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Selective Inhibition of Amygdala Neuronal Ensembles Encoding Nicotine-Associated Memories Inhibits Nicotine Preference and Relapse

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Abstract

BACKGROUND: Nicotine craving and relapse often occurs after reactivation of nicotine reward memories. We recently developed a memory retrieval–reconsolidation interference procedure in which reactivating nicotine reward memories by acute exposure to nicotine (the unconditioned stimulus [UCS]) and then pharmacologically interfering with memory reconsolidation decreased relapse to nicotine seeking in rats and nicotine craving in smokers. Here, we investigated underlying mechanisms.

METHODS: In the first series of experiments, we trained rats for nicotine-induced conditioned place preference (CPP) or nicotine self-administration and ventricularly microinjected them with the protein synthesis inhibitor anisomycin immediately after UCS-induced memory retrieval. In the second series of experiments, we used tyramide-amplified immunohistochemistry–fluorescence in situ hybridization to examine neural ensembles in the basolateral amygdala (BLA) reactivated by nicotine conditioned stimulus– or UCS-induced memory retrieval. We then used the Daun02 chemogenetic inactivation procedure to selectively inhibit the nicotine UCS-reactivated BLA neuronal ensembles.

RESULTS: Ventricular injections of the anisomycin immediately after nicotine UCS memory retrieval inhibited sub-sequent nicotine CPP and relapse to operant nicotine seeking after short or prolonged abstinence. More important, within BLA, distinct neuronal ensembles encoded pavlovian CPP and operant self-administration reward memories and nicotine (the UCS) injections

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in the home cage reactivated both neuronal ensembles. Daun02 chemogenetic inactivation of the nicotine-reactivated ensembles inhibited both nicotine CPP and relapse to nicotine seeking.

CONCLUSIONS: Results demonstrate that the nicotine UCS-induced memory retrieval manipulation reactivates multiple nicotine reward memories that are encoded by distinct BLA neuronal ensembles that play a role in nicotine preference and relapse.

Keywords

Daun02 inactivation; Drug seeking; Neuronal ensembles; Nicotine memories; Reconsolidation; Reinstatement; Relapse; Unconditioned stimulus

Nicotine addiction causes more than 6 million deaths each year (1). Two core features of this addiction are high relapse rates and persistent memories of nicotine (the unconditioned stimulus [UCS]) reward and nicotine-associated conditioned stimuli (CSs) (2–4). In humans (5) and animal models (6,7), key triggers of craving and relapse during abstinence are acute reexposure to nicotine (a smoking episode in humans or noncontingent nicotine priming injections in animal models) or nicotine-associated CSs, presumably due to reactivation of nicotine-associated memories (8,9).

Reconsolidation refers to a time-dependent process in which consolidated memories become transiently unstable after their reactivation (10–12). Studies using conditioned place preference (CPP) and drug self-administration (SA) procedures demonstrated that drug reward memories that were reactivated by drug-paired CSs undergo memory reconsolidation and that different manipulations that interfere with reconsolidation inhibit subsequent drug CPP and operant drug seeking and relapse (13–15). However, with few exceptions (15,16), the translation of these preclinical studies has not been successful. For example, the β -adrenoceptor blocker propranolol, which interferes with CS-induced retrieval and reconsolidation of drug reward memories in animal models (13,14,17,18), was ineffective in translational studies with human drug users (19–21).

We recently hypothesized (22,23) that a potential reason for the limited translational utility of the classical CS-induced retrieval and reconsolidation interference procedure is that the neuropharmacological manipulations interfere only with reconsolidation of memories selectively associated with the reactivated CS without affecting memories associated with other CSs or the UCS (the drug) itself. In the case of nicotine addiction, smoking is associated with multiple CSs that vary across individuals (24). Thus, it is not feasible to reactivate the memories of many different individual-specific nicotine-associated CSs and interfere with their reconsolidation in the clinic.

Based on these considerations and results from threat (fear) conditioning studies showing that interference with memory reconsolidation after memory retrieval by the UCS (aversive shock) prevents subsequent threat conditioning induced by multiple CSs (25–27), we recently developed a UCS-induced memory retrieval–reconsolidation interference procedure and demonstrated its efficacy in decreasing cocaine relapse and craving in rat models (22). However, a limitation of the procedure we introduced in Luo *et al.* (22), which is based on the memory retrieval–extinction memory reconsolidation procedure (28), is that it requires

multiple exposures to the UCS (cocaine priming) and subsequent time-locked extinction training (22). Based on this procedural limitation, we recently developed a simpler UCS-induced memory retrieval–reconsolidation interference procedure in which UCS-induced memory reactivation occurs only once in a nondrug context (23). In a rat-to-human translational study, we reactivated nicotine reward memories in rat models and in human smokers by acute exposure to nicotine (the UCS) and then interfered with memory reconsolidation using propranolol. We showed that in rats our procedure prevented both nicotine preference and nicotine relapse after both short and prolonged abstinence and that in humans the procedure prevented craving induced by diverse smoking-associated CSs (23). Dunbar and Taylor also recently reported that a drug named garcinol blocks the reconsolidation of multiple cocaine-paired cues after UCS reactivation (29).

