# Distinct Behavioral Responses of Chocolate and Morphine-Induced Conditioned Place Preference In Mice

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## Abstract

Addictive-like eating behavior has been studied to understand the underlying mechanisms of food addiction-induced obesity disease. The highly palatable food including chocolate can trigger addiction processing by activating the same drug-generated reward system in the brain. However, the influences of chocolate consumption especially without fasting condition on motivational reward effect in response to conditioned environmental cues have remained a controversy. Therefore, the purpose of this study was to determine the reward effect of chocolate-induced condition place preference (CPP) in comparison with the standard addictive drug, morphine. Male Swiss albino ICR mice (20-30 g) were used to examine reward-associated cues using a Y-shape CPP apparatus. The 27-day experiment consisted of habituation, pre-condition, condition, post-condition, extinction, extinction-test, and reinstatement periods. The condition induced-behaviors (e.g., CPP score, number of entries, locomotor) were analyzed to determine the motivational reward. The authors found that the sated animal did not reveal significant changes of chocolate condition induced-behaviors during all 3 conditions: post-condition, extinction-test, and reinstatement periods. In contrast, morphine treatment induced obvious changes in behavioral patterns associated with morphine condition including CPP score and the number of entries. In conclusion, these results indicated that chocolate cues might not be strong enough to trigger processing in the brain of sated mice.

***Keywords:*** *Addiction, Condition place preference, Palatable food, Chocolate, Morphine*

## 1. Introduction

A recently published report led by the World Obesity Federation has supported the definition of obesity as a "chronic disease." In 1997, the World Health Organization (WHO) declared obesity as a major public health problem and a global epidemic. Obesity is considered to be a public health concern and is characterized as the excessive accumulation of adipose tissue in the body, which is a risk factor for a variety of chronic diseases, including type II diabetes mellitus, cardiovascular diseases, and cancer ("WHO | Obesity: preventing and managing the global epidemic," 2015). The causes of increased obesity remain unclear. The main characteristics of obesity are the decrease in energy expenditure associated with the increase in food consumption, particularly high palatability and energy density of the food. Obesity itself is not triggered by a lack of motivation for weight loss, but some foods can trigger the addiction process by activating the same drug-generated reward system in the brain, the mesolimbic dopamine system (Campana, Brasiel, de Aguiar, & Dutra, 2019).

In general, food is a powerful reinforcer, especially palatable food. Motivation to obtain and consume palatable foods is an important factor in the control of food intake and plays a key role in the development and maintenance of obesity (Tracy, Wee, Hazeltine, & Carter, 2015). Palatable food typically contains high amounts of sugar, fat or salt, and exerts a hedonic effect that stimulates food intake in the absence of hunger, which is driven by the reward properties. Motivation-driven food intake is termed hedonic or non-homeostatic feeding while energy or nutrients-driven food intake is known as homeostatic feeding (Muñoz-Escobar, Guerrero-Vargas, & Escobar, 2019). The various experiments in animals have used chocolate as reward-associated learning. Chocolate that served as a highly palatable food reward, has been

used as the major method for motivating animals in basic learning experiments based on the reinforcing effects of food. Previous studies have indicated that consuming chocolate evokes pleasant feelings, reduces tension, and improves mood (Macht & Dettmer, 2006; Meier, Noll, & Molokwu, 2017). Furthermore, chocolate elicits unique brain activity compared to other high-sugar and high-fat foods, recruiting brain structures that respond to craving-inducing stimuli, and is, therefore, more likely to provoke an addictive-like eating response (Asmaro & Liotti, 2014).

The addictive-like behavior has been studied with various reward stimuli. Morphine is a standard addictive drug that has been popularly used to study the mechanism of the reward brain circuit. The mu-opioid receptor in the ventral tegmental area (VAT) is a key to the reward-related morphine pathway that activates the dopamine (DA) release in the reward circuit(Kim, Ham, Hong, Moon, & Im, 2016). Also, many reported indicated that chocolate seems to activate the circuit of reward and pleasure in the brain, through the release of dopamine similarities to addictive drugs. However, reward processing related to motivational-induced overconsumption of palatable food as well as chocolate has been unelucidated. Several methods have been implied to examine the rewarding effect of natural rewards (Suhaimi et al., 2016), addictive drugs(Leite-Morris et al., 2014), or palatable foods (Derman & Ferrario, 2018; Duarte et al., 2014). The condition place preference (CPP) model is one of the popular techniques that has been used to examine the reward properties of addictive substances in an animal model. The CPP paradigm is based on Pavlovian conditioning that determines the rewarding effect in association with an environmental cue. It's well known that the environmental cue can drive the animal to perform a learned behavior that animal to prefer one of the environments paired with a rewarding stimulus more than the others (Huston et al., 2013a).

Interestingly, the environmental cue is an important factor that activates food intake. It has been suggested the closely associated with the addictive-like behavior in both palatable foods (Herman & Polivy, 2008; Popkin, Duffey, & Gordon-Larsen, 2005), and addictive drugs (Gillman, Kosobud, & Timberlake, 2008). The study suggested that the environment can motivate the motivational properties that override the homeostatic control. The conditioned environment affects to stimulates eating in the sated state or inhibits eating in states of hunger (Petrovich, 2011). Even though, palatable food has been suggested to shares similar properties with the addictive drug. However, the mechanism underlying palatable food-induced addictive-like behavior remained unclear. Moreover, few studies investigated the effect of the environmental condition related to palatable food without fasting. Therefore, the purpose of this study was to determine the rewarding effect of chocolate-induced CPP in comparison with the standard addictive drug; morphine. The 27-day experiment consisted of habituation, pre-condition, condition, post-condition, extinction, extinction-test, and reinstatement period. The authors hypothesized that chocolate and morphine-induced CPP might reveal some distinct patterns of behavioral changes.

## 2. Objectives

The study aims to investigate the rewarding effect of chocolate in comparison to morphine by using the condition place preference (CPP) paradigm in mice.

## 3. Materials and Methods

### *3.1. Animals and chemical substances*

Male Swiss Albino (ICR) mice (7-8 weeks) were purchased from Nomura Siam International Co., Ltd. and kept in animal houses at the Prince of Songkla University. All mice were placed separately in laboratory animal houses at 23 ± 2 °C with 55 ± 10% relative humidity and 12 hours light/dark cycle with the lights off at 19:00 based on the Guidelines of the International Committee on Laboratory Animals. The animals were fed irradiation-sterilized pellet feed (No. CP082, Perfect Companion Group Co., Ltd., Bangkok, Thailand) and allowed access to distilled water ad libitum and acclimated to laboratory conditions for 7 days before the experiment. All the animal experimental procedures were authorized by the Prince of Songkla University Ethical Committee (MHESI 6800.11/124) and conducted under internationally accepted principles for laboratory animal use and care. The experiment was operated from 08:00 to 18:00. Morphine sulfate was

dissolved in saline solution and injected in subcutaneous (s.c.) with 15 mg/kg. Milk chocolate was used in this experiment and consisted of sugar 37%, skim milk powder 18%, cocoa mass 16%, and cocoa butter 13%.

*3.2. Experimental design*

Condition place preference (CPP) to place in Y-maze Plexiglas, as described previously by, that comprised three compartments including a neutral arm(stem), paired stimulus arm (CS+), and non-paired stimulus arm (CS-) as shown in Figure 1c. Each arm differentiated wall color and floor texture served as visual and contextual cues. The arms were connected with a triangular central zone. Prior to the testing period, mice were given 2 g of chocolate within their home cages to prevent a neophobia effect. Then, they were divided into 3 groups including the control (n=4), morphine (n=5), and chocolate (n=5) groups (Figure 1A).

*3.3. The condition place preference (CPP) procedure*

The CPP procedure includes 4 phases within 27 days as shown in Figure 1B. The schedule began with three days of habituation and was followed by a pre-conditioning day. On the pre-conditioning day, all mice were allowed to freely explore the chamber for 15 minutes. During the conditioning period, the animals were given a paired stimulus (chocolate or morphine injection) and immediately confined within the CS+ arm for 30 minutes (on C2, C4, C6, C8, and C10 day). For the alternative days, the mice were confined within CS-arm for 30 minutes (on C1, C3, C5, C7, and C9 day) and received either saline injection or food pellets. Therefore, the post-conditioning day was performed as same as the pre-conditioning day. In the extinction period, instead of received the paired stimuli (chocolate or morphine) and confine within CS+, all mice were given the saline injection or food pellets and confined within the CS+ arm for 30 minutes (on Ex2, Ex4, Ex6, Ex8, and Ex10 day) while they were confined within the CS- arm for 30 minutes on the alternative days (Ex1, Ex3, Ex5, Ex7, and Ex9 day). For the extinction test, all mice were given the saline injection and food pellets before explored thoroughly the chamber freely for 15 minutes. On the last day of the procedure, the reinstatement test, the mice were given the paired stimuli before explored thoroughly the chamber for 15 minutes.

*3.4. Statistical analysis*

Animal behavior was recorded on the pre-conditioning, post-conditioning, extinction test, and reinstatement test days by using a video camera mounted over the CPP apparatus. The videos were analyzed by the Autotyping toolbox (Gullotti et al., 2014) following these parameters: locomotor, number of entries, and time spent in each arm. The preference of each Y-maze arm was calculated as CPP score index following this formula:

$$\text{CPP score} = \frac{(\text{time spending on each arm})}{(\text{timeCS+}) + (\text{timeCs}-) + (\text{time stem})}.$$

All data were normalized by the differentiation with the pre-condition (Δ). For the statistic analysis, the data were averaged and displayed as mean ± Standard Error of Mean (S.E.M.). The significant differences among the treatment groups and conditions including, post-condition, extinction, and reinstatement were analyzed using two-way ANOVA repeated analysis since the experiment was designed as a repeated data collection from the same set of animals. Multiple comparisons using Tukey's post hoc were implied to indicate specific points of significance between groups. The differences were considered statistically significant at a p-value $< 0.05$.

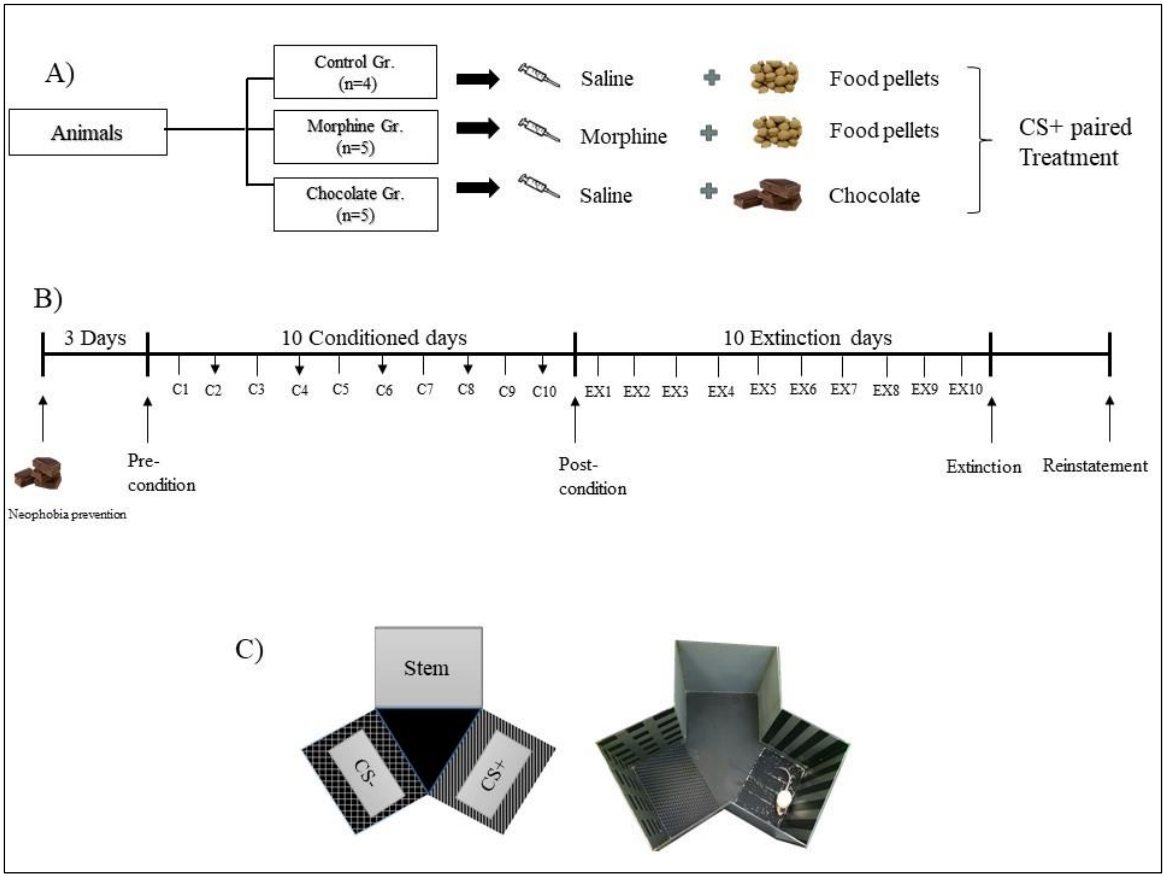


**Figure 1** Schematic diagram of the overview of experimental design. Animals were divided into 3 groups: control, morphine, and chocolate group (A). The schedule of the CPP procedure comprised 3 days of chocolate habituation to prevent chocolate neophobia for the chocolate group, pre-condition, condition, post-condition, extinction, and reinstatement (B). CPP procedure was performed using Y-shape CPP apparatus, which consists of 3 arms with different wall colors and floor textures (C). Each arm was randomly assigned as a paired side (CS+) or unpaired side (CS-) while the grey wall arm was assigned as a neutral side or stem arm.

## 4. Results and Discussion

### 4.1. Results

The CPP score of each arm was analyzed and shown in Figure 1C – E. Animal locomotion and position was tracked and represent as a heat map of time preferences as shown in Figure 1B. Form heat map, reinstatement of morphine group showed a pronounce time preference over CS+ arm indicated with high yellow tone color, while other condition and treatment did not differ. Therefore, the CPP score was calculated and normalized with the pre-condition to clarify the time or side preference. The CPP score was shown separately for each side preference including CS+ arm or paired-arm (Figure 1C), CS- arm or unpaired-arm (Figure 1D), and stem arm or neutral arm (Figure 1E). Two-way ANOVA repeated analysis of CPP score revealed a significant difference between treatment groups of the CS+ arm ($F_{2,11} = 7.837$, p-value < 0.01). Morphine treatment showed a significantly higher CPP score in reinstatement compare to chocolate (a) and control (b) treatment groups (Figure 1C). Within morphine treated-group, condition factors affect both CS+ arm ($F_{2,22} = 4.309$, p-value < 0.05) and stem arm (F2,22 = 4.038, p-value < 0.05). In the CS+ arm, the CPP score of morphine reinstatement was increased higher than post-condition and extinction (Figure 1C). In contrast, The stem preference score was significantly decreased in the reinstatement compared to the post-condition and extinction test.

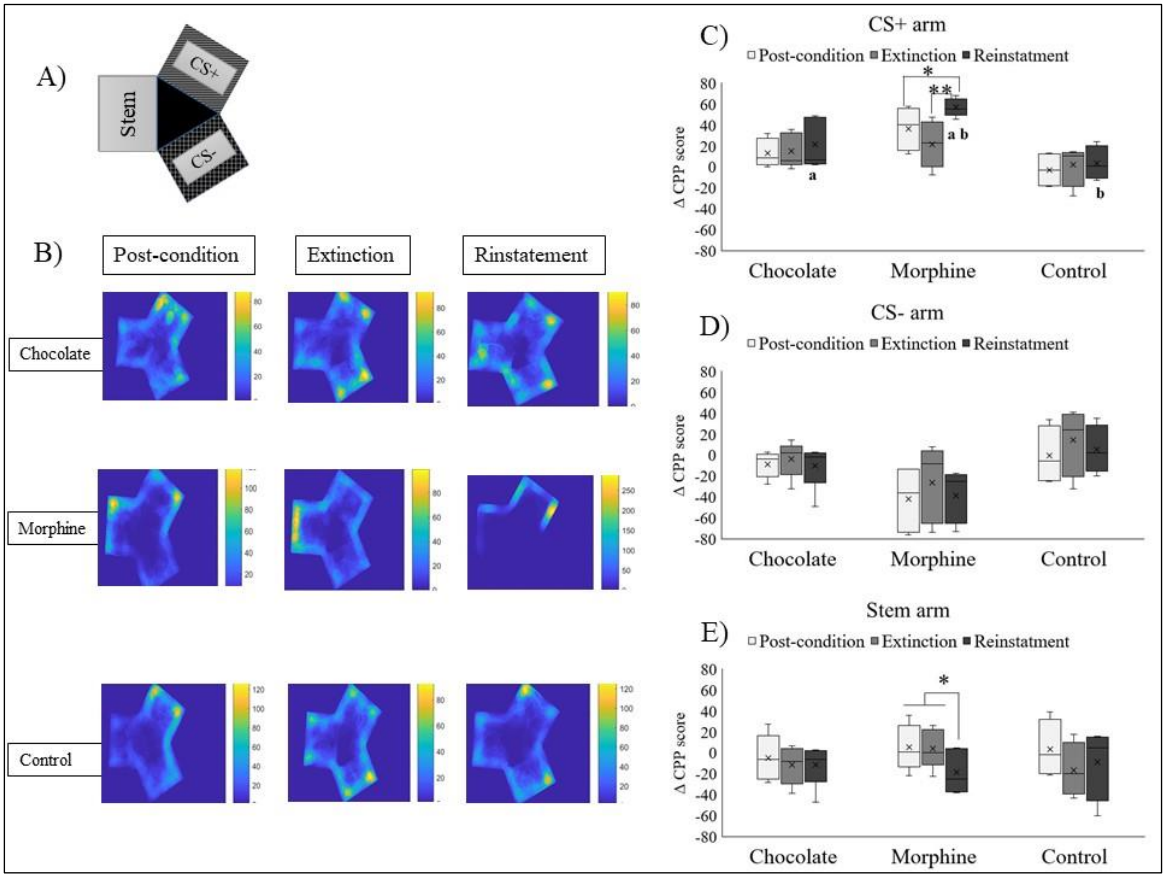


**Figure 2** The time preference of Y-maze CPP arm and CPP score analysis. The labeled arm of the Y-maze CPP apparatus (A). Representative heat map of individual animal position tracking of the 3 experimental groups: Chocolate, Morphine, and Control during the 3 different conditions: Post-condition, Extinction, and Reinstatement (B). The color represents the duration of time the animal visited this position, yellow tone color indicated high visiting while the blue tone color represents low visiting. Differential CPP score (ΔCPP score) of CS+ arm (C), CS- arm (D), and Stem arm (E) were analyzed. * p-value < 0.05, ** p-value < 0.01, significant different between treatment group [[a] morphine-chocolate, [b] morphine-control at p-value < 0.05].

To exclude hyperlocomotion affect the CPP score, locomotor activity was calculated during 15 minutes of exploring the CPP apparatus. Animal body and position were automatically detected and tracked by the Autotyping toolbox and individually displayed as shown in Figure 2B. The treatment groups and conditions including, post-condition, extinction, and reinstatement, factors were analyzed using two-way ANOVA repeated analysis. No significant difference was observed among all treatment groups as well as the 3 conditions.

The number of arm visiting represents a seeking behavior, which indicates an important character of reward behaviors. Animals tend to visit reward paired-arm. Therefore, the total number of each arm visiting during 15 minutes exploring the CPP apparatus were counted and calculated as a differential of pre-condition (ΔNo. of entries). The treatment groups and conditions including, post-condition, extinction, and reinstatement, factors were analyzed using two-way ANOVA repeated analysis. No significant difference was observed in CS+ and CS- arms. However, extinction and reinstatement of morphine treated group tend to increase No. of entries compare to control and chocolate group. In contrast, only stem arm revealed a

significant difference ($F_{2,22}$ = 13.415, p-value < 0.01). In morphine treated group, no. of entries during extinction test significantly higher than that of post-condition and reinstatement.

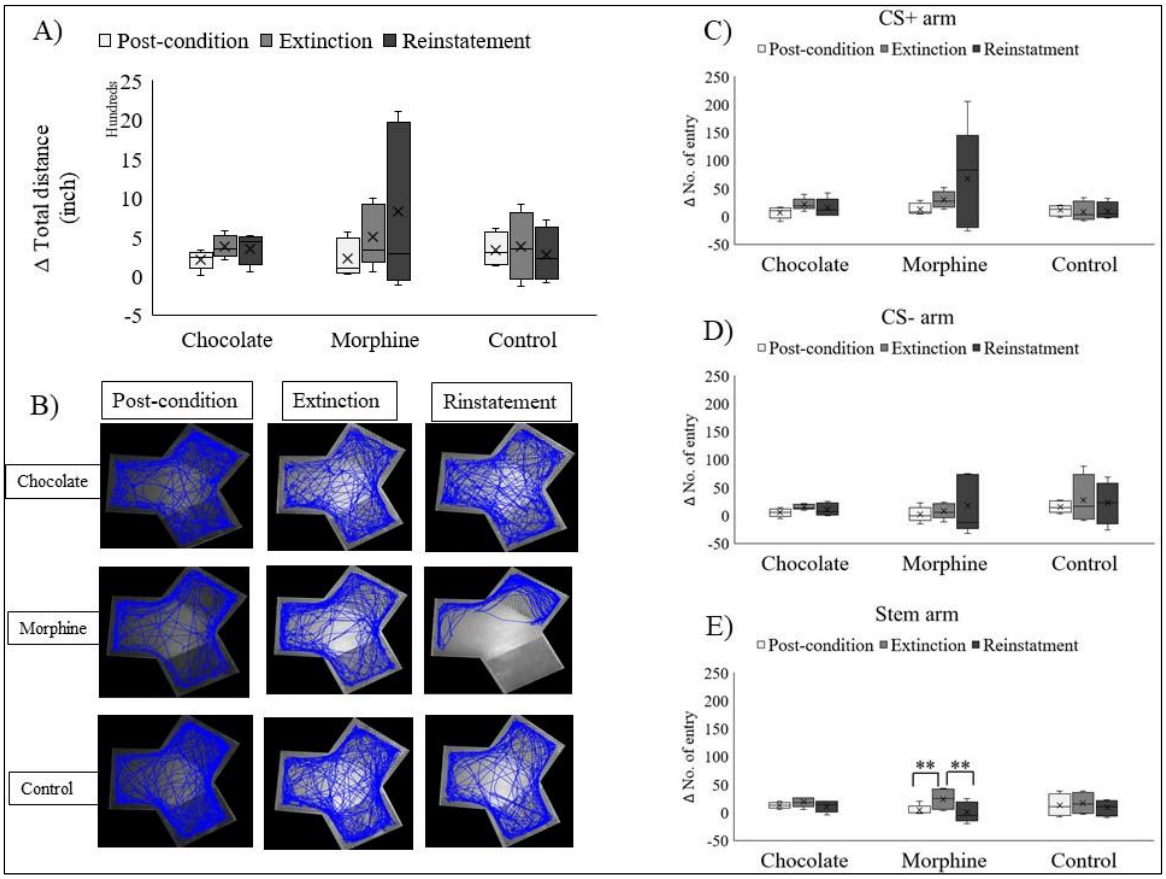


**Figure 3** Locomotor tracking activity and total No. of entries during CPP experiment. Total distance travel distance (inch) of chocolate, morphine, and control groups during the 3 conditions including post-condition, extinction, and reinstatement (A). Individual tracking line (blue line) of represented groups and conditions (B). Differential number of entries (ΔNo. of entry) of CS+ arm (C), CS- arm (D), and Stem arm (E) were analyzed. ** p-value < 0.01.

4.2. Discussion

The Y-maze apparatus is the simple behavioral paradigm that has been used to evaluate the short-term memory in rodents and therefore associated with reward and punishment. CPP score calculated from side preference has been used to investigate the reward memory in association with the environmental cue (White, Chai, & Hamdani, 2005) and craving incubation (Noye Tuplin & Holahan, 2019). In the present study, the authors found that the conditioned chocolate could not induce the increase of CPP score in the post-condition, extinction test, and reinstatement test, while morphine treatment increases CPP score in the CS+ arm and decrease in the stem arm during reinstatement condition. The previous study reported that no significant difference in the CPP score was observed from conditioned chocolate after the abstinent periods (24hr, 7, 14, and 28 days) (Noye Tuplin & Holahan, 2019). The finding from this study is consistent with the period of our study, 24 hours. After conditioning is a post-conditioning and 10 days after chocolate cessation or extinction period is extinction test. Moreover, the authors also confirm that chocolate did not reveal craving behaviors after chocolate priming and test during the reinstatement period.

Besides the CPP score, the number of entries could also represent a seeking behavior, which is one of the most important characters of reward responding behavior. The authors found that the number of entries of the chocolate treatment did not show significant changes in the post-condition, extinction test, and reinstatement test. In 2019, Noye Tuplin and Holahan reported that after animals absent from long-term chocolate intake, the number of entries into the paired arm of the CPP paradigm also did not significantly different in comparison to the unpaired arm. Since locomotor activity might affect reward responsible behavior, total distance traveled was also analyzed to investigate the effect of the condition on locomotor function. In condition chocolate treatment, no significant difference of locomotion was observed for all testing conditions. In contrast, morphine treatment showed obvious changes in both the CPP score and the number of entries, while no change in distance travel was observed. These findings suggested that the CPP score and the number of entries evaluated using the Y-maze CPP procedure specifically produced a conditioning reward effect but not general locomotor activity. However, a condition induced by chocolate and morphine in sated mice has a distinct reward mechanism.

Several studies have been reported the reward effect of palatable food. However, few studies investigated condition induced-reward effect of palatable food, and its mechanism underlying the brain reward circuit remained unelucidate. The seeking behavior and the craving incubation of palatable food are influenced by both homeostatic and non-homeostatic (Leigh & Morris, 2018). The previous study suggested that the anticipatory behavior of palatable food in rodents depended on daily food access. The previous experiment demonstrated that the anticipation of a scheduled food reward response influences the rat with restricted food access influences. This study suggested that the condition induced by palatable food might effective when the animal was in a deficit of an energy balance. Therefore, food reward-motivated partly via the modulation of energy balance while addictive drugs, including morphine, affect directly the reward brain circuit via opioid receptors and activate the addictive responses (Dejean, Boraud, & Le Moine, 2013)

Moreover, different macronutrient ingredients have reported differential effects. The previous study suggested a distinct behavioral pattern on the CPP paradigm induced by chocolate pellets (i.e., dark chocolate that is a low percentage of sugar and without milk powder mixed) and milk chocolate (i.e., sweet chocolate that is a high percentage of sugar and milk powder mixed). The chocolate pellets revealed behavior responses after 24 hr. and 28 days of abstinence but not in milk chocolate (Noye Tuplin & Holahan, 2019). Also, our study used a high percentage of sugar (37%) and milk powder (18%). Therefore, the present study demonstrated that the condition induced by using chocolate without fasting might not be strong enough to elicit reward behavior as seen in the morphine group. Besides the macronutrient ingredients, reward response also depends on the propensity of individual animals. The previous study reported that in some rats, food cues could reinstate food-seeking behavior (nose pokes response), while the hunger rat always displayed this behavior (Yager & Robinson, 2010).

Finally, the authors suggested that the effect of palatable food on reward behavior depends on various factors including energy balance, macronutrient ingredients, and even individual propensity. Even though several studies reported that the effect of palatable food can drive addictive-like behavior, however, various factors influence this behavior and depend on the individual genetic background.

## 5. Conclusion

This present study demonstrated the effect of chocolate cues (palatable food cues) on behavioral pattern changes in the Y-maze CPP paradigm compared with the standard addictive drug, morphine. Our finding revealed that the environmental cues (conditional stimuli, CS) paired with chocolate (unconditional stimuli, US) could not activate the addictive-like behaviors when animals were not restricted food access. Accordingly, our finding supported the evidence that the chocolate cues might not be strong enough to trigger reward processing in the brain of sated mice.

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