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Activation of 5-HT_{2A} receptor disrupts rat maternal behavior

Jun Gao [a, b, d, *, 1], Ruiyong Wu [c, d, 1], Collin Davis [d], Ming Li [a, b, d, **]

[a] Key Laboratory of Cognition and Personality (Southwest University), Ministry of Education, 400715, China
[b] Faculty of Psychology, Southwest University, Chongqing, 400715, China
[c] College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, Jiangsu 225009, China
[d] Department of Psychology, University of Nebraska–Lincoln, Lincoln, NE 68588-0308, USA

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ABSTRACT

Serotonin 5-HT_{2A} receptor is widely distributed in the central nervous system and plays an important role in sensorimotor function, emotion regulation, motivation, executive control, learning and memory. We investigated its role in rat maternal behavior, a naturalistic behavior encompassing many psychological functions that the 5-HT_{2A} receptor is involved in. We first showed that activation of 5-HT_{2A} receptor by TCB-2 (a highly selective 5-HT_{2A} agonist, 1, 2.5 or 5.0 mg/kg) disrupted maternal behavior dose-dependently, and this effect was reduced by pretreatment with a 5-HT_{2A} receptor antagonist MDL 100907, but exacerbated by pretreatment with a 5-HT_{2C} receptor antagonist SB242084 and a 5-HT_{2C} receptor agonist MK212, indicating that the maternal disruptive effect of 5-HT_{2A} activation is receptor-specific and can be modulated by 5-HT_{2C} receptor bidirectionally. We then microinjected TCB-2 into two brain regions important for the normal expression of maternal behavior: the medial prefrontal cortex (mPFC) and the medial preoptic area (mPOA) and found that only acute intra-mPFC infusion of TCB-2 suppressed pup retrieval, whereas intra-mPOA had no effect. Finally, using c-Fos immunohistochemistry, we identified that the ventral bed nucleus of stria terminalis (vBNST), the central amygdala (CeA), and the dorsal raphe (DR) were additionally involved in the maternal-disruptive effect of TCB-2. These findings suggest that the 5-HT_{2A} receptor in the mPFC and other maternally related regions is required for the normal expression of maternal behavior through its intrinsic action or interactions with other receptors (e.g. 5-HT_{2C}). Functional disruption of this neuroreceptor system might contribute to postpartum mental disorders (e.g. depression and psychosis) that impair the quality of maternal care.

1. Introduction

The 5-HT_{2A} receptor is densely expressed in various cortical areas (e.g. prefrontal cortex, piriform and enthorinal cortex) and subcortical areas, such as basal ganglia (e.g. caudate nucleus and accumbens) and the limbic system (Pompeiano et al., 1994). It plays an important role in a wide range of psychological functions, from sensorimotor function, emotion regulation, motivation, learning and memory to executive control (Zhang and Stackman, 2015). Dysfunctions of this receptor system have been suggested to contribute to several major neuropsychiatric disorders, such as schizophrenia, major depression, autism and Alzheimer's disease (Aznar and Hervig Mel, 2016; Fakhoury, 2016). Overall, the 5-HT_{2A} receptor appears to be an important neuromodulatory system that has a broad impact on basic brain functions.

Maternal behavior in rats is a naturally expressed and highly motivated behavior with its onset and maintenance critically dependent on many psychological functions that the 5-HT_{2A} receptor is intimately involved in. In addition, the 5-HT_{2A} receptor system and various sex hormones (e.g. estrogen, prolactin) that are fundamentally important for maternal behavior show various reciprocal interactions (Fink et al., 1996; Liang and Pan, 2000). Thus, it is logic to think that this receptor system should play a role in maternal behavior. This idea also fits well with the observation that the 5-HT_{2A} receptor is found in the brain regions important for olfactory processing (e.g. piriform and enthorinal cortex, endopiriform nucleus, and olfactory bulb/anterior olfactory nucleus), an important function necessary for maternal behavior. Surprisingly, at this time, there is no direct evidence supporting a role of 5-HT_{2A}

* Corresponding author. Faculty of Psychology, Southwest University, Chongqing, China.
** Corresponding author. Department of Psychology, University of Nebraska-Lincoln, 238 Burnett Hall, Lincoln, NE 68588-0308, USA.
E-mail addresses: gaojunscience@126.com (J. Gao), mli@unl.edu (M. Li).
[1] These authors contributed equally to this paper.

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receptor in the regulation of rat maternal behavior. The only available evidence is that drugs with a certain antagonist and agonist action on 5-HT_{2A} receptor, such as clozapine or DOI (2,5-dimethoxy-4-iodo-amphetamine), respectively, disrupt maternal behavior upon acute treatment (Li et al., 2004; Zhao and Li, 2009a, b; 2012). However, because both clozapine and DOI also have an action on 5-HT_{2C} receptor, and activation of 5-HT_{2C} alone is able to cause a severe disruption of maternal behavior care (Chen et al., 2014; Wu et al., 2016), it can be said that the maternal-disruptive effect of clozapine and DOI is due to their actions on 5-HT_{2C} receptor alone. Furthermore, recent work shows that selective blockade of 5-HT_{2A} receptor alone is ineffective to alter maternal care (Chen et al., 2014), casting a doubt on the involvement of the 5-HT_{2A} receptor in maternal behavior.