The goal of the current study was to identify neurobiological mechanisms that mediate drug reward memory reconsolidation following retrieval of the drug-associated memories by exposure to drug-associated CSs versus the UCS. We studied basolateral amygdala (BLA) neuronal ensembles based on the role of this brain area in drug memory reconsolidation (13,14). To identify and manipulate amygdala neuronal ensembles, we used two novel methods: Daun02 inactivation (30–32) and tyramide-amplified immunohistochemistry–fluorescence in situ hybridization (TAI-FISH) (33). These methods allow us to both identify neuronal ensembles encoding nicotine-associated cues and determine their causal role in nicotine preference and relapse.

METHODS AND MATERIALS

Subjects

We housed the male *Fos-lacZ* transgenic rats (32) and male Sprague Dawley rats (weighing 260–280 g; Beijing Vital River Laboratories, Beijing, China) in groups of five in a temperature-controlled (23 ± 2 °C) and humidity-controlled ($50 \pm 5\%$) animal facility with free access to food and water. Detailed information is provided in the Supplement.

Experimental Procedures

The CPP training, testing, extinction training, and drug priming–induced reinstatement of nicotine CPP are based on our previous studies (34–36). The training of intravenous nicotine SA and oral sucrose SA, extinction training, and nicotine priming–induced reinstatement of nicotine seeking are based on previous studies (23,37–39). The nicotine CPP–CS and nicotine operant–CS retrieval manipulations were identical to the one used in our previous studies (15,22). We injected the rats noncontingently with the previously self-administered drug (UCS) (nicotine, 0.15 mg/kg, subcutaneous) in their home cage (38,40,41). Detailed information is provided in the Supplement.

Stereotactic Surgery and Drug Infusions

We performed the stereotactic surgery and implanted 23 guide cannulas 1 mm above the lateral ventricle or BLA (22,34,42). We dissolved and injected anisomycin and Daun02 based on previous studies (32,42). Detailed information is provided in the Supplement.

TAI-FISH and Image Analysis

We anesthetized the rats with sodium pentobarbital and cut 30- μ m-thick coronal sections for subsequent examination of tyramide-amplified FISH and fluorescence immunohistochemistry. We performed tyramide-amplified FISH, fluorescence immunohistochemistry, and image analysis per previous studies (33,43,44). Detailed information is provided in the Supplement.

Statistical Analysis

We report the results as mean $\pm$ SEM and analyzed the data by analyses of variance with the appropriate between- and within-subjects factors for each experiment. Detailed information is provided in the Supplement.

RESULTS

Nicotine UCS Triggers Reconsolidation of Drug Reward Memories

In Experiment 1, we used a CPP procedure and the protein synthesis inhibitor anisomycin to determine whether exposure to nicotine UCS and nicotine CS retrieval (herein termed nicotine CPP-CS retrieval) manipulations would induce reconsolidation of the memories for nicotine reward (see Supplement for a detailed description of Experiments 1–7). CPP is a pavlovian conditioning procedure in which increased preference for the drug CS context serves as a measure of the drug's rewarding effects (45). Postretrieval brain injections of anisomycin within the reconsolidation windows (typically 1–2 hours) have been widely used in memory reconsolidation studies (11,13).

We injected anisomycin [400 μ g/rat, intracerebroventricular (42)] immediately after a short 5-minute exposure to the nicotine CPP-CS retrieval or nicotine UCS retrieval (22,23) (Supplemental Figure S1A). We found that anisomycin injections after either nicotine CPP-CS or nicotine UCS memory retrieval prevented nicotine CPP expression 1 day and 14 days later; anisomycin injections after nicotine CPP-CS or nicotine UCS retrieval also prevented nicotine priming-induced rein-statement of nicotine CPP after extinction (Supplemental Figure S1B, C). Anisomycin's effects on nicotine CPP expression were temporally and stimulus specific; anisomycin was ineffective when injected 9 hours after nicotine UCS retrieval (outside the reconsolidation window) or after vehicle (saline) injections (Supplemental Figure S1D, E).

In Experiment 2, we determined the generality of the inhibitory effect of anisomycin on reconsolidation after nicotine UCS retrieval using the intravenous drug SA procedure, in which drug intake is voluntary (46), and a rat model of relapse during forced abstinence, in which drug seeking is assessed in extinction tests in the presence of contextual and discrete CSs that were previously paired with drug SA (7). In the same rats, we also measured anisomycin's effects on nicotine priming-induced reinstatement after extinction using the established reinstatement model of drug relapse (6) (Supplemental Figure S1F). In both relapse models, a selective increase in nonreinforced responding on the device previously associated with drug infusions is interpreted to indicate relapse to drug seeking (7). We found that anisomycin injections immediately after nicotine UCS memory retrieval in the

home cage decreased relapse to drug seeking after short (2 days) or prolonged (32 days) abstinence (Supplemental Figure S1G) as well as nicotine priming-induced reinstatement after extinction (Supplemental Figure S1H).

The data from Experiments 1 and 2 with anisomycin confirm and extend previous studies that exposure to drug CSs (13,14,18) and drug UCS (22,23) retrieves drug-associated memories that undergo reconsolidation, and that interference with reconsolidation prevents subsequent drug preference and drug relapse.

BLA Neuronal Ensembles Encode Multiple Nicotine Reward Memories

In Experiments 3 to 7, we explored the brain mechanisms encoding the nicotine reward memories that are reactivated by the nicotine UCS retrieval manipulation and undergo reconsolidation. In Experiment 3, we asked whether both nicotine CS and UCS retrieval induce neuronal activation in BLA, a brain area previously implicated in reconsolidation of drug reward memories (13,14). We trained rats for nicotine CPP and removed their brains 90 minutes after nicotine CS or nicotine UCS retrieval (31) for detection of coexpression of Fos and NeuN (a marker of neurons) in both BLA and central nucleus of the amygdala (CeA) (Figure 1A, B), two major subregions of the amygdala (34). For BLA, we found that both nicotine CS and nicotine UCS retrieval increased Fos expression; double labeling of Fos with NeuN indicated that $5.5 \pm 0.7\%$ and $7.1 \pm 1.3\%$ of NeuN-labeled neurons were Fos positive (Figure 1C), respectively. For CeA, we found that nicotine UCS retrieval, but not nicotine CS retrieval, induced Fos expression in CeA; double labeling indicated that $2.5 \pm 0.8\%$ and $6.5 \pm 0.8\%$ of NeuN-labeled neurons were Fos positive for nicotine CS and UCS retrieval, respectively (Figure 1D). These results indicate that both nicotine CS and nicotine UCS can reactivate sparsely distributed neuronal ensembles in BLA that may encode the learned associations between nicotine rewarding effects and the drug-associated contexts and cues.

In Experiments 4 to 7, we explored whether the putative neuronal ensembles in BLA that were presumably reactivated by the nicotine UCS encode multiple nicotine reward memories by using two methods: Daun02 inactivation (30–32) and TAI–FISH (33). We used these methods to establish the following: 1) the degree of overlap between neuronal ensembles that encode nicotine pavlovian CPP reward memories and operant SA reward memories (Experiments 4 and 5), 2) whether nicotine UCS retrieval activates both forms of nicotine reward memories (Experiment 6), and 3) whether selective inhibition of nicotine UCS reactivated neuronal ensembles would prevent subsequent nicotine CPP and relapse to operant nicotine seeking (Experiment 7). The TAI–FISH method (33) is based on the temporal characteristics of the immediate early gene Fos after neuronal activation; messenger RNA (mRNA) expression is maximal after about 30 minutes, while protein expression is maximal after about 90 to 120 minutes (47). Accordingly, immunohistochemistry of Fos protein can identify previously activated neurons 90 minutes after exposure to stimulus 1 and in situ hybridization of *Fos* mRNA can identify previously activated neurons 30 minutes after exposure to stimulus 2, with 60 minutes separating stimulus 1 and stimulus 2 (Figure 2A). The Daun02 inactivation method employs *Fos-lacZ* transgenic rats in which β -galactosidase (β -gal) and Fos are coexpressed within behaviorally

activated neurons. Ninety minutes after the transgenic rats perform a behavioral or learning task, the previously activated neurons are inactivated by injecting the prodrug Daun02 into a given brain area. β -Gal within the behaviorally activated neurons converts Daun02 into daunorubicin, which inactivates and ablates these neurons (30,32). This method has been used in recent studies on the role of striatal and cortical neuronal ensembles in conditioned drug effects and drug relapse (31,48–50), including relapse to nicotine seeking (51).

In Experiment 4, we examined the time courses of *Fos* mRNA and Fos protein in BLA after nicotine CPP–CS or nicotine UCS retrieval using the TAI–FISH method (Figure 2B, C); we trained rats for nicotine CPP and dissected their brains 0, 15, 90, and 180 minutes after the nicotine CS or nicotine UCS retrieval manipulation. We found that the expression of *Fos* mRNA reached its peak 15 minutes after nicotine CS retrieval and returned to baseline 90 minutes later, while the expression of Fos protein reached its peak 90 minutes after nicotine CS retrieval (Figure 2D, E). The expression of *Fos* mRNA and protein exhibited a similar temporal pattern after nicotine UCS retrieval (Figure 2F, G). Together, these data indicate that the expression of *Fos* mRNA and Fos protein in BLA demonstrated distinct time courses after CS or UCS retrieval and can be used to represent different neural ensembles reactivated by nicotine CS- or UCS-induced memory retrieval.

