection), CPP was not suppressed (group VI, Fig. 2A and B). This finding shows that exposure to drug-associated environment was not sufficient for SL327 to erase CPP. However, in these conditions, mice were placed passively in the drug-paired environment. We therefore examined the effects of SL327 when mice were actively seeking the drug-paired compartment, i.e., during the CPP test (group IX, Fig. 2A). Injection of SL327 significantly decreased the expression of CPP (group IX, test 1, Fig. 2C). However, when these animals were tested 2 days later, CPP was restored (group IX, test 2, Fig. 2C). These results differ from those reported in rats, after bilateral injection of U0126, a MEK inhibitor, in NAcc core, which prevented both immediate and delayed expression of CPP (29). This discrepancy may be due to the different route of administration of the inhibitor. Intracerebral injections do not allow a precise control of the concentration of inhibitors in the injected structure, which may be far above those at which they are selective for their target kinase (35) and, thus, inhibit other pathways. Alternatively, the discrepancy between the two studies could be accounted for by minor differences in the CPP protocol, such as sensory modalities underlying the cues and strength of reactivation.

Blockade of ERK During Reexposure Erases CPP to Morphine. Despite different mechanisms of action, drugs of abuse share the ability (environment) and unconditioned (cocaine) stimuli. The ERK-dependent molecular mechanisms of the reactivation are not known but could be related to synaptic plasticity necessary to associate environmental cues with an increase in DA release and/or activity.
to raise extracellular DA concentration in the NAcc (1) and to increase ERK phosphorylation in this nucleus (11). It was important to determine whether inhibition of ERK activation erased only cocaine-CPP or whether this effect also applied to another drug acting through a different mechanism. We tested morphine in the same experimental paradigm as described in Fig. 1 for cocaine (Fig. 3A). Mice that were conditioned with morphine developed a strong place preference (group II, test 3, Fig. 3). The mice that had been reexposed without reexposure to morphine still displayed a significant CPP, although the response was less pronounced than when tested early (group II, test 3, Fig. 3). Finally, we examined whether priming by anisomycin suppressed the place preference when the animals were tested 24 h later (test 2, $F_{(2,32)} = 17.47, P < 0.01$). Data are expressed as mean ± SEM. Post hoc comparison by Bonferroni test was as follows: group I vs. group II to IV, * $P < 0.05$; **, $P < 0.01$; group III vs. group IV, $P < 0.01$; group IV vs. group III, ##, $P < 0.01$.

### The Effects of ERK Blockade on Cocaine- or Morphine-Induced CPP Are Mimicked by a Protein Synthesis Inhibitor

A major role of ERK activation is to control gene expression at both transcriptional (13) and translational (36) levels, and it is likely that its long-lasting consequences require protein synthesis. In addition, reactivation of memories during the reconsolidation phase makes them sensitive to protein synthesis inhibitors (25). We examined whether systemic administration of anisomycin, a protein synthesis inhibitor, after reexposure to morphine or cocaine was capable of erasing previously acquired CPP, using an experimental design similar to that described in Fig. 1 for SL327 (Fig. 4A). All mice conditioned to cocaine (groups b, d, and f) or morphine (groups c and e) did not display a similar CPP (test 1, Fig. 4B). Some of these mice were reexposed to the drug for which they had been conditioned, in the drug-paired environment, and then treated with vehicle (groups b and c, test 2, Fig. 4A and B) or anisomycin (groups d and e, Fig. 4A and B). CPP was absent the following day in mice in which reexposure was followed by anisomycin (groups d and e, test 2, Fig. 4B). In vehicle-treated mice CPP was not diminished (groups b and c, test 2, Fig. 4B). Importantly, injection of saline in the drug-paired compartment followed by anisomycin did not erase the CPP (group f, test 2, Fig. 4B), showing that a reexposure to the drug, and not a simple injection in the drug-paired compartment, was required to reactivate an anisomycin-sensitive form of memory. These results strongly support the hypothesis that ERK exerts its critical effect through regulation of protein synthesis, presumably by controlling transcription. They also show that simultaneous reexposure to drug and drug-associated context produces effects similar to the reconsolidation described for other types of memory (37).

**Locomotor Sensitization Does Not Undergo Protein-Synthesis Inhibitor-Sensitive Reconsolidation.** In addition to inducing CPP, single or repeated exposures enhance the locomotor effects of many...
drugs of abuse in rodents, a long-lasting behavioral alteration that may be relevant for some aspects of addiction (38). Sensitization is partly dependent on association of the locomotor effects of a drug with the environment in which it has been previously administered (39). Although the mechanisms underling locomotor sensitization are not fully understood, it is thought to reflect neuronal adaptations in several brain regions, including NAcc, prefrontal cortex, and DA neurons in the mesencephalon (40), that seem different from those of CPP (41).

We examined whether inhibition of protein synthesis was capable of altering locomotor sensitization using a single injection protocol (12, 42). We first evaluated whether anisomycin was able to prevent the induction of locomotor sensitization using a single injection paradigm (Fig. 5A). Mice received a first injection of cocaine (1st inj) and were challenged with a test injection 7 days later (test inj) (Fig. 5A). As previously reported (12), these mice displayed an increased locomotion in response to the second injection of cocaine (Fig. 5A). In contrast, mice that received anisomycin either 30 min before or immediately after the first injection of cocaine, did not display any locomotor sensitization a week later (Fig. 5A), demonstrating that protein synthesis is necessary for induction of this long-lasting effect of cocaine. This result confirms previous findings showing that locomotor sensitization was prevented by local application of anisomycin in the ventral tegmental area (43).