Because activation of 5-HT_{2A} receptor is capable of facilitating dopamine cell activity and dopamine release (Bortolozzi et al., 2003), and this action of dopamine is known to mediate rat maternal behavior, especially maternal motivation (Afonso et al., 2007; Febo et al., 2010; Li and Fleming, 2003; Numan, 2007), it is possible that activation of 5-HT_{2A} receptor, instead of blocking it, may cause alteration of maternal behavior. The present study tested this idea and examined the neurobiological mechanisms underlying the 5-HT_{2A} receptor effect in maternal behavior. We also explored the potential interactive effect of 5-HT_{2A} and 5-HT_{2C} receptors in maternal behavior in light of the findings that both 5-HT_{2A} and 5-HT_{2C} receptors are expressed throughout the mesolimbic and corticostriatal circuits (Bubar et al., 2011) and they often play *opposing roles* in various brain functions and psychological processes relevant to rat maternal behavior. Our findings revealed that the 5-HT_{2A} receptor, especially that expressed in the mPFC, is required for the normal expression of maternal behavior through its intrinsic action and/or interactions with other receptors (e.g. 5-HT_{2C}) or other neurotransmitter systems, such as dopamine. This work is scientifically significant as it enhances our understanding of the basic neurochemical basis of maternal behavior. It is also clinically significant, as it implies that dysregulation of frontal 5-HT_{2A} receptors may contribute to impaired maternal care as observed in people with postpartum depression who show elevated cortical 5-HT_{2A} receptor binding (Bhagwagar et al., 2006). Because many of these patients are treated with selective serotonin reuptake inhibitors (SSRIs), and chronic SSRI treatment could induce an up-regulation of the 5-HT_{2A} receptors (Hamon and Blier, 2013; Massou et al., 1997), this study also sheds light on the potential long-term negative impact of SSRI use during lactation on the quality of maternal care.

2. Materials and methods

2.1. Animals

Naïve pregnant female Sprague-Dawley rats (gestational days 6 upon arrival to the animal facility) were purchased from Charles River Inc. All rats were housed individually in 48.3 cm × 26.7 cm × 20.3 cm transparent polycarbonate cages under 12-h light/dark conditions (lights on at 6:30 a.m.), and had access to standard laboratory rat chow and tap water *ad libitum*. The colony was maintained with a controlled temperature (21 ± 1 °C) and a relative humidity of 45–60%. Experiments were conducted during the light cycle. All animal manipulations were reviewed and approved by the University of Nebraska Institutional Animal Care and Use Committee, 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 (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide) was used as the main pharmacological tool to investigate the role of the 5-HT_{2A} receptor in maternal behavior. It is highly selective on 5-HT_{2A} receptors, with no reported actions on other receptors (McLean et al., 2006). TCB-2, MK212 [6-Chloro-2-(l-piperazinyl) pyrazine hydrochloride] and SB242084 [6-Chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridiny]-1H-indole-1-carboxyamide dihydrochloride hydrate] were obtained from Tocris Bioscience (Ellisville, MO, USA). MDL100907 [(R)-(+)-a-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-pipidinemethanol] was purchased from Sigma-Aldrich (St. Louis, MO, USA). TCB-2 and MK212 was dissolved in 0.9% saline, while MDL100907 was dissolved in a minimal amount (up to 1%) glacial acetic acid and made up to volume with 0.9% saline. SB242084 was dissolved in 0.9% saline solution containing 8% hydroxypropyl-β-cyclodex-trin and 25 mM citric acid. All drugs were administrated subcutaneously except in Experiment 4 (an intracranial infusion). Doses of TCB-2, MDL100907, MK212, and SB242084 were chosen either based on our previous study (Chen et al., 2014) or several recent reports (Boulougouris et al., 2008; Burghardt et al., 2007; Fox et al., 2010; Strong et al., 2009). In Experiment 4, the bilateral microinjection (0.5 μl at 0.5 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. Basic procedure of the maternal behavior test

The basic procedure was identical to what has been described in our previous studies (Chen et al., 2014; Zhao and Li, 2009b). Starting 2 or 3 days prior to the first possible expected parturition date, the subjects were monitored in the morning and afternoon for signs of parturition. Once the dam was found with pups in the morning (that day was designated as postpartum day 1, PPD 1) or in the afternoon (PPD 0), two shredded paper towels were provided for nesting materials. On PPD 2, each litter was culled to 8 pups (4 males and 4 females with the most visible milk bands) and all subjects were changed to clean observation cages with their litters.

On the maternal behavior test days, pups were first removed from the dam and the nest was destroyed. Ten seconds later, the pups were placed back in the cage at the corner diagonal to the original nest site or dam sleeping corner. Each test was recorded by video cameras and analyzed manually using a computer with an event-recording program (JWatcher, http://www.jwatcher.ucla.edu). The raters were blind to each dam's treatment condition. The following behaviors were recorded and analyzed: pup retrieval (a rat picking up a pup in her mouth and carrying it back to the nest site), hovering over pup (a rat positioning herself over the pups with legs splayed to accommodate the pups, including hover, high and low crouching-over posture), pup licking (a rat placing its tongue on the anogenital area and the rest of a pup's body), nest building (a rat picking up nest material in her mouth and transporting it back to the nest site or pushing the material with her forepaws towards the nest site). The first pup retrieval latency was defined as the time elapsed from the first pup approach to the retrieval of the first pup into the nest. 600s was assigned to non-responders who did not approach or retrieve the testing pups. After the test, unretrieved pups were returned to the nest site. On PPD 2 or 3, to screen for baseline maternal performance and habituate dams to the testing procedure, we did a pup retrieval test (removing pups then return them 10 s later) for 10 min, and at the end of the 10-min period, unretrieved pups were returned to the nest site. Only those that retrieved all 8 pups were used in the

subsequent tests. Rats were typically tested at several time points before (−30 min) and after the drug administration (e.g. 30 min, 90 min). Frequency and/or duration of various maternal responses were recorded for 10 min.

2.4. Experiment 1: basic effects of 5-HT_{2A} activation by TCB-2 on maternal behavior

In this experiment, we tested 8 postpartum rats using a within-subjects Latin square design to determine whether 5-HT_{2A} receptor is involved in the regulation of maternal behavior expression and establish a minimum effective dose of TCB-2. Mother rats were randomly assigned to receive either vehicle (VEH), or one of the three doses of TCB-2 (5-HT_{2A} agonist, 1.0, 2.5 or 5.0 mg/kg, sc) on PPD 4, 6, 8, and 10. Maternal behavior was tested for 10 min at 30 min before and 30 min, 120 min, 24 h after the injection. Each rat was tested across the four treatment conditions, thus it served as its own control. Various measures of maternal behavior were recorded and quantified (e.g. frequency or duration).

2.5. Experiment 2: receptor specificity of TCB-2's maternal disruptive effect

This experiment was aimed to examine the receptor specificity of 5-HT_{2A} receptor and confirm TCB-2's effects on maternal behavior in Experiment 1 using a between-subjects design, since maternal behavior is known to decline with the progress of lactation. TCB-2 (2.5 mg/kg) was tested together with MDL100907 (a 5-HT_{2A} antagonist, 1.0, 2.0 mg/kg, sc) or saline. Specifically, on PPD 4, 6, 8, and 10, 32 mother rats (n = 8 per group) were randomly assigned to receive either a double injection of VEH (saline with 1% glacial acetic acid)+VEH (saline, n = 8), VEH (1% acid saline)+TCB-2 (n = 8), MDL100907−1.0+TCB-2 (n = 8), MDL100907−2.0+TCB-2 (n = 8). The first VEH or MDL100907 was injected 10 min before the second VEH or TCB-2 injection. Maternal behavior was tested for 10 min at 30 min before, 30 min, 90 min and 150 min after TCB-2 injection.

2.6. Experiment 3: interactions between 5-HT_{2A} receptor and 5-HT_{2C} receptor on maternal behavior

The 5-HT_{2A} and 5-HT_{2C} receptors often has opposing effects on behavior (Popova and Amstislavskaya, 2002; Robinson et al., 2008; Winstanley et al., 2004). In this experiment, we examined how the maternal disruptive effect of TCB-2 is altered by the blockade or activation of 5-HT_{2C} receptor in an attempt to elucidate the possible interaction between these two serotonin receptors on maternal behavior. TCB-2 (2.5 mg/kg) was tested together with either SB242084 (a 5-HT_{2C} antagonist, 0.6 and 1.0 mg/kg, sc), or MK212 (a 5-HT_{2C} agonist, 0.5, 1.0 mg/kg, sc). It should be noted that at the tested doses, SB242084 has no effect on maternal care (unpublished observations), whereas MK212 disrupts maternal behavior (Chen et al., 2014; Wu et al., 2016). On PPD 4, 6, 8, and 10, 29 mother rats (n = 7–8 per group) were randomly assigned to receive either SB242084−0.6+TCB-2 (n = 8), SB242084−1.0+TCB-2 (n = 7), MK212−0.5+TCB-2 (n = 7), or MK212−1.0+TCB-2 (n = 7), and their data were compared with those of the VEH + VEH (n = 8) and VEH + TCB-2 (n = 8) groups from Experiment 2. On each test day, the first VEH or SB242084 was injected at 10 min before the second VEH or TCB-2 injection, while MK212 was injected together with TCB-2 based on our previous studies showing that MK212's time course of action on maternal behavior is similar to that of TCB-2 (around 2 h) (Chen et al., 2014; Wu et al., 2016). Maternal behavior was tested for 10 min at 30 min before, 30 min, 90 min and 150 min after TCB-2 injection on PPD 4, 6, 8, and 10.

2.7. Experiment 4: neural basis of TCB-2's effect on maternal behavior: a microinjection study

In this study, we attempted to identify the brain regions where TCB-2 acts to disrupt maternal behavior by centrally infusing TCB-2 into the mPOA or mPFC. We chose the mPOA because it is the most critically important brain region for maternal behavior (Numan, 2007). We chose the mPFC because it is not only involved in maternal behavior (Afonso et al., 2007; Febo et al., 2010; Zhao and Li, 2012), but also appears to mediate the behavioral and neurochemical effects of 5-HT_{2A} activation (Feng et al., 2015; Kuroki et al., 2003). On day 11–13 of gestation, rats were anaesthetized using a mixture of ketamine HCl (90 mg/kg) and xylazine (4 mg/kg) (ip), and implanted with bilateral stainless-steel guide cannulas (22 gauge; Plastics One, Inc.) into the mPOA (n = 5) or mPFC (n = 26). The incisor bar was set at −3.4 mm. For the mPOA cannulation, the coordinates were set as: AP -0.5 mm, ML ± 0.75 mm, DV -6.5 mm (Small et al., 2003). For the mPFC, the coordinates were: AP + 3.0 mm, ML ± 0.75 mm, DV -2.2 mm (Febo et al., 2010).

In the mPOA experiment, we did a quick screening test on 5 postpartum females. They were tested for 10 min at 10 and 60 min after the central infusion of VEH, 0.1, 0.4, or 4.0 μg/side/0.5μl/side of TCB-2 on PPD 4, 6, 8, and 10, respectively. In the mPFC experiment, two batches of rats were tested. The first batch of 8 rats were tested identically as those in the mPOA experiment under the same dose range (VEH, 0.1, 0.4, or 4.0 μg/side/0.5μl/side). Based on the results from that study, we then used a between-subjects design and tested another batch of 18 rats randomly assigned to either the VEH (n = 8) or TCB-2 (n = 10) groups. Rats were tested for 10 min at 30 and 120 min after the central infusion of VEH or TCB-2 4.0 μg/side/0.5μl/side for 4 days on every other day from PPD 4 to 10. At the end of behavioral tests, rats were sacrificed and perfused. Their brains were sectioned and then stained with cresyl violet before viewing cannula placement as previously reported (Feng et al., 2015). The location of the injection site was mapped onto a stereotaxic atlas (Paxinos, 2005) (Fig. 4A).

2.8. Experiment 5: neural basis of TCB-2 effect on maternal behavior: a c-Fos immunohistochemistry study

To broaden our search of the relevant brain regions involved in the mediation of TCB-2's maternal disruptive effect, we used c-fos immunohistochemistry and examined the increased c-fos expressions in acute and repeated TCB-2-treated rats. A total of 46 postpartum rats were randomly divided into one of six groups: 5-day VEH (n = 10), 5-day 2.5 mg/kg TCB-2 (n = 9) and 5.0 mg/kg TCB-2 (n = 10) groups, as well as 1-day VEH (n = 6), 1-day 2.5 mg/kg TCB-2 (n = 6) and 5.0 mg/kg TCB-2 (n = 5) groups. For the 5-day groups, maternal behavior was tested for 10 min once daily from PPD 6 to 9 starting at 30 min after TCB-2 or vehicle injection. For the 1-day groups, maternal behavior was tested once daily from PPD 6 to 9, but no injection was done.

