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Abstract

Serotonin 5-HT_{2A} and 5-HT_{2C} receptors play important yet distinctive roles in the regulation of rat maternal behavior. The present study investigated their neural substrates and explored the possible behavioral mechanisms (i.e., behavioral organization or maternal motivation). Sprague-Dawley postpartum females were microinjected

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with either a selective 5-HT_{2A} agonist (TCB-2, 0.4 or 4.0 μg/side) or a 5-HT_{2C} agonist (MK212, 2.5 or 5.0 μg/side) into the medial prefrontal cortex (mPFC) or ventral tegmental area (VTA). Ten and 60 min later, their maternal activities were observed in the home cage; and their motivational responses towards pups were examined in a pup preference test and pup retrieval test throughout the first week of postpartum. In the mPFC, TCB-2 microinjection disrupted major components of maternal behavior (e.g., pup retrieval, pup crouching), as well as the sequential pup retrieval score (a measure of behavioral organization). In contrast, MK212 microinjection had a minimal disruption of maternal behavior. In the VTA, TCB-2 microinjection impaired pup retrieval, nest building, and pup crouching, whereas MK212 microinjection severely impaired pup retrieval, nest building and pup crouching. Moreover, only intra-VTA injection of MK212 significantly suppressed pup preference. Together, our data suggest that 5-HT_{2A} receptors in the mPFC and VTA may play an important role in the behavioral organization or executive control of maternal activities, but not in the motivational processing of the rewarding value of pups (maternal motivation). In contrast, 5-HT_{2C} receptors in the VTA play a critical role in maternal motivation, but not in the organization of maternal responses.

- Intra-mPFC infusion of a 5-HT_{2A} agonist TCB-2 disrupts maternal behavior organization.
- Intra-mPFC infusion of a 5-HT_{2C} agonist MK212 minimally disrupts maternal behavior.
- Intra-VTA infusion of either TCB-2 or MK212 disrupts maternal behavior.
- Only intra-VTA injection of MK212 suppressed pup preference.

Keywords: Maternal behavior, Serotonin, 5-HT2A, 5-HT2C, Medial prefrontal cortex, Ventral tegmental area

1. Introduction

Maternal behavior in rats is a major behavioral pattern of the mature female. Extensive research on the neurochemical basis of maternal behavior has focused mainly on the mesolimbic and mesocortical dopamine (DA) systems (Afonso et al., 2011, 2013; Li and Fleming, 2003; Numan and Young, 2016; Parada et al., 2008). Considerable evidence suggests that the mesolimbic DA system (ventral tegmental area [VTA], nucleus accumbens [NAc]) plays an important role in maternal behavior, especially the appetitive or motivational aspect (Hansen, 1994; Li, 2015; Li and Fleming, 2003; Olazabal et al., 2013; Pereira and Ferreira, 2006; Pereira et al., 2005; Stern and Keer, 1999; Zhao and Li, 2009b). Although the mesocortical pathway (VTA-medial prefrontal cortex [mPFC]) is speculated to play a role in executive control or behavioral organization of maternal activities (Afonso et al., 2007), empirical evidence is still limited (Leuner and Gould, 2010).

Serotonin (5-HT) is another important monoamine that plays a critical role in many psychological and physiological processes (e.g., prolactin release) relevant to rat maternal behavior (Barofsky and Harney, 1978; Barofsky et al., 1983; Cools et al., 2008; Graeff et al., 1996). Mechanistically, it can modulate the mesolimbic and mesocortical dopamine systems to achieve its regulation of maternal behavior, as serotonin receptors such as 5-HT_{2A} and 5-HT_{2C} are richly expressed in the mPFC, NAc and VTA (Celada et al., 2013; Hamon and Blier, 2013; Howell and Cunningham, 2015). Recent work shows that alterations of 5-HT neurotransmission via either lesioning serotonergic cell bodies in the dorsal raphe, or activating 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C}), or blocking 5-HT transporters by selective serotonin reuptake inhibitors (SSRI) cause maternal impairments (Chen et al., 2014; Holschbach et al., 2018; Li et al., 2018; Lonstein, 2018; Wu et al., 2018; Yang et al., 2015). Work focusing on 5-HT_{2A} and 5-HT_{2C} suggests that they are involved in distinctive behavioral processes that are critically important for the normal expression of rat maternal behavior, such as incentive motivation, aversive processing, and behavioral controls/organization (Chen et al., 2014; De Almeida and Lucion, 1994, 1997; Gao et al., 2018; Veiga et al., 2007; Wu et al., 2016; Zhao and Li, 2009a). Observational evidence seems to suggest that activating 5-HT_{2A} receptors may cause disruption in the *executive control of maternal activities (behavioral organization)*, while activating 5-HT_{2C} receptors may decrease *maternal motivation* (Chen et al., 2014; Gao et al., 2018; Li, 2015; Li et al., 2018; Wu et al., 2016, 2018). This hypothesis is consistent with many other animal behavioral studies showing that activation of 5-HT_{2A} receptors is often associated with the disruption of various executive functional processes such as attention, working memory, impulsivity, and behavioral inhibition (Aznar and Hervig Mel, 2016), whereas activation of 5-HT_{2C} receptors typically causes a decrease in DA function and a decrease in motivated behavior (Di Giovanni et al., 2000; Di Matteo et al., 2002; Filip et al., 2004, 2006; Manvich et al., 2012; McMahon et al., 2001; Nedergaard et al., 2004). However, direct evidence showing that 5-HT_{2A} activation does cause disorganized maternal responses is lacking. Moreover, although our initial studies show that the mPFC is one of the brain regions where 5-HT_{2A} receptors might be involved in maternal behavior (Gao et al., 2018; Wu et al., 2016), the central neural substrate underlying the maternal effect of 5-HT_{2C} receptors has not been explored.

The primary goal of the present study was to elucidate the behavioral and neural mechanisms underlying the maternal effects of 5- HT_{2A} and 5-HT_{2C} receptors. Behaviorally, we used two tests (i.e., the home-cage maternal behavior and pup preference) to analyze the behavioral mechanisms underlying the regulatory effects of 5-HT_{2A} and 5- HT_{2C} receptors, focusing on their influences on maternal motivation and behavioral organization of maternal activities. Neuroanatomically, we focused on the mPFC and VTA, as they are known for their roles in executive functions (Aznar and Hervig Mel, 2016; Celada et al., 2013; Martin-Ruiz et al., 2001; Vazquez-Borsetti et al., 2009), and emotional and motivational responses toward pups (Numan and Smith, 1984; Zhao and Li, 2012).

2. Materials and methods

2.1. Animals

Virgin female Sprague-Dawley rats weighing 220–260 g were purchased from Chongqing Tengxin Biological Technology Co., Ltd, China. They were initially housed in pairs in transparent cages (47 cm L×32 cmW×21 cm H) with corn-cob granule for bedding in a colony room on a 12-h light/dark cycle (lights on at 08:00). Room temperature was maintained at 22 ± 2 °C with a relative humidity of 45–75%. The rats had free access to food and water in their home cages. One week after arrival, each female rat was housed with a proven stud male for 10 days to ensure pregnancy, after which the pregnant female was singly housed until parturition. After parturition, the mother rat was housed together with her litter for the remainder of the experiment. Experiments were conducted during the light cycle when the maternal behavior is high (Grota and Ader, 1969; Jensen Pena and Champagne, 2013; Leon et al., 1984; Numan and Insel, 2003; Toki et al., 2007). Starting 2 or 3 days prior to the first possible parturition date, the subjects were monitored in the morning (9:00) and afternoon (16:00) for signs of parturition. The day when the pups were found in the morning or afternoon was designated as postpartum day 1 (PPD 1) or PPD 0, respectively. Two shredded paper towels were provided as nesting materials. On PPD 2, each litter was culled to 8 pups (4 males and 4 females with the most visible milk bands). All experimental procedures were approved by the local animal care and

use committee at Southwest University, China, and were carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs and choices of dosage

TCB-2 [a selective 5-HT_{2A} receptor agonist, 4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide] and MK212 [a selective 5-HT_{2C} receptor agonist, 6-Chloro-2-(l-piperazinyl) pyrazine hydrochloride] obtained from Tocris Bioscience (Ellisville, MO, USA) were used to selectively activate 5-HT_{2A} and 5-HT_{2C} receptors, respectively. MK212 and TCB-2 were dissolved in 0.9% saline. Doses of MK212 (2.5 or 5.0 μg/side) and TCB-2 (0.4 or 4.0 μg/side) and the test time were chosen based on our previous study (Gao et al., 2018; Wu et al., 2016). On each test day, bilateral intra-mPFC or intra-VTA microinjection (0.5 μl at 0.25 μl/min) started 1 min after the insertion of the injector, which remained in place for an additional 1 min before removal to allow for drug diffusion.

2.3. Stereotaxic surgeries

Stereotaxic implantation was performed in late gestation (GD 12–14), when pregnant females were anesthetized with 4% isoflurane (RWD Life Science, Shenzhen, China) and maintained with 1% isoflurane in 99.99% O2. A 22-gauge guide cannula (RWD Life Science, Shenzhen, China) was implanted into the mPFC (Experiments 1 and 2) or VTA (Experiments 3 and 4). The cannula was 1.5 or 2.0 mm above the target region in the mPFC or VTA, respectively. For the mPFC, the stereotaxic coordinates were: AP + 3.0 mm, ML±0.75 mm, DV -2.2 mm. For the VTA, they were set as: AP -5.8 mm, ML±0.7 mm, DV -6.0 mm. A stainless steel stylet was placed into the cannula to prevent occlusion. After the surgery, rats were individually housed and allowed to recover for at least 7 days in their home cages before behavioral tests. At the end of behavioral tests, rats were sacrificed and perfused. Their brains were sectioned and then stained with cresyl violet and the cannula placement was determined as previously reported (Feng et al., 2015; Gao et al., 2018). The location of the injection site was mapped onto a stereotaxic atlas (Paxinos, 2005) (**Fig. 1**).

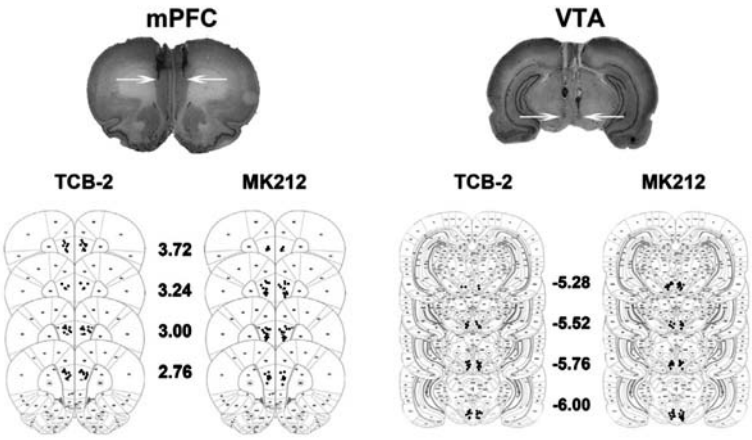

Fig. 1. Histological representations of microinjection sites and schematic diagrams showing the location of the injector tips in the VTA and mPFC. Data are reconstructed from Paxinos and Watson (Paxinos, 2005). *Numbers to the left of the sections* indicate anteroposterior distance from bregma in millimeters. The *arrow* in the histological representation section and *black dot* in the schematic diagrams denotes the infusion placement.

2.4. Experimental design

This study consisted of four independent experiments and examined how activation of 5-HT_{2A} or 5-HT_{2C} receptors in the mPFC (Experiments 1 and 2) or VTA (Experiments 3 and 4) altered maternal behavior in two behavioral testing paradigms described below. In Experiment 1 (5-HT_{2A}-mPFC), 26 rats were randomly assigned to one of the following three groups: VEH (n=8), TCB-2 0.4 (n=10), or TCB-2 4.0 (n=8) and tested, while Experiment 2 (5-HT_{2C}-mPFC) tested 27 rats in three groups: VEH (n=8), MK212 2.5 (n=8) or MK212 5.0 (n=11). Experiment 3 (5-HT_{2A}-VTA) tested 25 rats: VEH (n=9), TCB-2 0.4 (n=7), or TCB-2 4.0 (n=9), and Experiment 4 (5-HT_{2C}-VTA) tested 30 rats: VEH, MK212 2.5, or MK212 5.0 (n=10 per group). Home cage maternal behavior and pup preference test were conducted on PPD 3 and PPD 5, respectively. On each test day, the subject dams were tested twice (each test was 10 min) at 10 min and 60 min after the central infusion of VEH, TCB-2 or MK212 (**Fig. 2**). A total of 141 rats were used for stereotaxic surgeries. Eleven dams were excluded because they failed to retrieve 8 pups within 10 min in a quick pup retrieval test on PPD 2. Twenty-two dams were excluded because

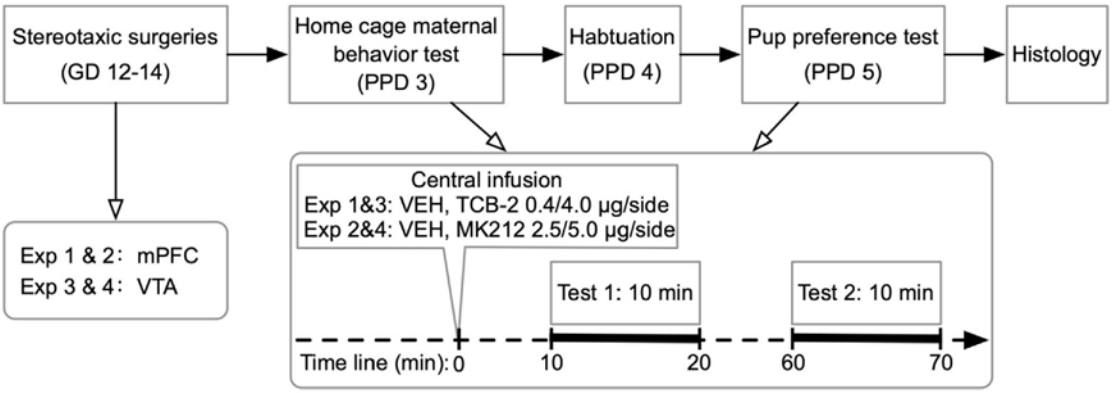

Fig. 2. A schematic illustration of the experimental procedure and treatments in experiments 1–4. Exp: experiment; GD: gestation day; PPD: postpartum day; VEH: vehicle.

they either showed cannula failure or misplacement, bad health or died before the first test. The final number of rats in different groups indicated above is the final number used for data analysis.

2.5. Home cage maternal behavior test

One day before the home cage maternal behavior test (PPD 2), we screened for baseline maternal performance and habituated dams to the testing procedure. A quick 10-min pup retrieval test (removing pups then returned them to the cage 10 s later) was conducted. Only those dams who retrieved all 8 pups within 10 min were used in the subsequent tests (11 dams were excluded).

On PPD 3, a formal maternal behavior test was conducted as described previously (Gao et al., 2018; Wu et al., 2016). Dams were tested for 10 min twice at 10 min and 60 min time points after the central infusion of VEH, TCB-2 or MK212. It started by taking the eight pups away from the mother and destroying the nest. Ten seconds later, the pups were placed in the corner of the cage diagonal to the nest site or dam sleeping corner. Each test was recorded for 10 min by video cameras and analyzed manually using a laptop computer with an event-recording program (Boris, http://www.boris.unito.it). At the end of the 10-min period, unretrieved pups were returned to the nest site. We recorded the following components of maternal behavior: (1) *pup retrieval* (a dam picking up a pup in her mouth and carrying it back to the nest site); (2)

pup licking (dam placing its tongue on the anogenital area and the rest of a pup' body); (3) *nest building* (dam picking up nest material in her mouth and transporting it back to the nest site); (4) *crouching over pup* (dam stands still over the litter, in high- or low-arched-back posture with its legs rigidly splayed and does not engage in other active behavior). Based on the retrieval performance, we also calculated three additional retrieval measures: (1) *sequential retrieval score* (the number of pup retrievals followed, within 15 s, by another pup retrieval, as a measure of executive function. First pup retrieval and pup retrieval occurred outside the 15 s interval were not counted. Thus with a maximum 8 pup retrievals, the sequential retrieval score ranges from 0 to 7) (Afonso et al., 2007); (2) fi*rst pup retrieval latency* (the time from the onset of the test until the first pup being deposited into the nest side); (3) *last pup retrieval latency*.