In Experiment 5, we used the TAI–FISH method to determine the degree of overlap in BLA neuronal ensembles encoding nicotine CPP and nicotine SA reward memories by using a modified training procedure in which we trained rats for both nicotine SA (14 days) and nicotine CPP (8 days), resulting in the formation of both pavlovian and operant nicotine reward memories in the same rat (Figure 3A). One day after training, we exposed the rats to the nicotine CPP–CS and nicotine or sucrose operant–CS retrieval 90 minutes apart (Figure 3A); we anesthetized the rats and dissected their brains 15 minutes after the second retrieval manipulation. We found that exposure to nicotine CPP–CS retrieval, nicotine operant–CS retrieval, and sucrose operant–CS retrieval led to similar activation of BLA (Figure 3B–D). More important, we found significantly higher protein + mRNA double labeling of Fos in rats exposed twice (90 minutes apart) to nicotine CPP–CS retrieval than to all other experimental manipulations in which the rats were exposed first to nicotine CPP–CS retrieval and 90 minutes later to a different retrieval manipulation (nicotine or sucrose operant–CS retrieval or home-cage control; Figure 3E, F). In addition, the number of double-labeled neurons in the group exposed first to nicotine CPP–CS and 90 minutes later to nicotine operant–CS retrieval was significantly higher than that of the group exposed first to nicotine CPP–CS and 90 minutes later to sucrose operant–CS retrieval (Figure 3E, F). Together, these data indicate that distinct but partially overlapping BLA neuronal ensembles encode nicotine CPP and SA reward memories.

In Experiment 6, we used the TAI–FISH method to determine whether UCS retrieval can simultaneously activate the distinct neuronal ensembles encoding nicotine CPP and nicotine SA reward memories (see Figure 4A for experimental design). We first exposed groups of rats to either their home cage, nicotine CPP–CS retrieval, nicotine operant–CS retrieval, sequential exposure to nicotine CPP–CS retrieval (5 minutes) + nicotine operant–CS retrieval (15 minutes), or sequential exposure to nicotine CPP–CS retrieval (5 minutes) + exposure to a novel environment (5 minutes). Ninety minutes later, we exposed the rats in

the different experimental conditions to either their home cage, nicotine UCS retrieval, sequential exposure to nicotine CPP–CS retrieval + nicotine operant–CS retrieval, or sucrose UCS retrieval (5 minutes of drinking 10% sucrose solution in the home cage). Fifteen minutes later, we anesthetized the rats and dissected their brains for assessment of Fos protein and mRNA expression in BLA (Figure 4B). We found that combined exposure to nicotine CPP–CS + nicotine operant–CS induced more Fos protein expression than exposure to each retrieval manipulation alone (Figure 4C). In addition, the nicotine UCS retrieval induced more *Fos* mRNA than the nicotine CPP–CS retrieval (Figure 4D). More important, the double-labeling analysis showed that the rats that were first exposed to nicotine CPP–CS retrieval + nicotine operant–CS retrieval and then exposed to nicotine UCS retrieval 90 minutes later had a higher percentage of overlapping activated neurons than the rats in all other conditions. In addition, the rats that were first exposed to nicotine CPP–CS retrieval + nicotine operant–CS retrieval and then exposed to sucrose UCS retrieval 90 minutes later had a lower percentage of overlapping activated neurons than the rats in all other conditions (Figure 4E, F). In addition, the rats that were first exposed to nicotine CPP–CS retrieval and then exposed to nicotine UCS retrieval 90 minutes later showed a similar percentage of overlapping activated neurons to that observed in rats exposed to nicotine CPP–CS retrieval + novel environment and then exposed to nicotine UCS retrieval 90 minutes later [Note that we used the novel environment as a nonspecific learning-independent manipulation that is known to increase Fos expression in the brain to a degree similar to or higher than that induced by conditioned drug cues (49,50)]. Together, these data indicate that nicotine UCS retrieval can simultaneously activate distinct BLA neuron ensembles that presumably encode both nicotine CPP and SA reward memories.

Selective Inhibition of Neuronal Ensembles Reactivated by Nicotine UCS Decreased Nicotine Preference and Relapse

In Experiment 7, we used the Daun02 inactivation method (Figure 5A and Supplemental Figure S2A) to determine whether selective inhibition of nicotine UCS reactivated neuronal ensembles would prevent both nicotine CPP expression and relapse to operant nicotine seeking. We found that in rats trained in the nicotine SA and CPP procedures (Figure 5B), Daun02 injections (2 µg/site) into BLA (Supplemental Figure S2B) 90 minutes after UCS retrieval prevented CPP expression 1 and 15 days later (Figure 5C) as well as operant relapse to nicotine seeking 2 and 16 days later (Figure 5D). BLA Daun02 injections after UCS-induced memory retrieval also decreased subsequent BLA (but not CeA) neuronal activity (assessed by β -gal expression) during the second operant extinction test session (Figure 5E). In contrast, Daun02 injections into BLA 90 minutes after nicotine CPP–CS retrieval (Figure 5F) prevented subsequent CPP expression (Figure 5G) but not operant relapse (Figure 5H).

Together, the results of Experiments 4 to 7 indicate that nicotine UCS retrieval reactivates distinct neuronal ensembles in BLA that encode nicotine reward memories that were formed during pavlovian CPP training and operant SA training and that selective inactivation of these neuronal ensembles caused long-lasting inhibition of nicotine CPP and relapse to operant nicotine seeking.

DISCUSSION

In the current study, we used a UCS-induced memory reconsolidation interference procedure in which we reactivated diverse nicotine reward memories by acute exposure to nicotine (the UCS) and then pharmacologically or pharmacogenetically interfered with the putative memory reconsolidation process (23). We found that ventricular injections of the protein synthesis inhibitor anisomycin immediately after nicotine UCS memory retrieval inhibited subsequent nicotine CPP and relapse to operant nicotine seeking after short or prolonged abstinence. This pharmacological manipulation had no effect on the different behavioral measures when administered 9 hours after UCS retrieval [outside the putative reconsolidation window (52)], supporting a memory reconsolidation account of the current results. More important, our results indicate that 1) within BLA, distinct neuronal ensembles encode pavlovian CPP and operant SA reward memories, 2) nicotine (the UCS) injections in the home cage activate both neuronal ensembles, and 3) Daun02 chemogenetic inactivation of the nicotine UCS-reactivated ensembles inhibited both nicotine CPP and relapse to nicotine seeking.

UCS Memory Retrieval Versus CS Memory Retrieval

Since 2005 (53,54), many studies using rats and mice have shown that consolidated drug-associated memories that were reactivated by drug-associated CSs undergo memory reconsolidation and that neuropharmacological or behavioral manipulations that interfere with reconsolidation inhibit subsequent drug CPP and relapse to operant drug seeking (13,14). However, as mentioned in the introductory paragraphs, with few exceptions (15,16), the translation of these preclinical studies to human addicts has not been successful (19–21). This state of affairs, and the realization that a major limitation of CS-induced retrieval and reconsolidation procedures is that the neuropharmacological manipulations interfere only with reconsolidation of memories selectively associated with the reactivated CS without affecting memories associated with other CSs or the UCS itself (25–27), inspired us to develop alternative memory reconsolidation interference procedures where the drug reward memory is reactivated by exposure to the drug itself (the UCS).

In a recent study (22), we introduced a UCS memory retrieval–extinction procedure and showed that unlike the established CS memory retrieval–extinction procedure (15,55), the UCS-induced procedure can prevent long-term relapse to cocaine seeking induced by multiple cocaine-associated CSs under a wide range of experimental conditions. However, from a clinical perspective, the UCS memory retrieval–extinction procedure may be difficult to implement because it requires multiple sessions of UCS retrieval plus extinction training. In contrast, in the novel UCS memory retrieval procedure described here and in our recent study (23), a single exposure to nicotine in the nondrug environment, followed by manipulations designed to interfere with memory reconsolidation, inhibited long-term nicotine CPP and operant nicotine seeking.

Together, the results from the current study and other studies (22,23,29) support the idea that UCS memory retrieval–reconsolidation interference procedures have superior relapse-prevention characteristics compared with the classical CS memory retrieval–reconsolidation interference procedures.

Methodological and Interpretation Issues

A consistent finding in our study was that anisomycin or Daun02 injections after UCS-induced memory retrieval completely blocked nicotine CPP but only partially inhibited operant nicotine seeking (see figures). We speculate that this pattern of results is likely due to the fact that operant drug SA is controlled by a complex interplay between operant and pavlovian conditioning processes (13,56), while drug CPP is primarily controlled by pavlovian conditioning processes. In this regard, there is evidence that reconsolidation of operant memories is more difficult to disrupt than reconsolidation of pavlovian memories (15,57).

Another issue to consider is that Fos induction in CeA was observed only after UCS-induced, but not CS-induced, memory retrieval. We speculate that Fos induction in CeA may be due to the acute pharmacological effect of nicotine (58,59). However, it is also possible that UCS-induced Fos induction in CeA is due to reactivation of neuronal ensembles that control nicotine seeking (51). Our experimental procedures were not designed to rule out this possibility. However, our results suggest that the inhibitory effect of BLA Daun02 injections on relapse is not due to diffusion into CeA because these injections selectively decreased subsequent relapse-associated neuronal activity (assessed by β -gal expression) in BLA but not in CeA (Figure 5E).

A methodological issue to consider is that in the TAI-FISH experiment we examined only the time course of mRNA expression after CS- or UCS-induced retrieval of CPP memory. We found that *Fos* mRNA returned to baseline level 90 minutes after exposure to these manipulations. However, we cannot exclude the possibility that *Fos* mRNA expression might not have returned to baseline 90 minutes after exposure to the other experimental manipulations (operant-CS memory retrieval and CPP-CS + operant-CS memory retrieval) and that some of the observed protein-mRNA double labeling under these conditions reflects the original stimulus exposure.

Basolateral Amygdala Neuronal Ensembles Encode Different Forms of Nicotine Reward Memories

Results from many studies suggest that BLA is critical for reconsolidation of drug reward memories after retrieval of these memories by drug-associated CSs (13,14). More recently, we reported that BLA neuronal activity is also critical for reconsolidation of cocaine reward memories after retrieval of these memories by a drug UCS (cocaine) exposure (22). An unresolved question from these studies is whether similar or different neurobiological mechanisms mediate drug reward memory reconsolidation following retrieval of the drug-associated memories by exposure to drug-associated CSs versus the drug (UCS) itself. Here, we used the TAI-FISH (33) and Daun02 inactivation (30,32) procedures to address this question. To the degree that Fos expression can serve as a marker of neuronal ensemble activation during learned behaviors (30,60), our TAI-FISH results indicate that distinct but partially overlapping BLA neuronal ensembles encode nicotine CPP versus operant SA reward memories and that the nicotine UCS retrieval manipulation can retrieve both forms of memories. Our Daun02 inactivation results indicate that selective inhibition of UCS reactivated neuronal ensembles inhibited both nicotine CPP expression and relapse to

operant nicotine seeking. In contrast, selective inhibition of nicotine CPP–CS reactivated neuronal ensembles inhibited nicotine CPP expression but not relapse to operant nicotine seeking (Figure 6).

Together, these results indicate that distinct BLA neuronal ensembles encode different forms of nicotine reward memories and that exposure to nicotine (the UCS) can simultaneously reactivate these memories. Although previous studies have demonstrated that neuronal ensembles in nucleus accumbens or prefrontal cortex mediate relapse to drug seeking (31,48,49,51), only a single drug memory (operant memory) was investigated. In the current study, we established an animal model of multiple drug memories (pavlovian and operant reward memories) and found that the different cue–drug associations are stored in different neuronal ensembles in BLA. The results from the TAI–FISH and Daun02 experiments provide additional support to the notion that UCS memory retrieval–reconsolidation interference procedures have superior relapse prevention characteristics compared with the classical CS memory retrieval–reconsolidation interference procedures.

CONCLUSIONS

We have used a novel UCS-based memory retrieval–reconsolidation interference procedure and demonstrated its efficacy in simultaneously preventing nicotine CPP and operant nicotine seeking during prolonged abstinence periods. We also showed that the UCS memory retrieval manipulation reactivates multiple nicotine reward memories that are encoded by distinct BLA neuronal ensembles. Our results suggest that the UCS-based memory retrieval–reconsolidation interference procedure can be a simple and promising method for decreasing nicotine craving and relapse to nicotine addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nicotine conditioned stimulus (CS) and nicotine unconditioned stimulus (UCS) memory retrieval activate neuronal ensembles in the basolateral amygdala (BLA). **(A)** Experimental procedures. We trained rats in the nicotine conditioned place preference (CPP) procedure. Twenty-four hours later, we exposed the rats to nicotine CPP-CS or nicotine UCS memory retrieval, perfused them, and dissected their brains 90 minutes later. **(B)** Schematic illustrating the location of BLA and central amygdala (CeA) with NeuN fluorescence immunohistochemistry. Scale bars represent 500 μ m. **(C, D)** Effect of the experimental manipulations on Fos protein and double labeling of Fos and NeuN. For BLA, both nicotine

UCS retrieval and nicotine CPP–CS retrieval increased Fos expression. For CeA, nicotine UCS retrieval, but not nicotine CPP–CS retrieval, increased Fos expression. $n = 4$ per experimental condition. Data are mean $\pm$ SEM of Fos-positive cells per square millimeter. Green and red arrows indicate Fos protein and NeuN signals, respectively. Scale bars represent 50 μm . *Different from the home cage (HC) group; one-way analysis of variance; $p < .05$.

Figure 2.

Using tyramide-amplified immunohistochemistry–fluorescence in situ hybridization to examine the time courses of protein and messenger RNA (mRNA) expression of Fos in basolateral amygdala induced by nicotine conditioned place preference (CPP)–conditioned stimulus (CS) or nicotine unconditioned stimulus (UCS) memory retrieval. **(A)** Schematic diagram showing the neural activation principle. **(B)** Experimental procedures. We trained rats in the nicotine CPP procedures. Twenty-four hours later, we exposed the rats to nicotine CPP–CS or nicotine UCS memory retrieval and performed perfusion and brain dissection 0, 15, 90, and 180 minutes later. **(C)** Representative images showing blue (4',6-diamidino-2-phenylindole [DAPI]), green (Fos protein), red (*Fos* mRNA), and merged channels of

double-label neurons in basolateral amygdala. Scale bars represent 100 μm . **(D)** Time courses of Fos protein and *Fos* mRNA expression after nicotine CPP–CS memory retrieval. **(E)** Representative images showing green (Fos protein), red (*Fos* mRNA), and merged channels of tyramide-amplified immunohistochemistry–fluorescence in situ hybridization double labeling after CS retrieval. $n = 6$ per experimental condition. **(F)** Time courses of Fos protein and *Fos* mRNA expression after nicotine UCS retrieval. **(G)** Representative images showing green (Fos protein), red (*Fos* mRNA), and merged channels of tyramide-amplified immunohistochemistry–fluorescence in situ hybridization double labeling after UCS retrieval. Nicotine UCS 0 minutes, $n = 5$; nicotine UCS 15 minutes, $n = 7$; nicotine UCS 90 minutes, $n = 7$; nicotine UCS 180 minutes, $n = 6$. Data are mean $\pm$ SEM of Fos-positive cells per square millimeter. Scale bars represent 50 μm . *Different from the protein level of 0-minute group; #Different from the mRNA levels of 0-minute group; one-way analysis of variance; $p < 0.05$. Max, maximum; Min, minimum; min, minute.

Figure 3.

Using tyramide-amplified immunohistochemistry–fluorescence in situ hybridization to map neural ensembles reactivated by the nicotine conditioned place preference (CPP)–conditioned stimulus (CS) and nicotine operant–CS memory retrieval. **(A)** Experimental procedures. We trained rats in the nicotine self-administration (nSA) and CPP procedures. Twenty-four hours later, we exposed the rats to the two different memory retrieval manipulations or control conditions 90 minutes apart. **(B)** Representative images showing green (Fos protein), red (*Fos* messenger RNA [mRNA]), and merged channels of tyramide-amplified immunohistochemistry–fluorescence in situ hybridization double labeling of the different experimental conditions. **(C, D)** Effect of the experimental manipulations on Fos protein and *Fos* mRNA expression. **(E)** Percentage of overlap in different experimental manipulations, as calculated by Y/G or Y/R. **(F)** Scaled Venn diagrams showing the mean

number of green, red, and yellow labeling cells per square millimeter in basolateral amygdala in the different experimental groups. The main finding is that the nicotine CPP-CS, nicotine operant-CS retrieval, and sucrose operant-CS memory retrieval manipulations led to similar activation of basolateral amygdala neurons. Protein + mRNA double labeling was significantly lower in rats exposed first to nicotine CPP-CS memory retrieval and 90 minutes later to nicotine or sucrose operant-CS retrieval than in rats exposed twice to CPP-CS retrieval. Fos serves as a putative marker of neuronal ensemble activation. Home cage (HC) → HC, $n=5$; HC → nSA, $n=6$; CPP → HC, $n=8$; CPP → CPP, $n=8$; CPP → nSA, $n=7$; CPP → sSA, $n=6$. Data are mean $\pm$ SEM of Fos-positive cells per square millimeter. Scale bars represent 50 μ m. *Different from the HC-HC group; #Different from the CPP-CS group; \$Different from the CPP-nSA group; one-way analysis of variance; $p < 0.05$. G, number of green labeling (Fos protein) cells; R, number of red labeling (Fos mRNA) cells; sSA, sucrose self-administration; Y, number of yellow labeling (Fos protein and Fos mRNA) cells.

Figure 4.

Nicotine unconditioned stimulus (UCS) memory retrieval activates basolateral amygdala neurons that overlap with neurons activated by both nicotine conditioned place preference (CPP)–conditioned stimulus (CS) and nicotine operant–CS memory retrieval. (A) Experimental timeline. We trained rats in the nicotine self-administration (SA) and CPP procedures. Twenty-four hours later, we exposed the rats to the different memory retrieval manipulations (nicotine CPP–CS retrieval, nicotine operant–CS retrieval, and nicotine UCS retrieval) or to the different control conditions (novel context or sucrose operant–CS retrieval) 90 minutes apart. (B) Schematic illustrating the location of basolateral amygdala with Fos messenger RNA (mRNA) fluorescence in situ hybridization. Scale bars represent 50 μ m. (C, D) Effect of the experimental manipulations on Fos protein and *Fos* mRNA expression. (E) Percentage of overlap in different experimental manipulations, as calculated by Y/G or Y/R. (F) Scaled Venn diagrams showing the number of green, red, and yellow

labeling cells per square millimeter in basolateral amygdala in different experimental groups. The main findings are that exposure nicotine UCS memory retrieval induced higher Fos expression than nicotine CPP–CS memory retrieval (termed CPP in the figure) or nicotine operant–CS memory retrieval (termed SA in the figure) and that *Fos* mRNA induced by nicotine UCS memory retrieval overlapped with Fos protein induced by both nicotine CPP–CS and nicotine operant–CS memory retrieval. Fos serves as a putative marker of neuronal ensemble activation. HC → HC, $n = 7$; CPP → CPP + SA, $n = 9$; CPP → UCS, $n = 8$; SA → UCS, $n = 6$; CPP + SA → UCS, $n = 7$; CPP + novelty → UCS, $n = 9$; CPP + SA → sucrose (refers to operant sucrose–CS retrieval), $n = 5$. Data are mean $\pm$ SEM of Fos-positive cells per square millimeter. *Different from the HC→HC group; #Different from the nicotine CPP→UCS group; \$Different from all other groups; one-way analysis of variance; $p < 0.05$. G, number of green labeling (Fos protein) cells; R, number of red labeling (*Fos* mRNA) cells; Y, number of yellow labeling (Fos protein and *Fos* mRNA) cells.

Figure 5.

Selective inactivation of unconditioned stimulus (UCS) reactivated basolateral amygdala (BLA) neuronal ensembles that encode nicotine reward memories inhibited subsequent nicotine conditioned place preference (CPP) and operant nicotine seeking. **(A)** The Daun02 inactivation method. The Daun02 inactivation method employs *Fos-lacZ* transgenic rats where β -galactosidase (β -gal) and Fos are coexpressed within behaviorally activated neurons. Ninety minutes after the transgenic rats perform a behavioral or learning task, the activated neurons are inactivated by injecting the prodrug Daun02 into a given brain area. β -Gal within the behaviorally activated neurons converts Daun02 into daunorubicin, which disrupts normal function of these neurons [adapted from (32)]. **(B)** Experimental timeline:

Nicotine UCS memory retrieval. We trained rats in the nicotine self-administration (SA) and CPP procedures. Twenty-four hours later, we exposed the rats to nicotine UCS-induced memory retrieval (0.15 mg/kg, in home cage), and 90 minutes after this we injected them with Daun02 (2 µg/side) or its vehicle into the BLA. We then tested them for CPP expression (CPP tests 3 and 4) and operant nicotine seeking (extinction [EX] tests 1 and 2). **(C, D)** BLA injections of Daun02 decreased subsequent expression of nicotine CPP and operant nicotine seeking in the extinction tests. Vehicle, $n = 8$; Daun02, $n = 8$. **(E)** Representative images showing expression of β -gal in BLA and central amygdala (CeA). Scale bars represent 50 µm. Daun02 injections into BLA immediately after UCS-induced memory retrieval decreased subsequent expression of β -gal in BLA but not CeA (brains were taken 90 minutes after EX test 2). Data are mean $\pm$ SEM of β -gal-positive cells per square millimeter. **(F)** Experimental timeline: Nicotine CPP-conditioned stimulus (CS) memory retrieval. The experimental conditions were the same as for the UCS memory retrieval groups except that we injected Daun02 or vehicle immediately after exposure to CPP-CS memory retrieval (a short 5-minute exposure to nicotine-paired CPP chamber). **(G, H)** BLA injections of Daun02 decreased subsequent expression of nicotine CPP but had no effect on operant nicotine seeking in the EX tests. Vehicle, $n = 7$; Daun02, $n = 8$. Data are mean $\pm$ SEM. *Different from the vehicle group; mixed analysis of variance; $p < 0.05$.

Figure 6.

Schematic model of the effect of nicotine unconditioned stimulus (UCS)-induced memory reconsolidation interference procedure. Nicotine exposures are associated with multiple conditioned stimuli (CSs) whose memories are encoded by multiple basolateral amygdala neuronal ensembles. In the CS-induced memory retrieval–reconsolidation procedure, a neuropharmacological manipulation interferes only with reconsolidation of memories selectively associated with the reactivated CS without affecting memories associated with other CSs or the UCS. In the UCS-induced memory retrieval and reconsolidation procedure, the neuro-pharmacological manipulation interferes with reconsolidation of memories associated with multiple CSs and the UCS, leading to a strong inhibitory effect on nicotine preference and relapse.