On PPD 10, all rats received either TCB-2 or VEH injection. One hour later, rats were overdosed and perfused as described in our previous work and their brains were extracted for c-Fos immunoreactivity staining (Zhao and Li, 2010, 2012). The number of positive cells characterized by clearly labeled nuclei was counted unilaterally in six serial sections with comparable anatomical levels across the treatment groups. We focused on the mPFC, nucleus accumbens shell (NAs) and nucleus accumbens core (NAc), dorsolateral striatum (DLSt), ventral lateral septum (LSv), mPOA, ventral tegmental area (VTA), and dorsa raphe (DR), because 5-$HT_{2A/2C}$ receptor agonist (DOI) and antagonist (clozapine and olanzapine) are shown to have effects on these regions in mother rats (Zhao and Li, 2010, 2012). DR was also chosen because it is a major serotonergic

brain site (Azmitia and Segal, 1978; Liu et al., 2000; Queree et al., 2009; Steinbusch, 1981). Other brain regions analyzed included the ventral bed nuclsus of the stria terminalis (vBNST), central (CeA) and medial amygdala (MeA), dentate gyrus (DG), and peri-aqueductal gray (PAG) (Paxinos, 2005).

2.9. Statistical analysis

Statistical analyses were performed using SPSS 20 software (SPSS Inc., Chicago, IL, USA). Maternal behavior data from each test day (PPD 4, 6, 8 and 10) 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 different test time points were further investigated using simple main effect tests (one-way ANOVA) followed by LSD post hoc tests for multiple comparisons when necessary. Data from the experiments with a Latin square or within-subjects design were analyzed using the Paired-Sample test. In Experiment 5, repeated measures ANOVAs were conducted to examine the effects of repeated TCB-2 administration from PPD 6 to 9. c-Fos data were analyzed using multivariate analysis of variance in a 2 × 2 design (drug and treatment condition as between-subjects factors, brain regions as within-subjects factors), and significant effects were followed up using the LSD post hoc test comparing the within-treatment condition across drug, and within-drug condition across treatment. Because the latency was not normally distributed, those data were analyzed using nonparametric Kruskal-Wallis test, and Mann-Whitney *U* test if the overall significant effects were determined. All data are presented as mean ± SEM. Differences were considered statistically significant if $p < 0.05$. In Experiment 3, 1 rat in the SB242084−1.0+TCB-2 group died unexpectedly before PPD 10, thus its data on that day were not included. Except for the data from Experiment 1, we only presented pup retrieval as a representation of the maternal disruptive effect of TCB-2 for simplicity reason. All other data (i.e. hovering over pup, licking and nest building) from Experiments 2, 3 and 4 are included in the supplementary materials.

3. Results

3.1. Experiment 1: basic effects of 5-HT_{2A} activation by TCB-2 on maternal behavior

Acute TCB-2 treatment dose-dependently disrupted various maternal responses. In comparison to the VEH-treated rats, rats under the influence of TCB-2 retrieved fewer pups into the nest at the 30 min ($p = 0.047$, 0.006, and 0.011 for TCB-2 1.0, 2.5, and 5.0 mg/kg) and 120 min time points ($p = 0.033$, 0.003, and 0.000 for TCB-2 1.0, 2.5, and 5.0 mg/kg) (Fig. 1A). This disruption disappeared at the 24 h time point ($p > 0.17$). In addition, TCB-2 dose-dependently prolonged the 1st pup retrieval latency, decreased amount of time spent on hovering over pup and nest building at the 30 min and 120 min points (all $p < 0.05$) (Fig. 1B, C and 1E). It also suppressed pup licking at the 120 min point ($p = 0.034$ and 0.050, for TCB-2 1.0 and 5.0 mg/kg respectively).

3.2. Experiment 2: receptor specificity of TCB-2's disruptive effect on maternal behavior

Once again, acute treatment of TCB-2 severely disrupted various components of maternal behavior on PPD 4. This confirmed the TCB-2's disruptive effect on maternal behaviors in Experiment 1 using the within-subjects design. As expected, pretreatment of the 5-HT_{2A} antagonist MDL100907 dose-dependently attenuated the TCB-2-induced maternal disruption. Fig. 2 shows the results of TCB-2 treatment alone, and MDL100907 (1.0 or 2.0 mg/kg) + TCB-2 treatment on pup retrieval at four test time points on PPD 4 in comparison to the vehicle control (data from PPD 6, 8 10 are similar to those from PPD 4. For simplicity, they are not presented). Both doses of MDL100907 were effective in reversing the effect of TCB-2 and 1.0 mg/kg dosage appears to be even more effective. Repeated measures ANOVA revealed a main effect of group [$F(3, 28) = 6.557$, $p = 0.002$], a main effect of test time [$F(3, 84) = 17.014$, $p = 0.000$], and a significant group × test time interaction [$F(9, 84) = 2.842$, $p = 0.006$]. Post hoc LSD tests indicated that the VEH + TCB-2 and MDL100907−2.0+TCB-2 groups retrieved fewer pups than the VEH + VEH group ($p < 0.001$ and $p = 0.004$, respectively), while the MDL100907−1.0+TCB-2 group actually retrieved more pups than the VEH + TCB-2 group ($p = 0.029$).

3.3. Experiment 3: interactions between 5-HT_{2A} receptor and 5-HT_{2C} receptor on maternal behavior

TCB-2-induced pup retrieval disruption was worsened by MK212 and SB242084 treatment, which activates and blocks 5-HT_{2C} receptor, respectively. Fig. 3A shows the pretreatment effect of SB242084 (0.6 and 1.0 mg/kg) on PPD 4. Repeated measures ANOVA revealed a main effect of group [$F(3, 27) = 45.633$, $p = 0.000$], a main effect of test time [$F(3, 81) = 100.785$, $p = 0.000$], and a significant group × test time interaction [$F(9, 81) = 12.813$, $p = 0.000$]. Post hoc LSD tests indicated that the two SB242084+TCB-2 groups retrieved fewer pups than the VEH + TCB-2 group ($p = 0.026$ and 0.009, respectively).

Fig. 3B shows the results of pretreatment with MK212 (0.5, 1.0 mg/kg) treatment on pup retrieval at four test time points on PPD 4. Repeated measures ANOVA revealed a main effect of group [$F(3, 26) = 37.187$, $p = 0.000$], a main effect of test time [$F(3, 78) = 52.172$, $p = 0.000$], and a significant group × test time interaction [$F(9, 78) = 6.457$, $p = 0.000$]. Post hoc LSD tests indicated that the VEH + TCB-2, MK212−0.5+TCB-2, and MK212−1.0+TCB-2 groups retrieved fewer pups than the VEH + VEH group (all $p < 0.001$). More importantly, the MK212−1.0+TCB-2 group retrieved fewer pups than the VEH + TCB-2 group ($p = 0.005$).

3.4. Experiment 4: neural basis of TCB-2's effect on maternal behavior: a microinjection study

To examine the neuroanatomical basis of action of TCB-2 in maternal behavior, we microinjected TCB-2 at 0.1, 0.4, or 4 μg/side into the mPOA or mPFC. All the injection sites were verified in the intended targeted areas. Results showed that intra-mPOA infusion of TCB-2 had no effect on pup retrieval (all $p > 0.241$) (Fig. 4B). However, intra-mPFC infusion of TCB-2 at 4 μg/side, but not at 0.1 or 0.4 μg/side, decreased the number of pups retrieved at 60 min time point ($p = 0.008$; Fig. 4C). This result from a within-subjects study was confirmed in the subsequent study with a between-subjects design. TCB-2 at 4 μg/side infused into the mPFC transiently suppressed pup retrieval at 30 min ($p = 0.006$) but not 120 min ($p = 0.144$) time points on PPD 4 (Fig. 4D). Such a disruption was also observed on PPD 6, 8, and 10, but the magnitude was reduced so that the group difference was found to be not significant (all $p > 0.051$), indicating a tolerance-like effect with TCB-2-induced activation of prefrontal 5-HT_{2A} receptor.

3.5. Experiment 5: neural basis of TCB-2 effect on maternal behavior: a c-Fos immunohistochemistry study

Behaviorally, acute TCB-2 treatment on PPD 6 dose-dependently reduced the number of pups retrieved ($p < 0.05$). With repeated

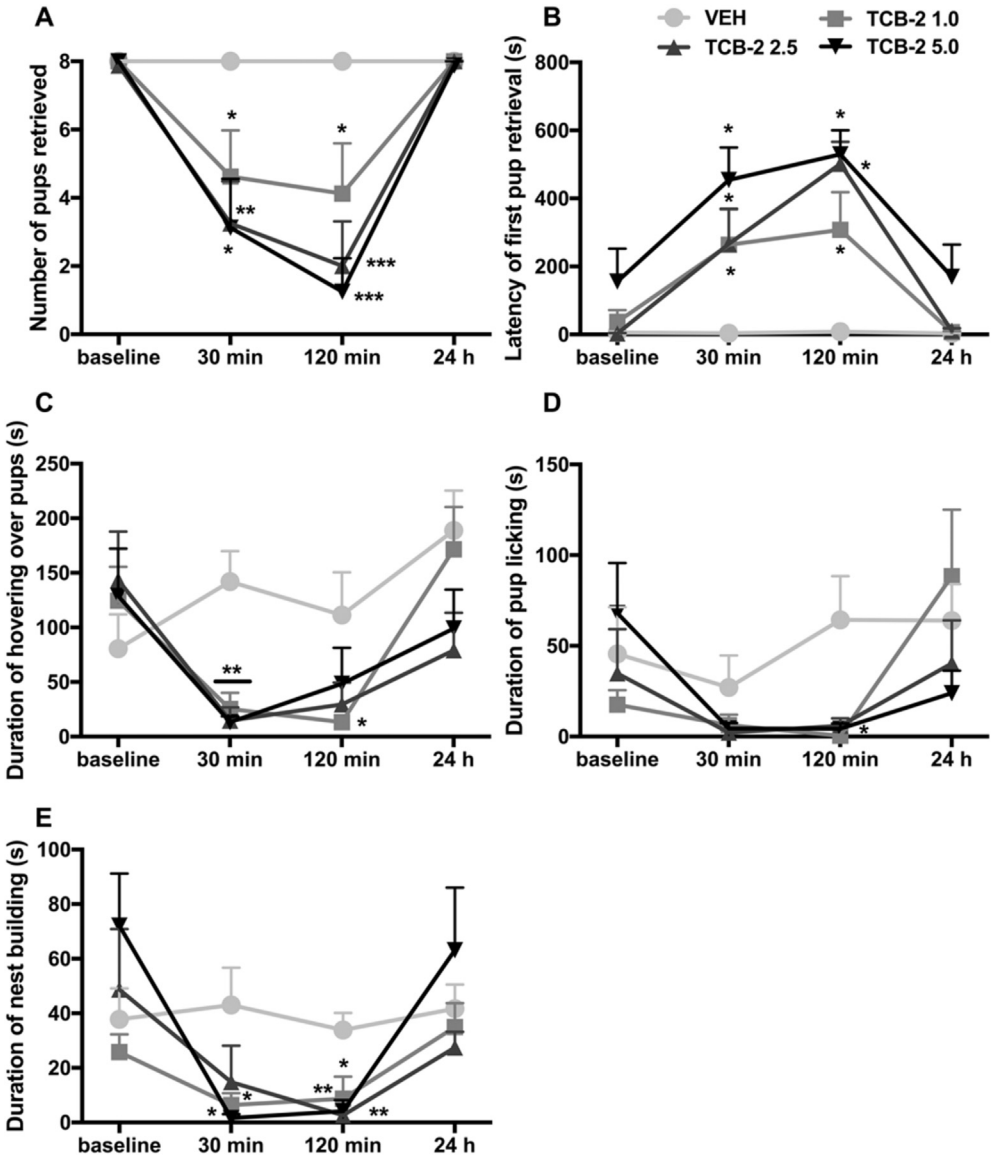

Fig. 1. Effects of systemic TCB-2 injection on maternal behavior. Mother rats were randomly assigned to receive an injection of vehicle (VEH), or TCB-2 (1.0, 2.5 or 5.0 mg/kg, sc) on PPD 4, 6, 8 and 10 when maternal behavior was observed for 10 min at 4 time points: 30 min before, 30 min, 120 min and 24 h after the injection. Data are presented and expressed as mean + SEM. **A**, number of pups retrieved; **B**, latency of first pup retrieval; **C**, duration of hovering over pups; **D**, duration of pup licking; **E**, duration of nest building. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different between the different drug administrations (VEH vs. TCB-2).

administration, TCB-2 gradually lost its disruption in a dose-dependent fashion (VEH vs. TCB-2 2.5, $p > 0.05$ on PP 7, 8, and 9; VEH vs. TCB-2 5.0, $p = 0.000$ on PP 7 and 8; $p > 0.05$ on PPD 9) (Fig. 5A). Repeated measures ANOVA revealed a significant main effect of drug treatment [$F(2, 26) = 18.629$, $p = 0.000$], test day [$F(3, 78) = 11.152$, $p = 0.000$] and interaction between the two factors [$F(6, 78) = 3.076$, $p = 0.009$].

With the acute treatment, TCB-2 significantly altered c-Fos immunoreactivity in the vBNST [$F(2, 14) = 5.463$, $p = 0.018$], CeA [$F(2, 14) = 6.634$, $p = 0.009$] and DR [$F(2, 14) = 4.902$, $p = 0.024$], with no significant change in other examined regions (all $p > 0.05$). Specifically, in comparison to the VEH treatment, TCB-2 at 5.0 mg/kg significantly increased c-Fos immunoreactivity in the vBNST, CeA and DR (all $p < 0.05$), whereas TCB-2 at 2.5 mg/kg increased c-Fos immunoreactivity only in the vBNST and DR (all $p < 0.05$), showing a dose-dependent effect in the CeA (Fig. 5B and D). The two TCB-2 groups did not differ from each other. With the repeated TCB-2 treatment, TCB-2 failed to alter c-Fos immunoreactivity in the regions where acute TCB-2 had an effect, including the vBNST [$F(2, 18) = 0.093$, $p > 0.05$], CeA [$F(2, 18) = 2.005$, $p > 0.05$] and DR [$F(2, 18) = 0.625$, $p > 0.05$], and also failed to show c-Fos immunoreactivity in other examined areas (Fig. 5C). The lack of change in c-Fos immunoreactivity matches well with the lack of behavioral effects of repeated TCB-2 treatment, indicating that the c-Fos signal is valid in revealing brain regions targeted by TCB-2.

4. Discussion

This is the first study to provide direct evidence that 5-HT_{2A} is critically important for the regulation of maternal behavior in rats. Behaviorally, activation of the 5-HT_{2A} receptor by TCB-2 dose-dependently disrupted various maternal responses, especially pup retrieval and hovering over pups. Neurochemically, we showed that the effect of TCB-2 was receptor-specific, as only pretreatment of a

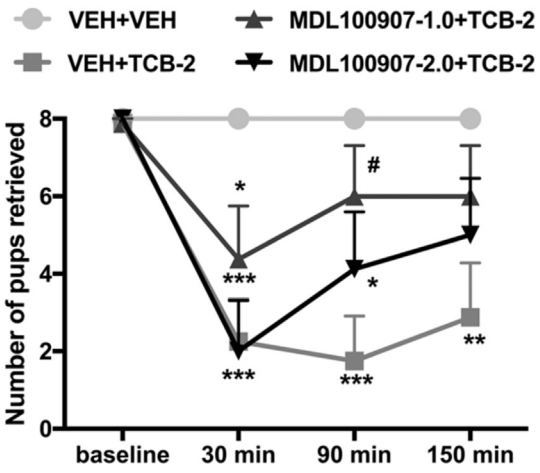

Fig. 2. Effects of MDL100907 (1.0, 2.0 mg/kg) pretreatment on TCB-2 (2.5 mg/kg)'s disruptive effect on pup retrieval. On PPD 4, the first VEH and MDL100907 was injected 10 min before the second VEH and TCB-2 injection. Pup retrieval was tested for 10 min at 30 min before (baseline), 30 min, 90 min and 150 min after TCB-2 injection. Number of pups retrieved in each test is expressed as mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different between the VEH + VEH and +TCB-2 groups. $\#p < 0.05$ significantly different between MDL100907-(1.0 or 2.0)+TCB-2 and VEH + TCB-2 groups.

emotional processing, incentive motivation, and memory, are involved in the support of these behavioral responses. In light of the current findings, one obvious question is: how does TCB-2 affect basic psychological processes to cause maternal disruption? Our recent work suggests that the TCB-2's induced disruption is not likely caused by the drug's effect on mothers' motivational and emotional processing of the incentive salience of pups, as dams treated with TCB-2 actually increased their preference to pups over a male conspecific in a pup preference test (Wu et al., under review). This enhanced emotional and motivational responses towards pups might be due to the stimulating effect of TCB-2 on dopamine neurotransmission in the mesocortical and mesolimbic dopamine systems (Di Giovanni et al., 2000; Di Matteo et al., 2002). TCB-2 also does not appear to cause a disruption of maternal behavior by simply increasing motor activity and stereotypical behaviors. However, previous work suggests that just increasing locomotor behavior and stereotypies in rats does not significantly alter their ability to be maternal (Johns et al., 1998). Also in a related study, we did not find any significant change in motor activity in dams treated with TCB-2 (Wu et al., under review). One possible mechanism may involve the action of TCB-2 on behavioral organization, as stimulation of 5-HT_{2A} receptor is shown to increase impulsive response while suppression of 5-HT_{2A} receptor decrease it (Winstanley et al., 2004). Similar to DOI, a selective 5-$HT_{2A/2C}$

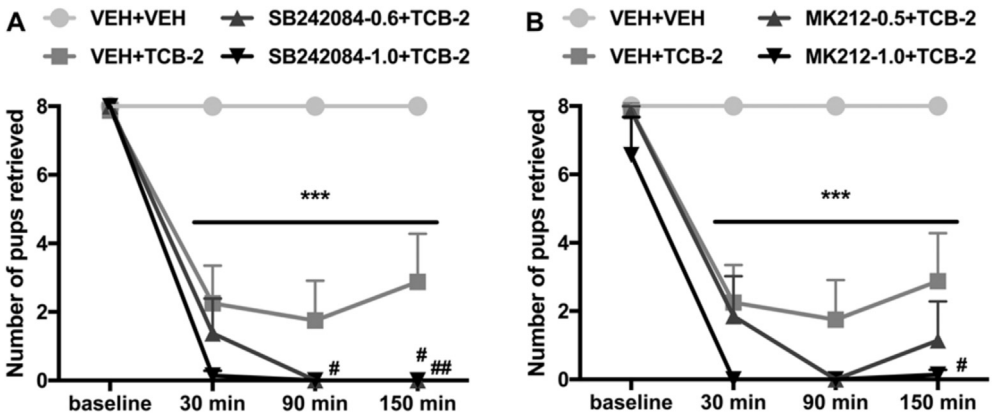

Fig. 3. Effects of SB242084 (0.6, 1.0 mg/kg) (**A**) and MK212 (0.5, 1.0 mg/kg) (**B**) pretreatment on TCB-2 (2.5 mg/kg)'s disruptive effect on pup retrieval. SB242084 (0.6, 1.0 mg/kg) was injected 10 min before the second VEH and TCB-2 injection, while MK212 (0.5, 1.0 mg/kg) was injected together with TCB-2. Pup retrieval was tested for 10 min at 30 min before (baseline), 30 min, 90 min and 150 min after TCB-2 injection. Number of pups retrieved in each test is expressed as mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different between the VEH + VEH and TCB-2 groups. $\#p < 0.05$, $\#\#p < 0.01$ significantly different between SB242084-(0.6 or 1.0)+TCB-2 or MK212-(0.5, or 1.0) and VEH + TCB-2 groups.

selective 5-HT_{2A} receptor antagonist MDL100907 reduced the maternal disruptive effect of TCB-2, while pretreatment with 5-HT_{2C} receptor drugs, SB242084 (a 5-HT_{2C} antagonist) or MK212 (a 5-HT_{2C} agonist), exacerbated it. This result also suggests that the maternal disruptive effect of 5-HT_{2A} activation is modulated by 5-HT_{2C} receptor bidirectionally. Central action of TCB-2 points out that the 5-HT_{2A} receptors, especially those in the mPFC, vBNST, CeA and DR, are likely involved in the mediation of maternal behavior. The 5-HT_{2A} receptors in the mPOA did not appear to be involved in the effect of TCB-2, as results from both the microinjection and c-fos studies did not find any effect when the MPOA was manipulated or examined. However, this conclusion needs to be further examined because of the small size.

Maternal behavior is a cluster of observable behavioral responses (e.g. pup retrieval, pup licking, nursing and nest building) organized seamlessly to ensure the survival of the young. Various psychological processes such as sensorimotor function, attention, agonist which we have shown to exert a disruptive effect on rat maternal behavior via its action on 5-HT_{2A} receptor (Zhao and Li, 2010), TCB-2, like other 5-HT_{2A} agonists (Gonzalez-Maeso et al., 2007; Krebs-Thomson et al., 1998), is a hallucinogen (e.g. inducing head twitches) (Fox et al., 2010) that could disrupt the organization of behavioral response patterns (e.g. increased fragmentation and premature, or 'impulsive' responding) necessary for the normal expression of maternal responses. In other words, TCB-2 might have disrupted the behavioral organization aspect of the executive function. In our tests, we did observe that rats treated with TCB-2 often exhibited interrupted normal sequence of pup-directed responses (e.g. pup retrieval and pup licking, fragmentation), indicating a disruption of the organization of microregulatory maternal responses. This idea is also consistent with our later finding that the mPFC is one critical site for the action of TCB-2 (Fig. 5), and the mPFC is known for its role in the executive control (Chudasama, 2011). Future work needs to employ other highly