2.6. Pup preference test

One day before the test (PPD 4), the subject rats were habituated to the testing room and arena for 30 min. They were brought into the testing room, removed from their home cages and placed into an open-field arena made of black acrylic (76 cm L×76 cmW×50 cm H). Inside the arena, there were two cylinder-shape cages made of transparent acrylic (top and bottom) with stainless metal bars spaced in between (8.0 cm H×7.5 cm in diameter). This cage was used to confine 4 pups or 4 erasers in the subsequent pup preference tests. The two cages were empty during the habituated test day (PPD 4). On the PPD 5, the subject rats were brought into the testing room, 30 min later, they were microinfused with VEH, TCB-2 or MK212. Ten min and 60 min later, they were placed into the arena and their exploration time with one of two stimuli were recorded for 10 min. One stimulus was 4 pups confined into the small cage and another one was cage with 4 pink erasers used as a novel object. The pup containing cage and object were placed at the center of two adjacent quadrants at the backend of the arena. While the subject rats were placed midway at the frontend. They could see, hear and smell the pups but not contact them physically. The total time spent exploring the two stimuli during the 10 min period was recorded using a digital video camera and analyzed using the software of Noldus EthoVision XT 8.5 (Wageningen, The Netherlands). Exploration was defined as sniffing or touching the cage/object with the nose and/or forepaws. The arena

and the cages were cleaned and deodorized with a 75% ethanol solution between subjects and after each test. Pup preference is calculated as the ratio of time spent exploring the pup cage over the total amount of time spent exploring the cage and object.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 20 software (SPSS Inc., Chicago, IL, USA). All data (except the latency data) from each test day (PPD 3 and 5) were analyzed separately using a factorial repeated measures analysis of variance (ANOVA), with group as the between-subjects factor and test time point as the within-subjects factor. Group differences at each time point were further analyzed using a simple main effect test (one-way ANOVA) followed by LSD post hoc tests for multiple comparisons if necessary. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Experiment 1: Effect of activation of 5-HT$_{2A}$ receptors in the mPFC by TCB-2 on maternal behavior

3.1.1. Home cage maternal behavior test

As shown in **Fig. 3**, intra-mPFC infusion of TCB-2 dose-dependently disrupted pup retrieval (Fig. 3A). Repeated measures ANOVA revealed a main effect of group [$F(2, 23)=7.055$, $p=0.004$], but no main effect of test time [$F(1, 23)=0.11$, $p=0.744$], nor group × test time interaction [$F(2, 23)=0.797$, $p=0.463$]. Post hoc LSD test showed that TCB-2 4.0 group retrieved fewer pups than the VEH and TCB-2 0.4 groups ($p=0.003$ and 0.004, respectively). Intra-mPFC infusion of TCB-2 also decreased the sequential retrieval score (Fig. 3B). Repeated measures ANOVA revealed a main effect of group [$F(2, 23)=5.29$, $p=0.013$], but no main effect of test time [$F(1, 23)=0.24$, $p=0.629$], nor group × test time interaction [$F(2, 23)=0.125$, $p=0.883$]. Further analyses showed that the TCB-2 4.0 group had significantly lower sequential retrieval scores than the VEH and TCB-2 0.4 groups at these time points ($p=0.005$ and 0.022, respectively).

On other maternal activities, intra-mPFC TCB-2 significantly decreased the duration of crouching [$F(2, 23)=5.368$, $p=0.012$] (Fig. 3D).

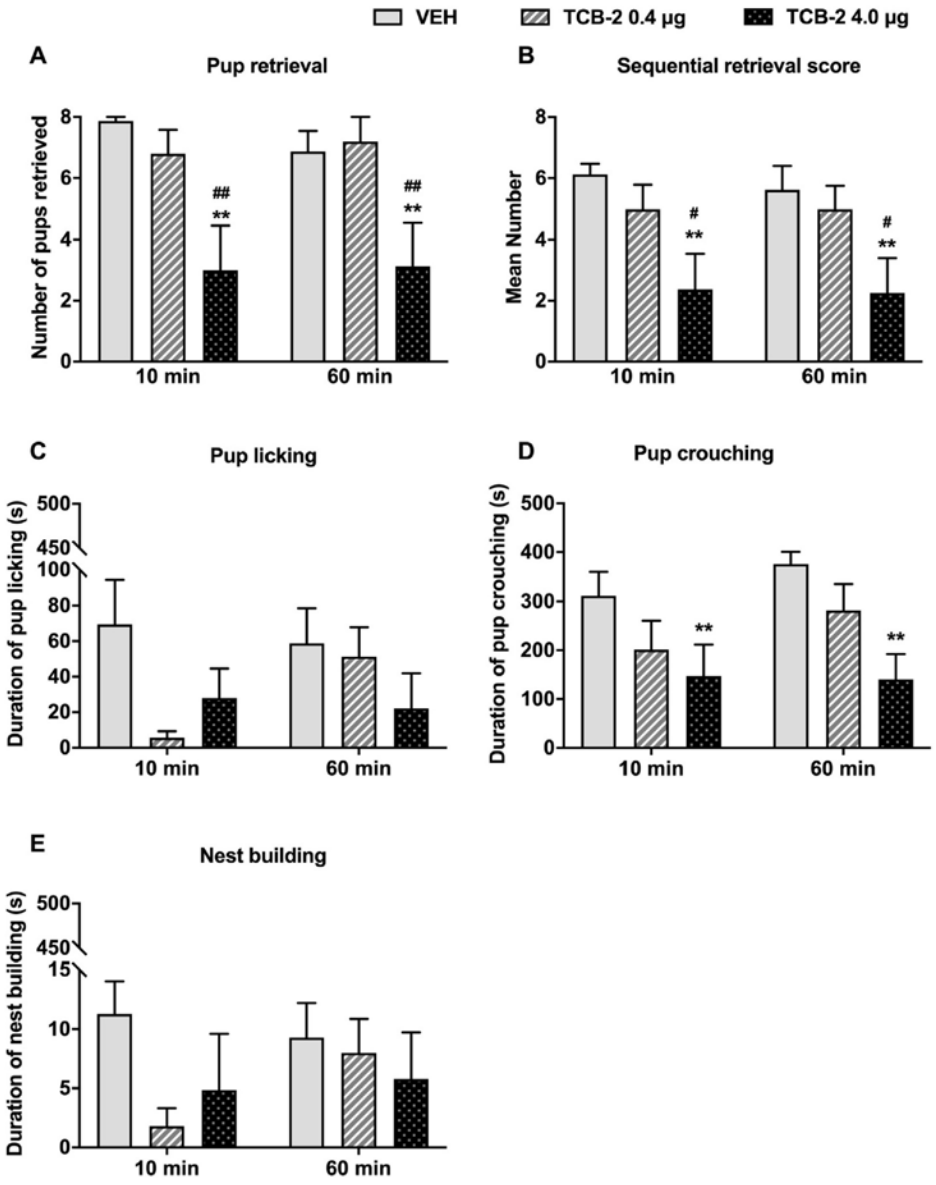

Fig. 3. Effects of TCB-2 microinfused into the medial prefrontal cortex (mPFC) on maternal behavior in home cage test on PPD 3. A, number of pups retrieved; B, sequential retrieval score; C, duration of pup licking; D, duration of pup crouching; E, duration of nest building. All data are expressed as mean + SEM. $^{**}p < 0.01$ significantly different between the VEH and TCB-2 groups. $\#p < 0.05$, $\#\#p < 0.01$ significantly different between the two TCB-2 groups.

Its effects on pup licking [F (2, 23)=2.981, p=0.071] (Fig. 3C) and nest building [F (2, 23)=1.225, p=0.312] (Fig. 3E) were not significant. Repeated measures ANOVA revealed no main effect of test time (all p > 0.245), nor group × test time interaction (all p > 0.200). Post hoc LSD test showed that TCB-2 at 4.0 μg/side (p=0.003) suppressed the crouching behavior at 60 min time point.

3.1.2. Pup preference test

Fig. 4 shows the exploration time with the pup cage and object, and the pup preference ratio in mother rats treated with TCB-2 into the mPFC. Repeated measures ANOVA revealed that intra-mPFC TCB-2 had no effect on the exploration time of pup [F (2, 23)=0.669, p=0.522], object [F (2, 23)=0987, p=0.388], nor the pup preference ratio [F (2,

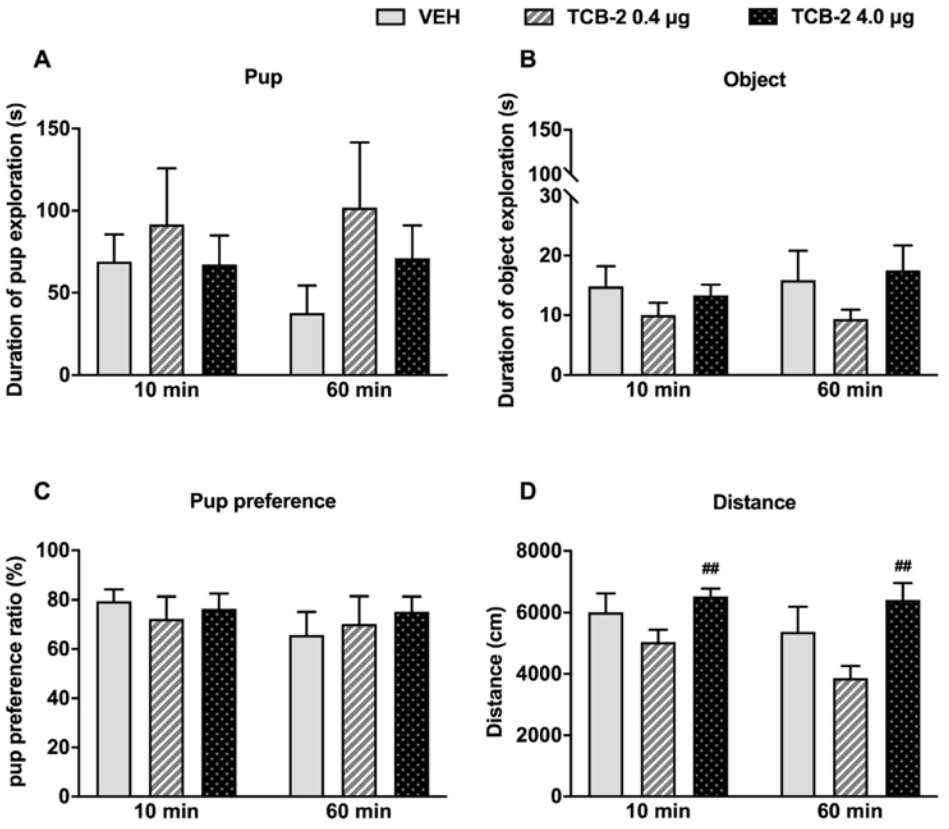

Fig. 4. Effects of TCB-2 microinfused into the medial prefrontal cortex (mPFC) on pup preference on PPD 5. Duration of pup exploration time (A), object exploration time (B), pup preference ratio (C), and distance travelled (D) are expressed as mean + SEM. ##*p* < 0.01 significantly different between the two TCB-2 groups.

23)=0.09, p=0.914]. Intra-mPFC TCB-2 only affected the distance travelled [F (2, 23)=5.342, p=0.012] (Fig. 4D). Repeated measures ANOVA revealed a main effect of test time on the object exploration time [F (1, 23)=10.797, p=0.003] and distance travelled [F (1, 23)=4.6, p=0.043], but no main effect of test time on the pup exploration time [F (1, 23)=0.574, p=0.457] or pup preference ratio [F (1, 23)=1.858, p=0.186]. Moreover, there was no group × test time interaction (all p > 0.078). Further analyses showed that the difference in the distance travelled was between TCB-2 0.4 and 4.0 groups (p=0.004). Overall, intra-mPFC TCB-2 treatment had a broader disruption of various maternal activities, including pup retrieval and crouching. However, it did not affect pup preference, indicating that the emotional processing of the rewarding value of pups was not mediated by mPFC 5-HT_{2A} receptors.

3.2. Experiment 2: Effect of activation of 5-HT_{2C} receptors in the mPFC by MK212 on maternal behavior

3.2.1. Home cage maternal behavior test

As shown in **Fig. 5**, intra-mPFC infusion of MK212 had no effect on pup retrieval [F (2, 24)=0.267, p=0.768; Fig. 5A], retrieval score [F (2, 24)=0.185, p=0.323; Fig. 5B], pup licking [F (2, 24)=1.745, p=0.196; Fig. 5C], pup crouching [F (2, 24)=0.261, p=0.772; Fig. 5D], and nest building [F (2, 24)=0.969, p=0.394; Fig. 5E], as there was no main effect of group, or group × test time interaction (all p > 0.083). Repeated measures ANOVA only revealed a main effect of test time on pup retrieval [F (1, 24)=5.762, p=0.024], retrieval score [F (1, 24)=6.095, p=0.021], pup licking [F (1, 24)=26.417, p < 0.001], pup crouching [F (1, 24)=12.158, p=0.002], but not on nest building [F (1, 24)=1.106, p=0.303].

3.2.2. Pup preference test

Fig. 6 shows the exploration time with the pup cage and object, and the pup preference ratio in mother rats treated with intra-mPFC MK212. Repeated measures ANOVA revealed that intra-mPFC MK212 had no effect on any measure from this test. There was no main effect of MK212 on the exploration time of pup [F (2, 24)=0.084, p=0.92], object [F (2, 24)=0.195, p=0.824], the pup preference ratio [F (2, 24)=0.479, p=0.625], and the distance travelled [F (2, 24)=0.152, p=0.86]. Repeated measures ANOVA revealed a main effect of test time on object exploration time [F (1, 24)=18.025, p < 0.001], but not on pup exploration time [F (1, 24)=0.032,

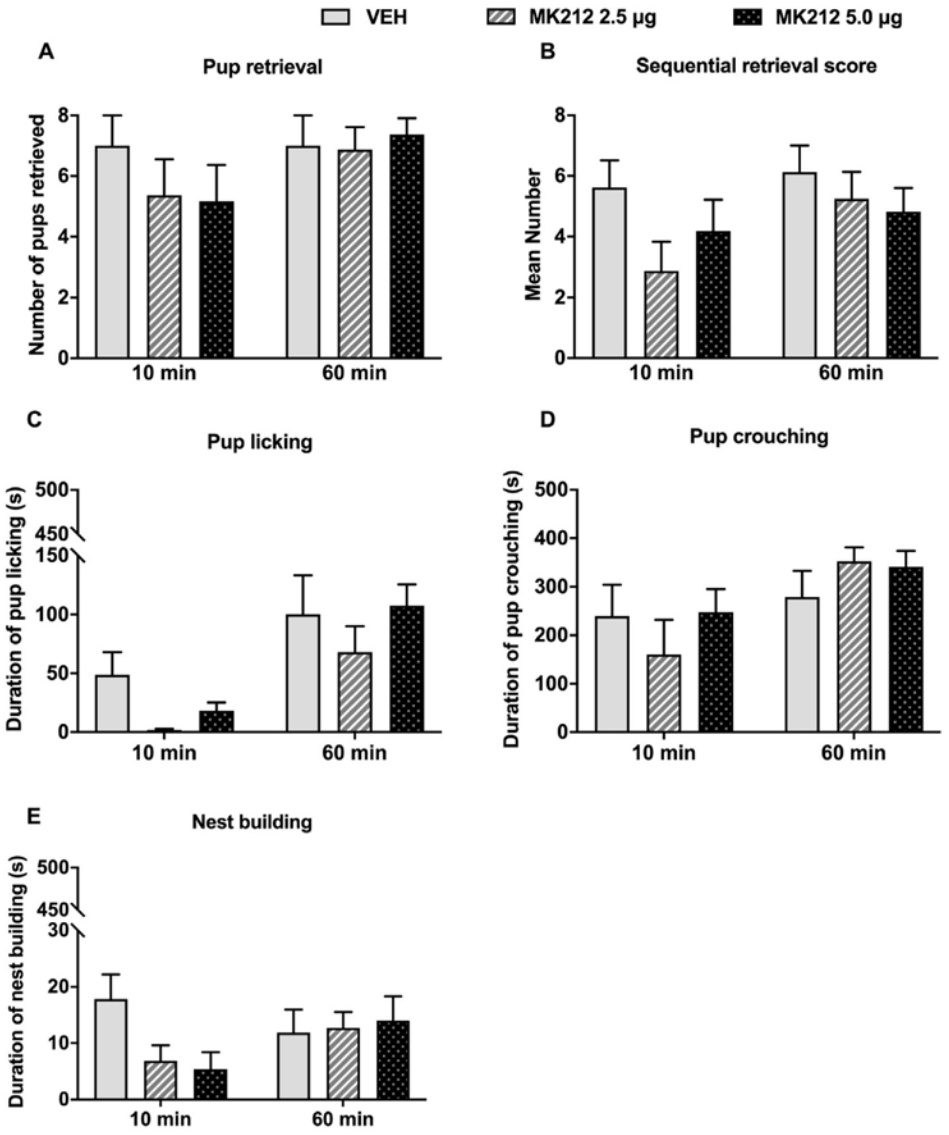

Fig. 5. Effects of MK212 microinfused into the medial prefrontal cortex (mPFC) on maternal behavior in home cage test on PPD 3. A, number of pups retrieved; B, sequential retrieval score; C, duration of pup licking; D, duration of pup crouching; E, duration of nest building. All data are expressed as mean + SEM.

p=0.859], pup preference ratio [F (1, 24)=2.18, p=0.153], or distance travelled [F (1, 24)=3.94, p=0.059]. Additionally, there was no group × test time interaction (all $p > 0.104$). Overall results indicate that activation of mPFC 5-HT_{2C} receptors by MK212 had a minimal disruption of maternal behavior in comparison to the effect of activation of mPFC 5-HT_{2A} receptors by TCB-2. Both treatment failed to alter pup preference.

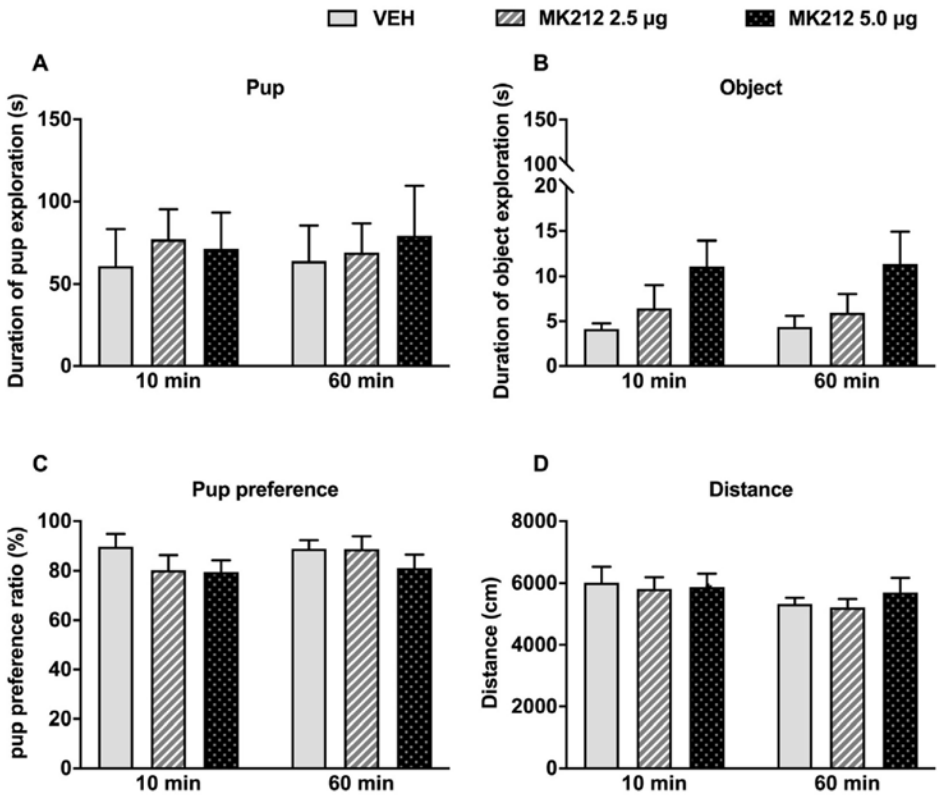

Fig. 6. Effects of MK212 microinfused into the medial prefrontal cortex (mPFC) on pup preference on PPD 5. Duration of pup exploration time (A), object exploration time (B), pup preference ratio (C), and distance travelled (D) are expressed as mean + SEM.

3.3. Experiment 3: Effect of activation of 5-HT$_{2A}$ receptors in the VTA by TCB-2 on maternal behavior

3.3.1. Home cage maternal behavior test

As shown in **Fig. 7**, intra-VTA infusion of TCB-2 dose-dependently disrupted pup retrieval. Repeated measures ANOVA revealed a main effect of group [F (2, 22)=6.233, p=0.007], but no main effect of test time [F (1, 22)=0.012, p=0.915], nor group × test time interaction [F (2, 22)=2.265, p=0.128]. Post hoc LSD test showed that TCB-2 at 4 μg/side, but not at 0.4 μg/side, decreased the number of pups retrieved (p=0.002; Fig. 7A). Intra-VTA infusion of TCB-2 also decreased the sequential retrieval score, as supported by the main effect of group [F (2, 22)=9.698, p=0.001]. Repeated measures ANOVA did not find the main effect of test time [F (1, 22)=3.556, p=0.073] or group × test time interaction [F (2, 22)=1.956,

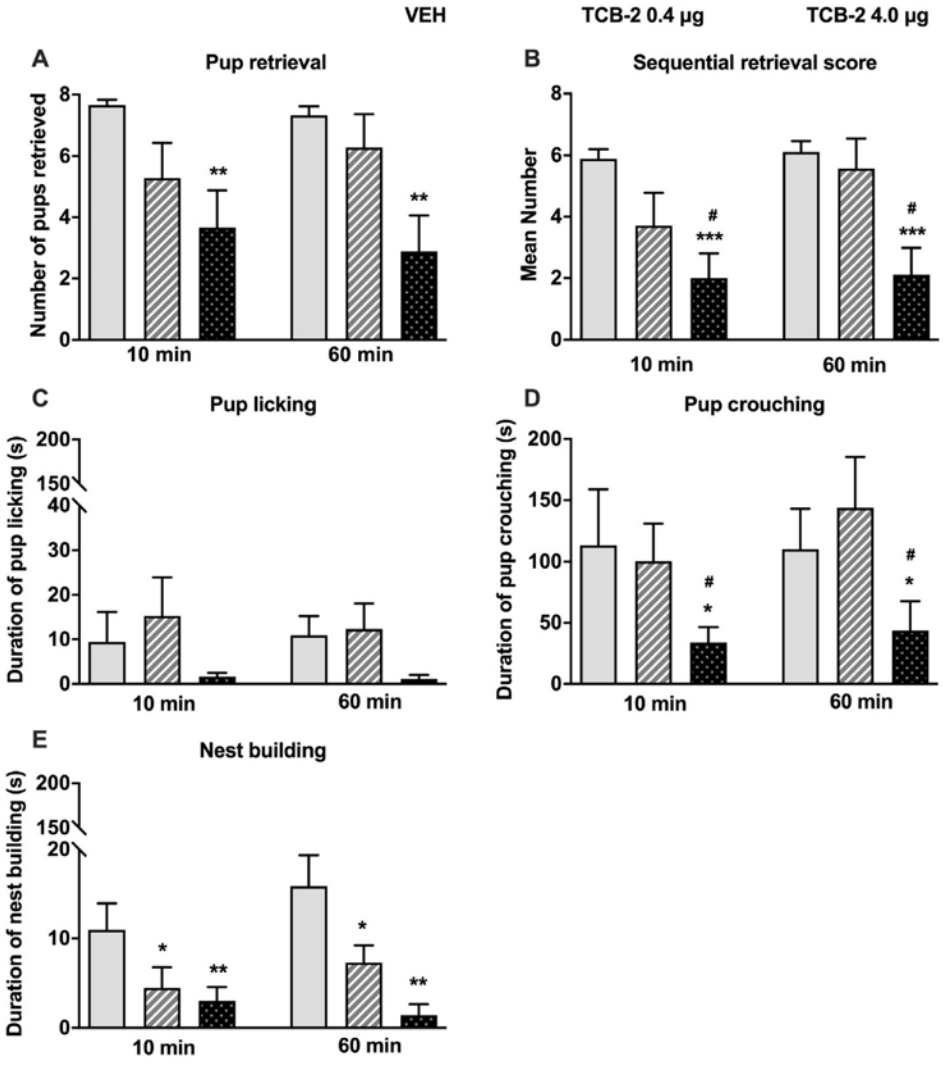

Fig. 7. Effects of TCB-2 microinfused into the ventral tegmental area (VTA) on maternal behavior in home cage test on PPD 3. A, number of pups retrieved; B, sequential retrieval score; C, duration of pup licking; D, duration of pup crouching; E, duration of nest building. All data are expressed as mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different between the VEH and TCB-2 groups. #$p < 0.05$ significantly different between the two TCB-2 groups.

p=0.165] (Fig. 7B). Further analyses showed that only the TCB-2 4.0 group had a significantly lower sequential retrieval score than the VEH group ($p < 0.001$). Additionally, the TCB-2 4.0 group had significantly lower sequential retrieval score than the TCB-2 0.4 group (p=0.014). Intra-VTA TCB-2 had no effect on the duration of pup licking [repeated measures ANOVA, F (2, 22)=2.49, p=0.106] (Fig. 7C), but significantly

disrupted pup crouching [F (2, 22)=3.932, p=0.035] (Fig. 7D), and nest building [F (2, 22)=8.082, p=0.002] (Fig. 7E). The main effect of test time (all p > 0.180), or group × test time interaction (all p > 0.179) were not significant. Further analyses showed that dams treated with TCB-2 at 0.4 μg/side (p=0.021) and 4.0 μg/ side (p=0.001) had lower nest building activity than the VEH group, while dams treated with TCB-2 at 4.0 μg/side had lower pup crouching activity than the VEH group (p=0.03) and TCB-2 0.4 group (p=0.021).

3.3.2. Pup preference test

Fig. 8 shows the effects of intra-VTA TCB-2 on pup preference. Repeated measures ANOVA showed that TCB-2 only decreased the pup exploration time [F (2, 22)=3.924, p=0.036], but did not affect the object exploration time [F (2, 22)=0.176, p=0.84], pup preference ratio [F (2,

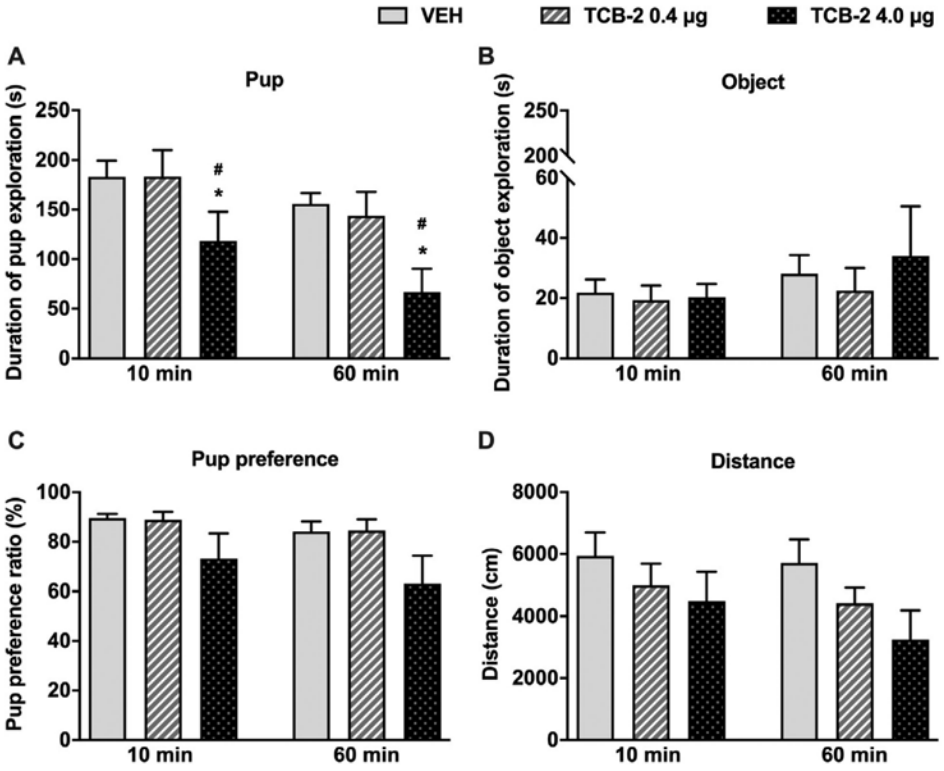

Fig. 8. Effects of TCB-2 microinfused into the ventral tegmental area (VTA) on pup preference on PPD 5. Duration of pup exploration time (A), object exploration time (B), pup preference ratio (C), and distance travelled (D) are expressed as mean + SEM. **p* < 0.05 significantly different between the VEH and TCB-2 groups. #*p* < 0.05 significantly different between the two TCB-2 groups.

22)=2.271, p=0.128], and distance moved [F (2, 22)=1.802, p=0.19]. The main effect of test time on the pup exploration time [F (1, 22)=15.204, p=0.001] and pup preference ratio [F (1, 22)=7.323, p=0.013] was significant, but its effects on the object exploration time [F (1, 22)=1.511, p=0.233] and distance moved [F (1, 22)=1.972, p=0.175] were not. Moreover, there was no group × test time interaction (all p > 0.219). Further analyses showed that dams treated with TCB-2 at 4.0 μg/side had lower pup exploration time than the VEH group (p=0.025) and TCB-2 0.4 group (p=0.028). Overall results from this experiment show that activation of 5-HT_{2A} receptors in the VTA primarily impaired pup retrieval, pup crouching and nest building, but did not impair pup preference. Also, the maternal disruptive effect of 5-HT_{2A} activation in the VTA was much less severe than the effect of 5-HT_{2A} activation in the mPFC (Experiment 1).

3.4. Experiment 4: Effect of activation of 5-HT_{2C} receptors in the VTA by MK212 on maternal behavior

3.4.1. Home cage maternal behavior test

As shown in **Fig. 9**, intra-VTA infusion of MK212 disrupted various maternal responses. Repeated measures ANOVA on pup retrieval revealed a main effect of group [F (2, 27)=12.883, p < 0.001], test time [F (1, 27)=13.47, p=0.001], and a significant interaction between the two [F (2, 27)=8.401, p=0.001]. Post hoc LSD test showed that intra-VTA MK212 2.5 and 5.0 μg/side (p=0.025 and p < 0.001, respectively) significantly disrupted pup retrieval at 10 min time points (Fig. 9A). In addition, the MK212 5.0 group also had significantly fewer pup retrievals than the MK212 2.5 group at 10 min time point (p=0.001). Intra-VTA infusion of MK212 also significantly decrease the sequential retrieval score (Fig. 9B). Repeated measures ANOVA revealed a main effect of group [F (2, 27)=6.496, p=0.001], test time [F (1, 27)=15.078, p=0.001], but no significant group × test time interaction [F (2, 27)=2.548, p=0.097]. Post hoc LSD test showed that the MK212 5.0 group had a significantly lower sequential retrieval score than the VEH group (p < 0.001) and MK212 2.5 group (p=0.008).

Repeated measures ANOVA revealed that intra-VTA MK212 also significantly disrupted pup crouching [F (2, 27)=3.883, p=0.033; Fig. 9D], nest building [F (2, 27)=9.452, p=0.001] (Fig. 9E), but it had no effect on pup licking [F (2, 27)=2.616, p=0.092; Fig. 9C]. The effects of test time (all p > 0.056) and group × test time interaction (all p > 0.225) were not

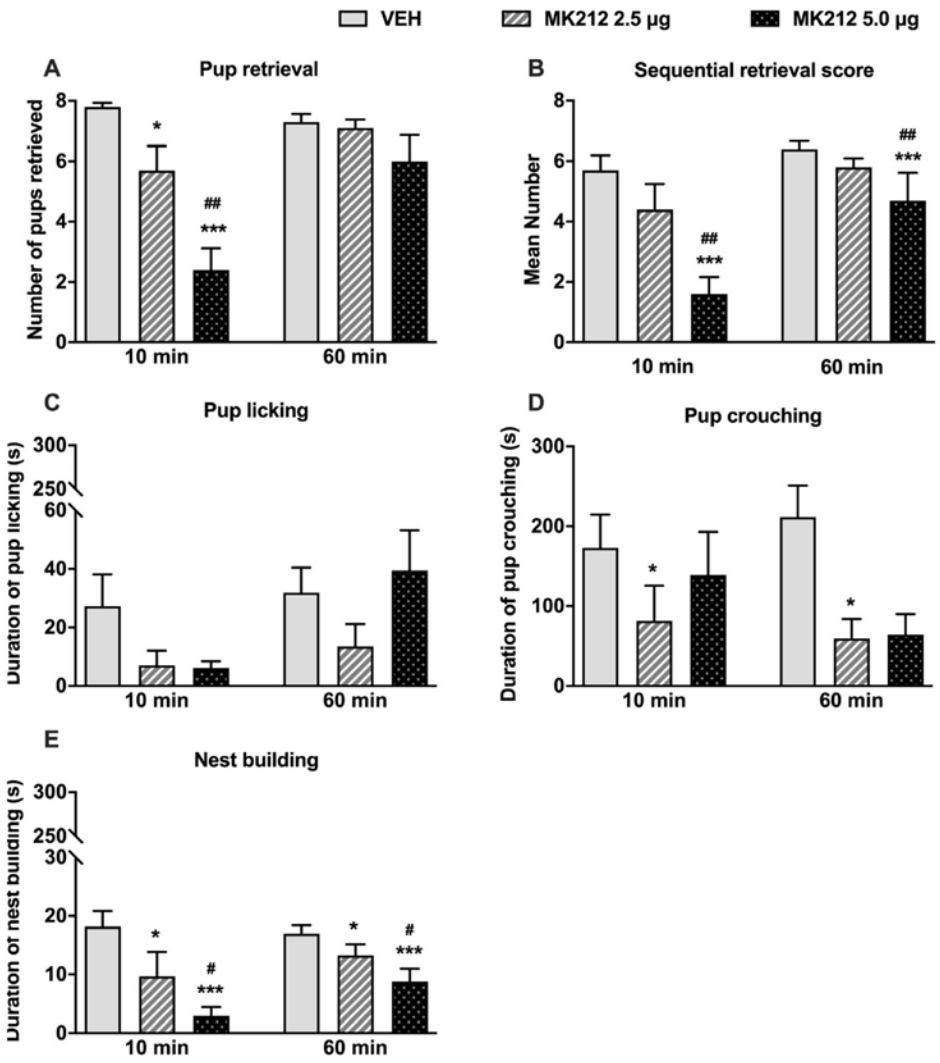

Fig. 9. Effects of MK212 microinfused into the ventral tegmental area (VTA) on maternal behavior in home cage test on PPD 3. A, number of pups retrieved; B, sequential retrieval score; C, duration of pup licking; D, duration of pup crouching; E, duration of nest building. All data are expressed as mean + SEM. *$p < 0.05$, ***$p < 0.001$ significantly different between the VEH and MK212 groups. ##$p < 0.01$ significantly different between the two MK212 groups.

significant. Further analyses with post hoc LSD test revealed that MK212 at 2.5 μg/side significantly suppressed pup crouching time (p=0.012). MK212 2.5 and 5.0 groups also had a significant fewer nest building activities than the VEH group (p=0.032, p < 0.001, respectively), and this effect was dose-dependent (i.e., the MK212 5.0 group had a significant fewer nest building than the MK212 2.5 group, p=0.046).

3.4.2. Pup preference test

As showed in **Fig. 10**, MK212 microinjected into the VTA significantly decreased pup preference ratio. Repeated measures ANOVA revealed a main effect of group ($F(2, 27)=8.329$, $p=0.002$] (Fig. 10C). However, intra-VTA MK212 did not decrease the pup and object exploration time, as well as the distance moved (all $p > 0.133$). The main effect of test time was significant only on distance moved [$F(1, 27)=8.523$, $p=0.007$], but not on the pup and object exploration time or pup preference ratio (all $p > 0.113$). Additionally, there was no group × test time interaction on these measures (all $p > 0.057$). Most importantly, post hoc LSD tests revealed that the MK212 5.0 group had a significantly lower the pup preference ratio than the VEH and MK212 2.5 group ($p < 0.001$, $p=0.015$, respectively). Thus, in comparison to the effect of activation of 5-HT_{2A} receptors in the VTA, activation of 5-HT_{2C} receptors had a broader maternal disruptive effect, as it not only suppressed pup retrieval and nest building, but also pup crouching and pup preference.

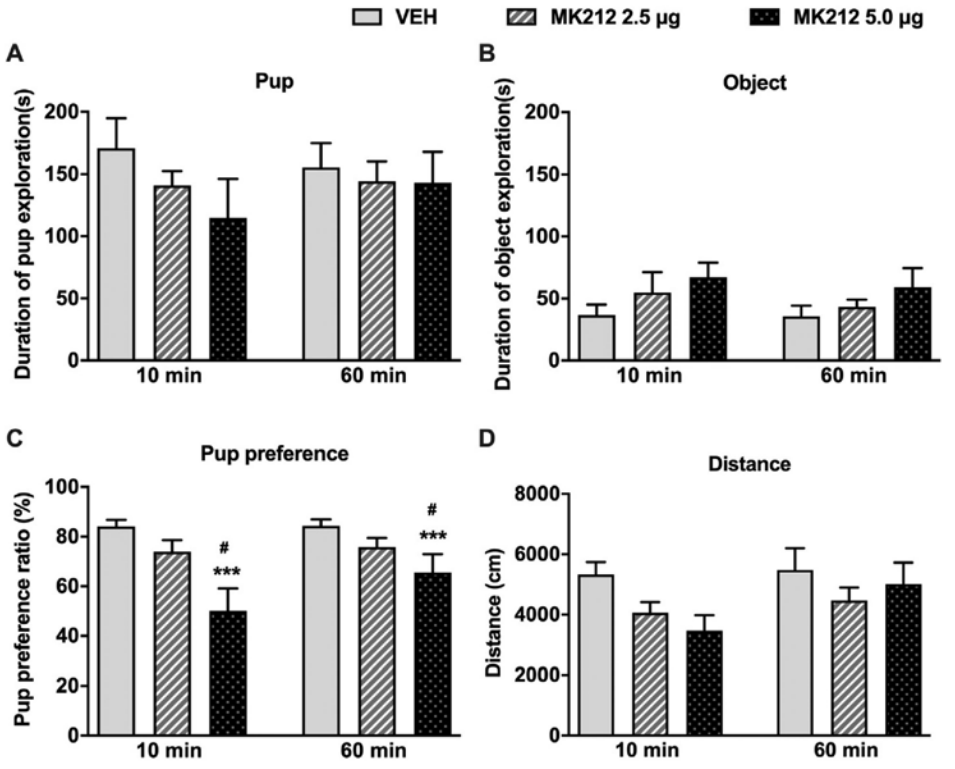

Fig. 10. Effects of MK212 microinfused into the ventral tegmental area (VTA) on pup preference on PPD 5. Duration of pup exploration time (A), object exploration time (B), pup preference ratio (C), and distance travelled (D) are expressed as mean + SEM. ***$p < 0.001$ significantly different between the VEH and MK212 groups. #$p < 0.05$ significantly different between the two MK212 groups.

4. Discussion

The present study systemically investigated how serotonin acts through its two primary receptors 5-HT_{2A} and 5-HT_{2C} in the mPFC and VTA to regulate maternal behavior in the rat. We showed that intra-mPFC injection of TCB-2 dose-dependently disrupted major components of maternal behavior (e.g., pup retrieval, pup crouching), as well as the sequential pup retrieval score (a measure of behavioral organization). In contrast, intra-mPFC injection of MK212 had a minimal disruption of maternal behavior. However, none of the drug treatments affected pup preference, suggesting that 5-HT_{2A} and 5-HT_{2C} in the mPFC are not involved in the pup reward processing or maternal motivation. In the VTA, MK212 microinjection severely impaired pup retrieval, nest building and pup crouching. It also significantly suppressed pup preference. In contrast, TCB-2 microinjection impaired pup retrieval, pup crouching, and nest building, but not pup licking. Collectively, these results appear to suggest a doubly dissociated behavioral function between mPFC 5-HT_{2A} and VTA 5-HT_{2C} receptors in the regulation of maternal behavior: 5-HT_{2A} receptors in the mPFC appear to play an important role in the behavioral organization or executive control of maternal activities, but not in maternal motivation. In contrast, 5-HT_{2C} receptors in the VTA seem to play a critical role in maternal motivation, but not in the maternal behavioral organization.

Early work from our laboratory has shown that systemic activation of 5-HT_{2A} receptors by TCB-2 (1, 2.5, or 5.0 mg/kg, sc) or activation of 5-HT_{2C} receptors by MK 212 (agonist, 0.5, 1.0, or 2.0 mg/kg, sc) dose-dependently disrupts maternal behavior, while blockade of both receptors has no effect (Chen et al., 2014; Gao et al., 2018). We speculated that one possible molecular mechanism through which 5-HT_{2A} and 5-HT_{2C} could influence maternal behavior is their modulation of the mesolimbic and mesocortical DA systems, as 5-HT_{2A} activation facilitates, whereas 5-HT_{2C} activation inhibits DA cell activity and DA release (Bubar et al., 2011; Howell and Cunningham, 2015). Both effects on DA could impair maternal behavior, as considerable evidence has clearly shown that balanced DA neurotransmission is critical for the normal display of maternal behavior (Numan, 2007; Zhao and Li, 2010). Thus, we focused on the mesolimbic and mesocortical DA system as the possible neural substrate underlying the maternal regulatory effects of 5-HT_{2A} and 5-HT_{2C} activation. Our initial work demonstrates that the mPFC is indeed one brain region implicated in the maternal effects of 5-HT_{2A} receptors, but not of

5-HT_{2C} receptors, as microinjection of TCB-2 (0.1, 0.4, or 4.0 μg/0.5μl/side) into the mPFC suppressed pup retrieval (Gao et al., 2018). In contrast, intra-mPFC infusion of MK212 in a broad dose range (25 and 250 ng, 1, 2, 5, and 10 μg/0.5 μl/side) had no effect on pup retrieval (Wu et al., 2016). The effect of TCB-2 is anatomically specific, as microinjection into other brain regions such as the medial preoptic area (MPOA, a critical brain region for maternal behavior) has no effect (Gao et al., 2018). The present study not only replicated these findings, but also extended to the examination of their behavioral mechanisms. We used the pup preference test and found that activation of either receptor type did not affect pup preference, thus, the mPFC 5-HT_{2A} and 5-HT_{2C} receptors do not appear to have a disruptive effect on the motivational responses toward pups. Rather, activation of the mPFC 5-HT_{2A} receptors severely disrupted the executive control of maternal activities. This is supported by the finding that intra-mPFC injection of TCB-2 significantly decreased the sequential retrieval score, as well as other maternal responses. The sequential retrieval score was first used in Afonso et al. (2007) and is suggested to be a valid measure of executive function of dams, as it reflects the ability of dams to complete multiple pup retrievals in a timely fashion (within 15 s of each other) when they face distractions or mild stress. Its validity as a measure of executive function is also supported by the finding that it is selectively disrupted by the mPFC lesions (Afonso et al., 2007). However, as pointed out by Nie et al. (2018), one potential problem with this idea is that other psychological processes such as maternal motivation and anxiety levels could change the sequential pup retrieval score. Thus, it should be best interpreted with caution and better used together with anatomical information. In this case, it was the mPFC that was being manipulated, and the mPFC is well documented to play a central role in top-down control of many higher-order functions, such as working memory, attention, emotion regulation, inhibitory control, and cognitive flexibility (Anastasio et al., 2015; Moghaddam and Homayoun, 2008; Puig and Gulledge, 2011; Puig et al., 2015). Additionally, informal observations suggested that dams that were treated with intra-mPFC TCB-2 spent more time on rearing, walking, and remaining immobile.

The mPFC has reciprocal connections with the raphe nuclei (Celada et al., 2001, 2002). The robust anatomical and functional connections between the mPFC and the raphe nuclei suggest that 5-HT could play an important role in mPFC-mediated executive functions, primarily via its action on 5-HT_{2A} receptors and others (e.g., 5-HT_{1A}) (Aznar and Hervig

Mel, 2016; Carli and Invernizzi, 2014; Celada et al., 2013; Martin-Ruiz et al., 2001; Vazquez-Borsetti et al., 2009). Although 5-HT_{2A} and 5- HT_{2C} receptors are all expressed in the mPFC, 5-HT_{2A} receptors are by far the most abundant subtype in this region (Lopez-Gimenez et al., 2013; Pompeiano et al., 1994; Yadav et al., 2011). In addition, 5-HT_{2C} receptors are mostly concentrated in the deep layers (layers V and VI) and expressed predominantly on the *GABAergic neurons* (> 70% of cells) (Amitai et al., 2012; Nocjar et al., 2015), whereas 5-HT_{2A} receptors are primarily expressed in the pyramidal neurons in layers II/III and V (Amargos-Bosch et al., 2004; Santana et al., 2004). More importantly, behavioral studies suggest that activation of prefrontal 5- HT_{2C} receptors does not disrupt but actually improves executive function, an effect opposite to that seen in activation of 5-HT_{2A} receptors (Aznar and Hervig Mel, 2016; Nocjar et al., 2015). Our current finding that only 5-HT_{2A} but not 5-HT_{2C} receptors in the mPFC is involved in the mediation of maternal behavior is also consistent with the observation that prefrontal 5-HT_{2C} receptors do not modulate cortical pyramidal neurons (Celada et al., 2013).

For the first time, the present study identified the neural substrate that supports 5-HT_{2C}'s maternal effects. In our previous study, we microinjected MK212 into several brain regions, including the mPFC, NAc shell, and MPOA, however, none of these manipulations affected pup retrieval or other maternal responses (Wu et al., 2016). The present study found that intra-VTA microinjection of MK212 severely impaired various maternal responses, including pup retrieval, nest building and pup crouching. Moreover, intra-VTA injection of MK212 also significantly suppressed pup preference, suggesting that 5-HT_{2C} in the VTA may play a role in maternal behavior by altering maternal motivation. Informal observations suggested that dams treated with intra-VTA MK212 ignored the pups, and spent less time rearing and more time immobile. The VTA receives direct inputs from the raphe region (McDevitt et al., 2014; Watabe-Uchida et al., 2012), and 5-HT_{2C} receptors have been found in various VTA neurons, mainly the local GABA interneurons and more than half of the DA and GABA VTA neurons that project to the NAc (Bubar and Cunningham, 2007; Bubar et al., 2011; Xu et al., 2017). One major and well-documented effect of VTA 5-HT_{2C} activation is to inhibit DA cell firing and consequent DA release into the NAc (Howell and Cunningham, 2015), which is primarily achieved by activating local GABA neurons (Bubar et al., 2011; Di Giovanni et al., 2000; Di Matteo et al., 2002; Howell and Cunningham, 2015). This supports the idea that activating VTA

5-HT_{2C} receptors suppresses incentive motivation (Fletcher et al., 2004; Valencia-Torres et al., 2017), and is consistent with our current finding on decreasing pup preference. In contrast, activating 5-HT_{2C} receptors expressed on the VTA DA neurons would be expected to *stimulate* the output of DA through an intracellular cascade that results in neuronal depolarization (Sheldon and Aghajanian, 1991), causing an *increase* in DA cell firing and DA release (Bubar and Cunningham, 2007; Bubar et al., 2011). This action is likely to cause an increase in maternal motivation, an outcome opposite to its action on local VTA GABA neurons. In fact, a recent paper reports that the VTA DA neurons are transiently activated during pup retrieval (Fang et al., 2018). Based on these findings, we can tentatively conclude that activation of 5-HT_{2C} localized on the VTA GABA neurons causes a disruption of maternal motivation. This action is responsible for the maternal disruptive effect of MK212.

The present study also found that activation of 5-HT_{2A} receptors in the VTA impaired pup retrieval, pup crouching and nest building, but did not impair pup preference. Like 5-HT_{2C} receptors, the 5-HT_{2A} receptors in the VTA are localized to both DA and non-DA neurons (Doherty and Pickel, 2000; Nocjar et al., 2002; Rodriguez et al., 2000), and appear to have a stimulating effect on DA activity, as microinfusion of the preferential 5-HT_{2A} agonist 1-(2,5-dimethoxy-4-iodo)-2-aminopropane (DOI) into the VTA evokes hyperactivity in rats, and overexpression of 5-HT_{2A} receptors in the VTA enhances cocaine-evoked hyperactivity (Herin et al., 2013). Thus, activation of 5-HT_{2A} receptors in the VTA may play a role in maternal behavior by altering the DA mesocortical circuit. The specific behavioral mechanism underlying this effect of VTA 5-HT_{2A} receptors is not clear. Because intra-VTA infusion of TCB-2 also decreased the sequential retrieval score, but did not affect pup preference, this disruption might be due to a disruption of executive control of maternal activities. We had observed that a higher percentage of dams treated with intra-VTA TCB-2 often picked up pups in their mouths, then moved around in the cage before dropping them off, an activity highly consistent with this executive function view of the VTA 5-HT_{2A} receptors.

In conclusion, this study demonstrates that serotonin 5-HT_{2A} and 5-HT_{2C} receptors expressed in the mesocortical dopamine system regulate maternal behavior in the rat through distinct behavioral mechanisms. We found a doubly dissociated behavioral function involving the mPFC 5-HT_{2A} and VTA 5-HT_{2C} receptors. If confirmed in the future research, this knowledge could enhance our understanding of functional

roles of serotonin in the regulation of maternal behavior, and how dysregulation of 5-HT, especially the 5-HT_{2A} (Meyer, 2012) and 5-HT_{2C} receptor systems (Gurevich et al., 2002) may contribute to postpartum mental disorders (Gavin et al., 2005).

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Competing interests — None.

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