We then tested whether protein synthesis inhibition after reexposure to cocaine could erase previously induced locomotor sensitization (Fig. 5B). For this purpose, after the first exposure to cocaine, mice received no treatment on day 3 or were reexposed to cocaine in the drug-associated environment (actimeter) and then received an injection of vehicle or anisomycin (Fig. 5B). As expected, mice not reexposed, or reexposed to cocaine and treated with vehicle, showed a robust locomotor sensitization (Fig. 5B). Mice reexposed to cocaine and treated with anisomycin (group III) also developed significant sensitization (Fig. 5B). These results demonstrate that, once induced, locomotor sensitization cannot be reversed by cocaine reexposure in the presence of anisomycin. These results contrast with those reported above for CPP and further support that these two behavioral responses depend on different mechanisms.

Role of ERK in Reconsolidation of Drug-Environment Association. The present study provides strong evidence that mechanisms similar to memory reconsolidation are operating during repeated drug administration in the same environment. Moreover, it demonstrates that these mechanisms can be manipulated by simple systemic pharmacological treatments. These results extend the recent reports on the effects of local manipulation of the ERK pathway in NAcc or of immediate early gene Zif268 in basolateral amygdala (17, 44). Thus, the ERK pathway plays a central role in the effects of drugs of abuse at several stages. This pathway is activated in the NAcc, the extended amygdala, and other brain regions during the first administration of a variety of drugs of abuse (9, 45–47). It is also activated in response to repeated administrations of the same drug (7, 9, 47) or, after conditioning, in response to drug-associated cues (16, 29). We found that, when mice are placed in the CPP test conditions, ERK activation was more widespread than previously reported in rats (29), including the DStr and prefrontal cortex. We also found that an important substrate of cAMP-dependent protein kinase (PKA), GluR1, was phosphorylated in NAcc and DStr, an effect that may be important for the behavioral response.

Peripheral administration of a pharmacological inhibitor of MEK, which crosses the blood–brain barrier, prevents the induction (9, 14) and expression (present study) of drug-environment associations, as well as a process similar to reconsolidation (see ref. 48 for a recent discussion of reconsolidation). In contrast to reconsolidation of other types of memory achieved by exposure to the conditioned stimulus alone, inhibitor-sensitive reactivation of drug-related memory seemed to require the association of both uncontaminated (drug injection) and conditioned (drug-paired environment) stimuli. When SL327-pretreated mice were injected with saline in the drug-paired compartment or when they received cocaine in their home cage, CPP was not suppressed. Moreover, although the administration of a MEK inhibitor before the test blocked the expression of CPP acutely, it did not prevent its reexpression 2 days later. This observation differs from a recent report in rat (29). However, it is possible that, by acutely blocking the expression of CPP on the test day, SL327 also prevented contextual memory reactivation. Therefore, further exploration is needed to determine to what extent conditioned stimuli are sufficient for reconsolidation in this context. Interestingly, as in reconsolidation of other types of memory (25, 28), that observed in the present study was inhibited by both a MEK inhibitor and a protein synthesis inhibitor. This finding suggests that a major role of ERK in this context is the control of gene expression, although we cannot rule out that the two inhibitors achieve similar effects through unrelated mechanisms.

Our study does not allow identifying in which brain structures inhibitor-sensitive reactivation is important for the maintenance of drug-induced conditioning. It is likely that several brain regions, including basolateral amygdala (44) and NAcc (29), and possibly others, are all necessary for drug-related memory reconsolidation. On the other hand, the present study provides evidence that a unique systemic injection of a kinase inhibitor has long-lasting behavioral effects, if it is specifically administered before reexposure to the drug in the previously drug-paired environment. The resulting erasure of the previously learned CPP lasts for at least 2 weeks and does not seem to be
related to an extinction mechanism. It is not known, however, whether well learned drug associations after a long training protocol would be sensitive to inhibitors. Thus, our results provide an incentive for further exploring possible therapeutic strategies using kinase inhibitors in specific conditions to reverse previously acquired drug-induced conditioning.

Materials and Methods

Experiments were in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (decree 87849, license 01499) in 8-week-old male C57BL/6J mice. Cocaine-HCl and morphine sulfate (Sigma-Aldrich) were dissolved in 0.9% (wt/vol) NaCl (saline); SL327 (Biaffin, Kassel, Germany) dissolved in DMSO was diluted twice in water; and anisomycin (Sigma-Aldrich) in 1 M HCl was diluted in saline, and adjusted to pH7. For immunoblotting, microdisks were prepared from sections of rapidly frozen brains at the end of CPP and lysed in a 1% SDS (vol/vol) solution at 100°C as described (12). CPP was done in a two-compartment apparatus (Imetronic, Pessac, France) with different patterns on floors and walls, separated by a central neutral area (41). Preconditioning phase (day 1, pretest, 18 min) was as follows: mice were placed in the central neutral area and allowed to explore both compartments. Mice were randomly assigned to the various experimental groups (unbiased protocol). Conditioning (days 2–7) was as follows: