Cycloheximide Enhances Maintenance of Methamphetamine-Induced Conditioned Place Preference

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Abstract

Accrued evidence demonstrated the necessity of protein synthesis at acquisition, consolidation and expression stages in conditioning /learning tasks, while the underlying mechanisms of the maintenance of memory remained less explored. This study was designed to characterize the maintenance of methamphetamine-induced conditioned place preference, a drug-induced learning and memory. In addition, cycloheximide, a protein synthesis inhibitor, was used to examine the involvement of protein synthesis in the maintenance of such place preference memory. We found that the maintenance of the rapidly-established methamphetamine (2 mg/kg, i.p.) -induced conditioned place preference could be long-lasting and even over fifty days under the present protocol of extinction. Moreover, it was of interest to note the undulating expression of this conditioned place preference throughout the extinction protocol. Most importantly, as the methamphetamine-induced conditioned place preference was acquired and expressed by mice, the saline-pretreated control mice underwent numbers of intermittent extinction across a long-term retention test period, while cycloheximide-pretreated mice exhibited unaltered methamphetamine -induced conditioned place preference throughout the same retention test period. Taken together, we conclude that [1] methamphetamine-induced conditioned place preference could last for a long period of time, and such place preference memory is reluctant to extinguish even animals' repeated exposure to the previous conditioned environment at a drug-free status, and [2] blockade of protein synthesis may enhance the maintenance of the methamphetamine-induced conditioned place preference.

Key Words: methamphetamine, cycloheximide, protein, extinction, conditioned place preference

Introduction

Memory formation *via* information encoding (learning) and the subsequent stabilization of such experiences (consolidation) required activation of a variety of intracellular signaling molecules (1). Some of these message transduction cascades ultimately resulted in gene expression and protein synthesis, which have been considered necessary for certain long-term memory (16, 21). The protein synthetic process and various forms of long-term memory formation, thus, can be suppressed by protein synthesis inhibitors. This notion has been validated in many species, ranging from rodents to hermissenda, in many forms of memory (14, 27, 41).

However, the roles of protein synthesis in the extinction of an established memory remained scarcely studied. A spontaneous recovery of the learned responses after a period of time lapse (39) and a rapid speed in relearning the responses (35) have been reported even following a complete extinction of the learned responses. These findings strongly suggested that memory extinction may not be intuitively viewed as a forgetting or an erasing process but an extinction in memory expression (1). A few lines of evidence have demonstrated that the learned responses disappeared, whereas the memory trace seemed

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to remain intact (8, 36). Paradoxically, in an attempt to monitor the explicit extinction of the learned responses, retention tests are inevitably performed. These retention tests themselves, are a new situation with no specific response reinforced. Thus, the extinction of the learned responses could involve the acquisition of this new, distinctive experience, and the consequential, adaptive expression of the lately-learned nonreinforced responses in the retention tests (13). Given the documented characteristics of learning and memory formation, protein synthesis involvement was reasonably suspected in this regard. Not surprisingly, protein synthesis inhibition beginning from the retention tests could suppress the memory formation of such lately-learned nonreforced responses while spare the previously-learned responses. For example, infusion of a protein synthesis inhibitor, anisomysin, into the insular cortex blocked the extinction of conditioned taste aversion (6). Moreover, microinjection of anisomysin and messenger RNA synthesis inhibitors, DRB and alpha-amanitin, into the hippocampal CA1 region blocked the response extinction in the inhibitory avoidance task (46, 47).

Conditioned place preference (CPP) was a frequently-used behavioral paradigm to evaluate the reinforcing efficacy (2, 5, 44), maintenance, and reinstatement in various drugs of abuse, including stimulants, dopaminergic drugs, opiates, ethanol and nicotine/cholinergic drugs (7, 15, 24, 25, 29, 32, 40). Acquired drug-induced CPP, through a conditioning/learning process, extinguished progressively with repeated retention tests at a drug free status (4, 29) and completely following the forced extinction training protocol (3, 9, 22). The repeated retention tests and/or forced extinction trainings, in this regard, could serve as a new and distinctive learning/conditioning in this drug-induced CPP paradigm. As such, protein synthesis was speculated to be involved in the extinction of the drug-induced CPP. Thus, this study aimed to examine the effects of cycloheximide, a protein synthesis inhibitor, on the extinction of the methamphetamine (MA)-induced CPP. The blocking effects of cycloheximide on the extinction of MA-induced CPP were anticipated due to the cycloheximide-associated deteriorating effects on the lately-learned responses associated with the retention tests.

The maintenance of an acquired CPP following the repeated daily retention tests ranged from 6 to 12 days in cocaine (20, 38), whereas the maintenance of other learning and memory paradigms following the similar extinction protocol ranged 1-5 days, including inhibitory avoidance (10, 37, 47), context-elicited fear (13, 23), conditioned taste aversion (6) and spatial memory of Morris water maze (23). In this study, a long-term repeated daily retention test was conducted throughout the extinction process of the MA-induced CPP to delineate the characteristics of the long-enduring maintenance of the MA-induced CPP.

Materials and Methods

Subjects

Male C57BL/6NCrj mice, aged 7-8 wks of age (NCKU Lab Animal Center, Tainan, Taiwan), were used with access to food and water *ad libitum*. The colony room was temperature- and humidity-controlled and maintained on a 12 h light/dark cycle (lights on at 0700). All experiments were performed in a laboratory with the temperature maintained at 21±1°C during the light cycle. This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the local Animal Care Committee at National Cheng Kung University College of Medicine.

Place Preference Apparatus

Drug conditioned pairings and drug-induced CPP tests were performed in four identical, commercial place preference apparatus designed for mice (Med Associates Inc., Georgia, Vermont, USA), each containing three compartments separated by automatically-controlled guillotine doors. The two end compartments ($17 \times 13 \times 13$ cm) were interconnected by a center compartment ($9 \times 13 \times 13$ cm). The end compartments were used for drug conditioning, while the center compartment was used only for the CPP tests. There were three distinctive cues among the three compartments: color of walls, texture of floors and the degree of illumination. One end compartment had opaque white walls, a wire-mesh floor and medium (approximately 160 Lux) or dim (approximately 40 Lux) light illumination, and the other end compartment had opaque black walls, a steel bar grid floor and dim or medium light illumination. The center compartment had opaque gray wall, a gray PVC plastic platform floor and the brightest light illumination. There was a 5×6 cm vaulted doorway cut between the end and center compartments that could be occluded by the guillotine doors. All floors were raised 2.5 cm above the table to reduce urine and feces accumulation. Mouse location in the compartment was monitored by photocell detectors aligned 1.5 cm above the floor across three compartments with 3 cm apart, connected *via* interface card to IBM-compatible PC and the time spent in each compartment was recorded by the MED-PC software for Windows. Our preliminary data indicated that naive animals, when allowed to freely explore all the compartments, showed no biased preference for either end compartments, although they spent the least amount of time in the center compartment.

Place Preference Conditioning and Extinction Protocol

Except the mice used in Experiment 1, all mice were

submitted to three phases of experimental manipulation: MA-induced CPP trainings, CPP tests and CPP extinction with repeated daily retention tests. MA-induced CPP trainings consisted of totally six days of pairings, three MA (2 mg/kg) and three saline pairings, respectively. During the daily 30-min pairings, each animal was confined in one end compartment (MA-paired side) immediately following intraperitoneal (i.p.) administration of MA on days 1, 3 and 5, whereas confined in the other end compartment (saline-paired side) after saline injection (i.p.) on alternate days 2, 4 and 6. Twenty-four hours after the conclusion of MA-induced CPP trainings, the mice underwent the CPP tests. They were placed into the center compartment at the beginning of the CPP tests with free access to the three compartments for a 15-min period at a drug-free status. Successful expression of the MA-induced CPP was determined for each mouse. Since the time the mice spent in center compartments may affect the CPP expression across different trials in mice, preference indices, as calculated in the formula, [(the time spent on MA-paired side) - (the time spent on saline-paired side) / (the time spent on both side)] × 100, were standardized for the comparisons in the magnitude of the MA-induced CPP between animals across the tests in different experiments. The CPP extinction referred to a significantly lower preference index observed during the repeated retention tests when compared to the preference index obtained in the CPP tests.

Experiment 1. The Exploratory Test for Saline-Pretreated Mice after Saline-Paired Trainings in the CPP Apparatus

Although our preliminary data indicated that naive animals, when allowed to freely explore in the commercial CPP apparatus, showed no biased preference for either end compartment, MA-induced CPP and its modulation involved injections as routes for drug and vehicle delivery. To avoid the administration confounds, twenty-two mice received six days of saline (i.p.) pairings. Thirty minutes before each saline pairing, a subcutaneous (s.c.) injection of saline was given. Following the CPP trainings, they underwent CPP tests and their preference indices obtained in this experiment served as the preference index baseline for both Experiments 2 and 3.

Experiment 2. MA-Induced CPP Extinction with Repeated Daily Retention Tests

Eighteen mice underwent MA-induced CPP trainings, followed by the CPP tests to assure their acquisition in MA-induced CPP. Then, they were randomly assigned to two groups, Repeat-test group and Nonrepeat-test group. One day after the CPP tests, the mice in Repeat-test group started daily 15-min retention tests for seventeen consecutive days. Then, they underwent another retention test on day 50 after their CPP tests. Mice of Nonrepeat-test group were left in their home cages without experiencing daily retention test except on day 17 after their CPP tests.

Experiment 3. The Effects of Cycloheximide on Maintaining the MA-Induced CPP

Thirty-two mice were used and the detailed procedure in MA-induced CPP and CPP tests were performed as previously described. They all acquired MA-induced CPP and were randomly divided into two groups, the cycloheximide-pretreated (n = 16) and the saline-pretreated groups (n = 16). Twenty-four hours after the CPP tests, eight mice in cycloheximide-pretreated group received a 15 mg/kg cycloheximide injection (s.c.) while the remaining eight mice received a 30 mg/kg cycloheximide injection (s.c.) before the retention tests on a daily basis. Two saline-pretreated groups (n = 8 for each group), serving as the corresponding controls, received an equivalent volume of saline administration (s.c.) under a similar treatment regimen. Significantly, the daily dose of 15 mg/kg cycloheximide did not affect the locomotor activities in mice for at least 5 consecutive days (data not shown), while 30 mg/kg cycloheximide started to severely impair the locomotor activities on day 3 after use.

Drugs

Methamphetamine hydrochloride was purchased from National Bureau of Controlled Drugs in Taiwan. Cycloheximide was obtained from Sigma (St. Louis, MO, U.S.A) and stored in a 4°C refrigerator before use. Both drugs were dissolved in 0.9 % saline.

Statistical Analyses

One way ANOVA was employed to examine the mean differences in preference indices obtained from the exploratory and CPP tests. To examine the blocking effects of cycloheximide on the extinction of MA-induced CPP, a mixed-design repeated measure ANOVA was used, followed by Fisher's protected least significant difference (Fisher's PLSD) for post-hoc multiple comparisons if appropriate. The levels of statistical significance were set at $P<0.05$.

Results

Experiment 1. Saline-pretreated Mice Did Not Exhibit Biased Place Preference between Two End Compartments under the Saline-Paired Trainings

The repeated measure ANOVA for end compartments revealed that saline-pretreated mice spent comparable amount of time (mean±S.E.M. in second)

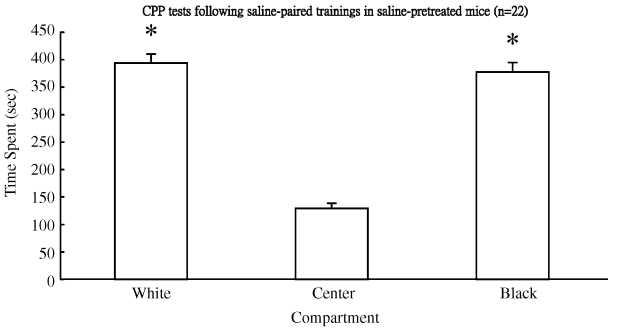

Fig. 1. No preference bias was observed between the end compartments in saline-pretreated mice under a saline-paired training protocol. Time spent (in seconds) in the white, center, and black compartments was represented as mean ± SEM. *Significantly greater than the time spent in the center compartment, $P<0.05$.

in two end compartments (393.7±16.9 and 377.3±17.1) and less time in the center compartment (129.0±9.6) ($F(2, 44)=65.915$, $P<0.01$) after the saline-paired trainings (Fig. 1). Likewise, our preliminary data has demonstrated that naive mice exhibited an indistinctive preference to explore the end compartments. These results, taken together, indicate the unbiased design of the commercial CPP apparatus and our training protocol. Thus, the preference index obtained in this Experiment can be utilized as the baseline against which the preference indices derived from the acquisition and extinction of MA-induced CPP in Experiments 2 and 3 were determined.

Experiment 2. MA-Induced CPP Maintained Unaltered over a Long Period in the Repeated Daily Retention Tests

Repeat-test and Nonrepeat-test groups demonstrated comparable preference indices in CPP tests and both group indices were greater than the index obtained from mice in Experiment 1 ($F(2,37)=8.751$, $P<0.01$) (Fig. 2), indicating an established MA (2 mg/kg)-induced CPP in these mice. Furthermore, one way repeated measure ANOVA revealed that the preference indices fluctuated across the retention test period ($F(18,144)=2.286$, $P<0.01$) with mice showing a significantly lower preference index only on day 15 following the CPP tests (Fig. 2). These results suggest that the acquired MA-induced CPP resisted extinguishing under a repeated daily retention test protocol. Finally, the preference index in Nonrepeat-test group remained unchanged on day 17 following the CPP tests, implying that the MA-induced CPP could be sustained/intact for at least 17 days even if repeated daily retention tests were omitted.

Experiment 3. The Effects of Cycloheximide on Maintaining the MA-induced CPP

In CPP tests, one-way ANOVAs revealed group differences in preference indices ($F(2,37)=14.92$, $P<0.001$; $F(2,35)=6.58$, $P<0.01$) with the saline- and cycloheximide-pretreated groups exhibiting comparable preference indices and both indices were greater than the baseline index ($P<0.01$ and $P<0.01$) (Fig. 3A and Fig. 4). These results revealed that these two groups of mice acquired the MA-induced CPP. Moreover, two-way mixed-design repeated measure ANOVA revealed a day by group interaction ($F(16, 224)=2.158$, $P<0.01$). The preference indices in saline-pretreated group seemed to progressively decrease, while the indices in cycloheximide (15 mg/kg)-pretreated group maintained unaltered, if not increased, throughout the retention test days (Fig. 3A). Preference indices on days 10, 15 and 16 (two consecutive days) in saline-pretreated group were significantly lower than their indices in CPP tests ($P<0.05$). The repeated extinctions observed for two consecutive days in these animals seemed to represent a reliable extinction in such MA-induced CPP. Most importantly, the preference indices in the cycloheximide-pretreated group were significantly greater than the indices in the saline-pretreated group on days 8, 10, 12, 14 and 16 ($P<0.01$) (Fig. 3A). The time the mice spent in three compartments was depicted for cycloheximide (15 mg/kg)- and saline-pretreated mice, respectively, across the repeated daily test period (Fig. 3B and 3C). In parallel with the above-mentioned analyses in preference indices, the saline-pretreated mice demonstrated greater time spent in the MA-paired compartment than in the saline-paired compartment except on days 10, 15, and 16. In addition, cycloheximide-pretreated mice exhibited greater time spent in MA-paired compartment than in saline-paired compartment across the retention test period. Likewise, two-way mixed-design repeated measure ANOVA revealed a day by group interaction ($F(10, 140)=1.904$, $P<0.05$), indicating that the differences in preference indices between cycloheximide (30 mg/kg)- and saline-pretreated group progressively increased across the retention test days (Fig. 4). The preference index on day 4 in saline-pretreated group was lower than the index in their CPP tests ($P<0.05$). The preference indices on days 4, 8, 9 and 10 in cycloheximide-pretreated mice were greater than the corresponding indices in saline-pretreated group ($P<0.05$). It was interesting that cycloheximide (30 mg/kg)-pretreated mice exhibited greater preference indices on days 5, 8, 9, and 10 than their indices obtained in the CPP tests ($P<0.05$).

Discussion

Methamphetamine-induced CPP, once established, was reluctant to extinguish over a long period of time regardless of the insertion of the repeated,

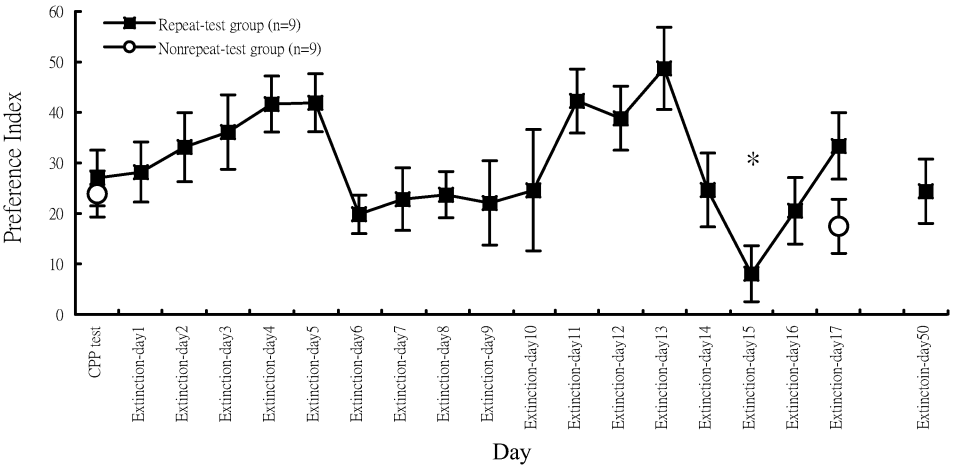

Fig. 2. Methamphetamine (MA, 2 mg/kg)-induced conditioned place preference (CPP) and its maintenance across a repeated daily retention test protocol. Repeat-test group underwent daily retention tests following the CPP test, while Nonrepeat-test group experienced the retention test only on day 17 after their CPP tests. Nonrepeat-test group exhibited a comparable preference index on day 17 as it on the CPP test. Repeat-test group exhibited MA-induced CPP on day 50 after the CPP test under a condition of retention test omission from days 18 to 49. *Significantly lower than the preference index obtained in the CPP test, $P<0.05$.

non-reinforced retention tests. Although the mice, which have acquired and expressed the MA-induced CPP, demonstrated their initial extinction on day 15 after the CPP tests under the repeated daily test protocol in Experiment 2, such occasional CPP extinction might not be a reliable and irreversible extinction. Two lines of evidence support the notion that occasional extinction can not appropriately predict a stable decrease in learned performance. First, the preference indices of the mice in Experiment 2, returned to a similar level as their baseline obtained in the CPP test for the following two days (days 16 and 17). Second, saline-pretreated mice, serving as cycloheximide (15 mg/kg)-pretreated controls in Experiment 3, showed their initial extinction on day 10 after the CPP tests, while their preference indices rebounded to an indistinctive level as their baseline for the next few days (days 11, 12, 13). Likewise, in Experiment 3 another group of saline-pretreated mice exhibited their initial extinction as early as on day 4 after the CPP tests, while their preference indices remained at a comparable level with their baseline for the following test days (days 5-10). We, thus, suggest that two or more consecutive days of extinction can be used for reliably determining the extinction of MA-induced CPP. Nonetheless, even continuous extinction in CPP performance may not guarantee a permanent extinction of this drug-induced CPP since we found that mice were capable of maintaining the MA-induced CPP for over 50 days as revealed in Experiment 2. These findings are of importance in two-fold. First, when the adaptive mechanisms of MA abstinence and relapse are studied, an extraordinarily long time lapse is expected in achieving the appropriate paradigms. Second, since acquired MA-induced CPP may sustain without re-exposure to MA, it is reasonable to suspect that the MA-related environmental cues could acquire and maintain their reinforcing effects in a long-term manner. In fact, the conditioned emotional significance of the drug-paired environment (the conditioned stimulus) was reported to persist over a long period of time (28). The lapse of time alone, accordingly, might not be sufficient to disrupt the emotional salience and/or reinforcing effects of the drug-related environmental cues.

Several neuronal substrates and intracellular molecules have been reported involved in the acquisition, consolidation, and expression of abuse drug-induced CPP. Calcium channel inhibitors have been demonstrated to block cocaine- and amphetamine-induced CPP in rats (30, 33, 34). A non-competitive NMDA receptor antagonist, MK-801 blocked the acquisition of cocaine- and morphine-induced CPP (12, 45), whereas a competitive NMDA receptor antagonist, CGP37849, attenuated the morphine-induced CPP (45). An irreversible inhibitor of GABA transaminase, gamma-vinyl GABA, blocked the expression and acquisition of cocaine-, nicotine- and heroin-induced CPP (17, 18, 31). A non-selective inhibitor of protein kinases, H7, reduced the consolidation of cocaine-induced CPP (11). Inhibition of calcium-calmodulin kinase-II attenuated both the acquisition and reinstatement of morphine-induced CPP (19, 26) and the acquisition of amphetamine-induced CPP (42). Suppression of c-fos induction by injection of an antisense oligodeoxynucleotide into the nucleus accumbens blocked the acquisition of morphine-induced CPP (43). However, the roles of down-stream mRNA and protein expression in the maintenance and/or extinction of the MA-induced

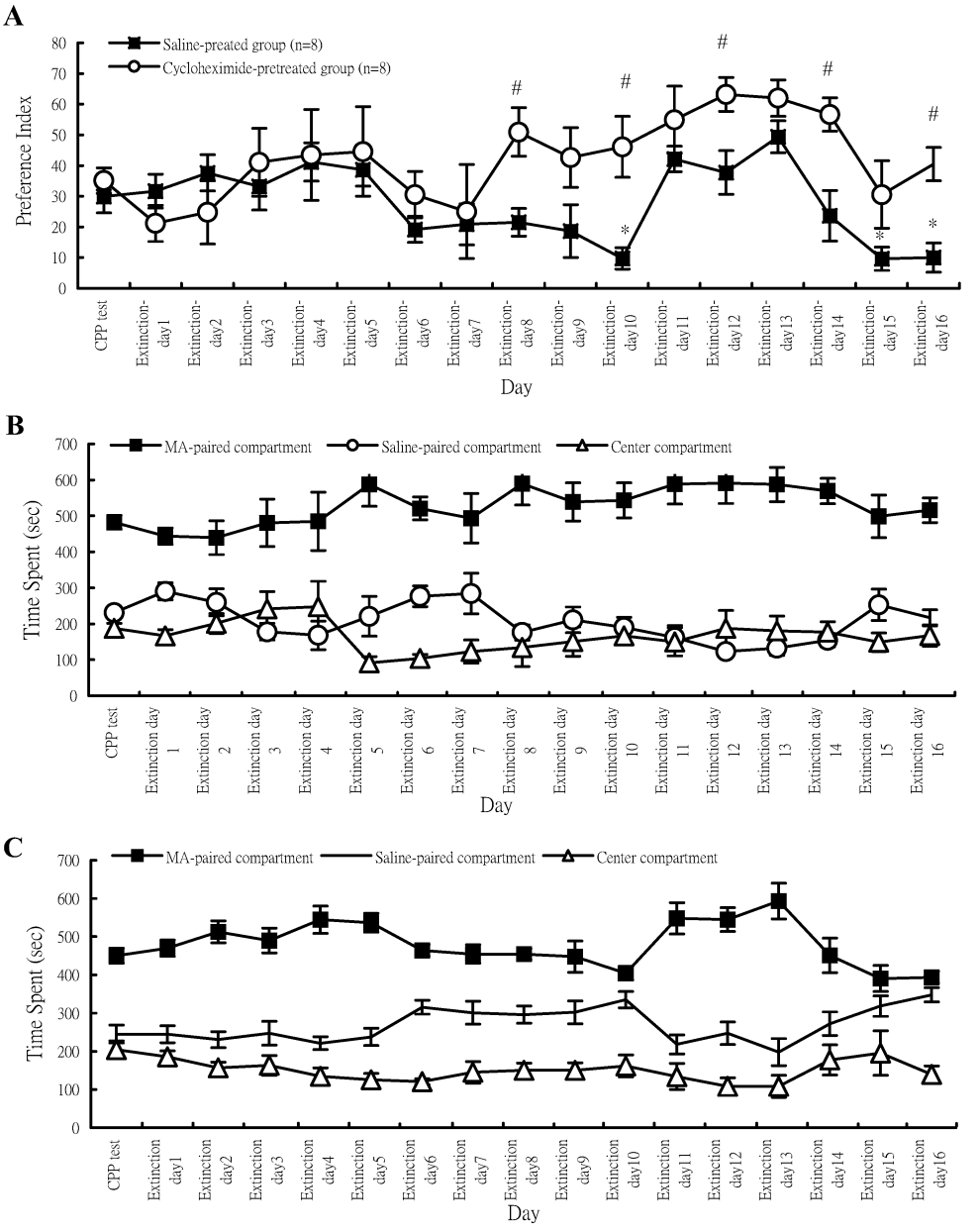

Fig. 3. MA-induced CPP and the blocking effects of cycloheximide on the extinction of such CPP across the repeated daily retention tests. Panel A depicts the effects of cycloheximide (15 mg/kg) and an equivalent volume of saline, i.p. injected 30 min before each retention test, on the extinction (indexed by preference indices) of the MA-induced CPP. *Significantly lower than their preference index observed in the CPP test, $P<0.05$. #Significantly greater than the corresponding indices observed in saline-pretreated group, $P<0.05$. Panel B depicts the time spent (in seconds) in each compartment for the cycloheximide-pretreated mice, whereas panel C depicts the time spent in each compartment for the saline-pretreated mice across the 16-day retention tests. All the time spent in MA-paired and saline-paired compartment were different except on days 10, 15, and 16 in saline-pretreated mice.

CPP have yet been vigorously studied. We, hereby for the first time, reported that protein synthesis blockade played a pivotal role in maintaining and/or avoiding the extinction of MA-induced CPP.

Mice, as the retention test progressed, demonstrated observable, though unreliable, extinction in MA-induced CPP, implying that the nonreinforced retention test could be a distinctive form of learning from the MA-induced CPP trainings. Though similar context cues are shared, these two forms of learning are substantially different in many ways, including confined space for exploration, subjective status for MA or no injections, and the training period. The acquisition and/or consolidation of these retention test-associated nonreinforced responses may require protein synthesis. Thus, cycloheximide pretreatment may block the protein synthesis necessary for the acquisition and consolidation of such responses

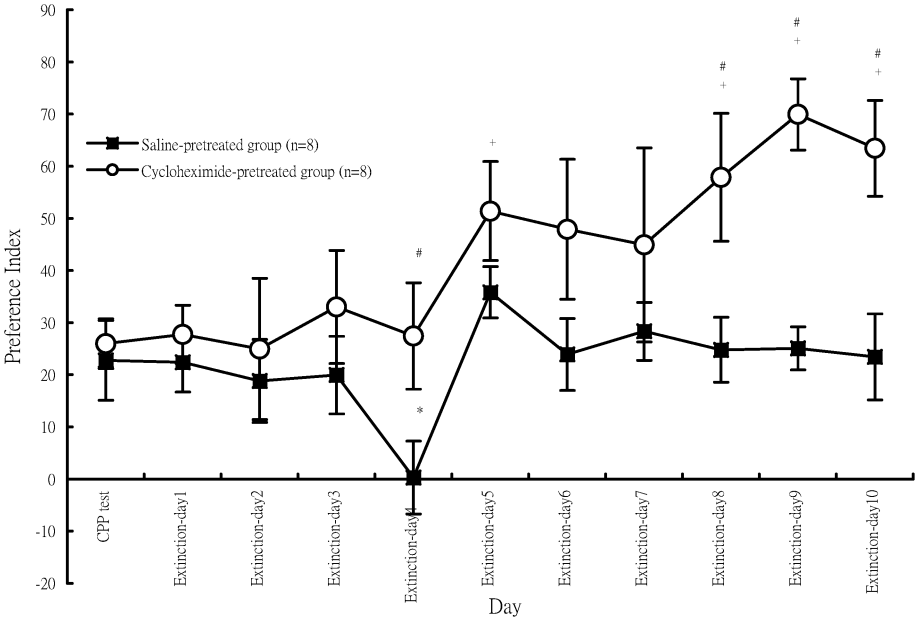

Fig. 4. MA-induced CPP and the blocking effects of cycloheximide (30 mg/kg, i.p. injected 30 min before each retention test) on the extinction of such CPP across the 10-day retention tests. All data after day 10 were excluded for further analysis due to the mortality seen in the cycloheximide-pretreated group. *Significantly lower than their preference index observed in the CPP test, $P<0.05$. #Significantly greater than the corresponding indices observed in saline-pretreated group, $P<0.05$. +Significantly greater than their preference indices observed in the CPP test, $P<0.05$.

associated with the retention tests, whereas the MA-induced CPP remains intact. However, alternative explanations might predict the same observations. Cycloheximide pretreatment before the daily retention tests could interrupt the expression of CPP extinction. This possibility is less likely due to our observations that although cycloheximide-pretreated mice may exhibit MA-induced CPP after a forced extinction protocol, they did not demonstrate CPP extinction immediately when cycloheximide pretreatment was replaced by saline injection. Another explanation involves the immediate and/or long-lasting, motor-impairing effects of cycloheximide pretreatment and thus the formed MA-induced CPP can be sustained. Our preliminary data have shown that 5 consecutive days of cycloheximide treatment (15 mg/kg/day, i.p.) did not affect mouse locomotor activities. Moreover, accumulated and long-lasting motor-impairing effects of cycloheximide were not evident since a relatively constant level of exploration time was observed in the center compartment for the cycloheximide-pretreated mice (Fig. 3B). Finally, cycloheximide could enhance the MA-induced CPP reconsolidation, as indicated in both cycloheximide (15 and 30 mg/kg)-pretreated groups (Fig. 3A and Fig. 4). However, no such effect can be seen in the initial few days in either cycloheximide-pretreated group. Even more so, the preference indices could be greater than their index baseline obtained in the CPP tests on one day, while drop back to the baseline the next day. These findings, thus, do not support the reconsolidation-enhancing role of cycloheximide in this MA-induced CPP paradigm.

In sum, we conclude that 1) MA-induced CPP, once established, was reluctant to extinguish over a long period of time in the previous conditioned environment regardless of the insertion of repeated, non-reinforced retention tests, and (2) blockade of the protein synthesis can enhance the maintenance of the MA-induced CPP or avoid the extinction of the MA-induced CPP.

Acknowledgments

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