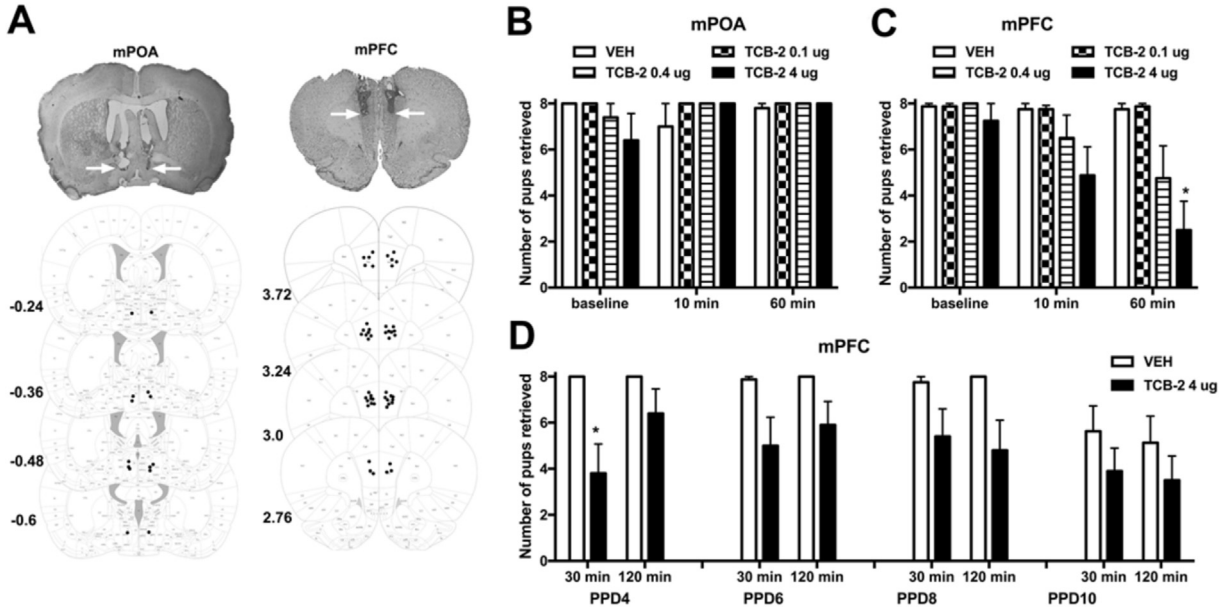

Fig. 4. **(A)** Histological representations of microinjection sites and schematic diagrams showing the location of the injector tips in the mPOA and mPFC. Data are reconstructed from Paxinos and Watson (Paxinos, 2005). *Numbers to the left of the sections* indicate anteroposterior distance from bregma in millimeters. The *arrow* in the histological representation section and *black dot* in the schematic diagrams denotes the infusion placement. Effects of TCB-2 microinfused into the medial preoptic area (mPOA, **B**), or medial prefrontal cortex (mPFC, **C** and **D**) on pup retrieval throughout the four test days (PPD 4, 6, 8 and 10). Number of pups retrieved in each test is expressed as mean + SEM. $^{*}p < 0.05$ significantly different between the VEH and TCB-2 groups.

selective 5-HT_{2A} receptor agonists, coupled with detailed behavioral analysis of specific executive functions to further test this hypothesis.

Our previous work fails to show any maternal effect with blockade of 5-HT_{2A} receptor, as acute and repeated treatment of MDL100907 does not alter maternal behavior at the behaviorally active doses (Chen et al., 2014). In the present study, MDL100907 pretreatment attenuated the maternal disruptive effect of TCB-2, confirming that the mechanism of action of TCB-2 is through stimulation of 5-HT_{2A} receptor. A more interesting and unexpected finding is that both 5-HT_{2C} agonist and antagonist potentiated the maternal disruptive effect of TCB-2. Although the 5-HT_{2A} and the 5-HT_{2C} receptors are closely related members of the G-protein-coupled receptors that share the highest degree of sequence homology (about 50% overall sequence identity) and cellular signaling pathways (Becamel et al., 2004), they often play opposing roles in various brain functions and psychological processes. Overall evidence seems to suggest that activation of 5-HT_{2A} receptors has a similar function to blockade of 5-HT_{2C} receptors, whereas activation of 5-HT_{2C} receptors is functionally equivalent to blockade of 5-HT_{2A} receptors. These opposing effects between 5-HT_{2A} and 5-HT_{2C} receptors are found in the modulation of dopamine release and cell firing (Di Giovanni et al., 2002; Di Matteo et al., 2002; Ichikawa et al., 2001; Millan et al., 1998), in behavioral inhibition and impulsivity (Robinson et al., 2008; Winstanley et al., 2004), reversal learning (Boulougouris et al., 2008), head-twitch response (Vickers et al., 2001), sexual behavior (Popova and Amstislavskaya, 2002), as well as in drug-motivated behaviors (Filip et al., 2004; McMahon et al., 2001). Based on these observations, we originally expected to see the 5-HT_{2C} antagonist SB240084 to enhance while the 5-HT_{2C} agonist MK212 to reverse the maternal disruptive effect of TCB-2. Indeed, the behavioral enhancement effect of SB240084 was confirmed. SB240084 may do so to further increase TCB-2-induced dopamine release in the mPFC and NA and cell firing in the ventral tegmental area, causing disruption of *behavioral organization* (fragmentation) (Di Giovanni et al., 2002; Di Matteo et al., 2002; Ichikawa et al., 2001; Millan et al., 1998). In contrast, MK212, as a 5-HT_{2C} agonist, was supposed to counteract the TCB-2-induced increase in dopamine release (Di Giovanni et al., 2000; Di Matteo et al., 2002) to reduce the maternal disruptive effect of TCB-2. The lack of such an effect is important, as it indicates that TCB-2 could disrupt maternal behavior through other mechanisms, such as by influencing the glutamatergic function in the mPFC, in addition to its known impacts on the VTA dopaminergic cells. Our microinjection work showing that intra-mPFC injection of TCB-2 suppressed pup retrieval, is consistent with this idea. This idea is also supported by the observation that the mPFC glutamatergic neurons do express 5-HT_{2A} (Nocjar et al., 2015); lesions of the mPFC can cause pup retrieval deficits (Afonso et al., 2007); and inactivation or inhibition of neuronal activity in the mPFC disrupt maternal behavior (Febo et al., 2010).

The direct interaction between the 5-HT_{2A} and 5-HT_{2C} in the mPFC has been reported in motor impulsivity (Anastasio et al., 2015). Existing evidence suggests that the mPFC glutamatergic neurons predominantly express 5-HT_{2A}, whereas the mPFC GABAergic interneurons predominantly express 5-HT_{2C} (Nocjar et al., 2015). Therefore, TCB-2 could stimulate the 5-HT_{2A} receptors on the glutamatergic neurons to disrupt maternal behavior, while MK212 could stimulate the 5-HT_{2C} receptors on the GABAergic interneurons to cause a disinhibition of mPFC glutamatergic neurons, leading to a further exacerbation of maternal disruption. This is an intriguing idea and worth further investigation.

Our previous c-Fos studies show that the 5-$HT_{2A/2C}$ agonist DOI and antagonists clozapine and olanzapine, though not selective to 5-HT_{2C} receptors, increased c-Fos expression in the mPFC (Zhao and Li, 2010, 2012). In the present study, although we did observe an increase in c-Fos expression in the mPFC by acute TCB-2, the effect did not reach a statistically significant level. The c-Fos results reveal several other brain sites where TCB-2 might have an action, including the vBNST, CeA and DR, all of which has been implicated in the regulation of certain aspect of maternal behavior (Barofsky

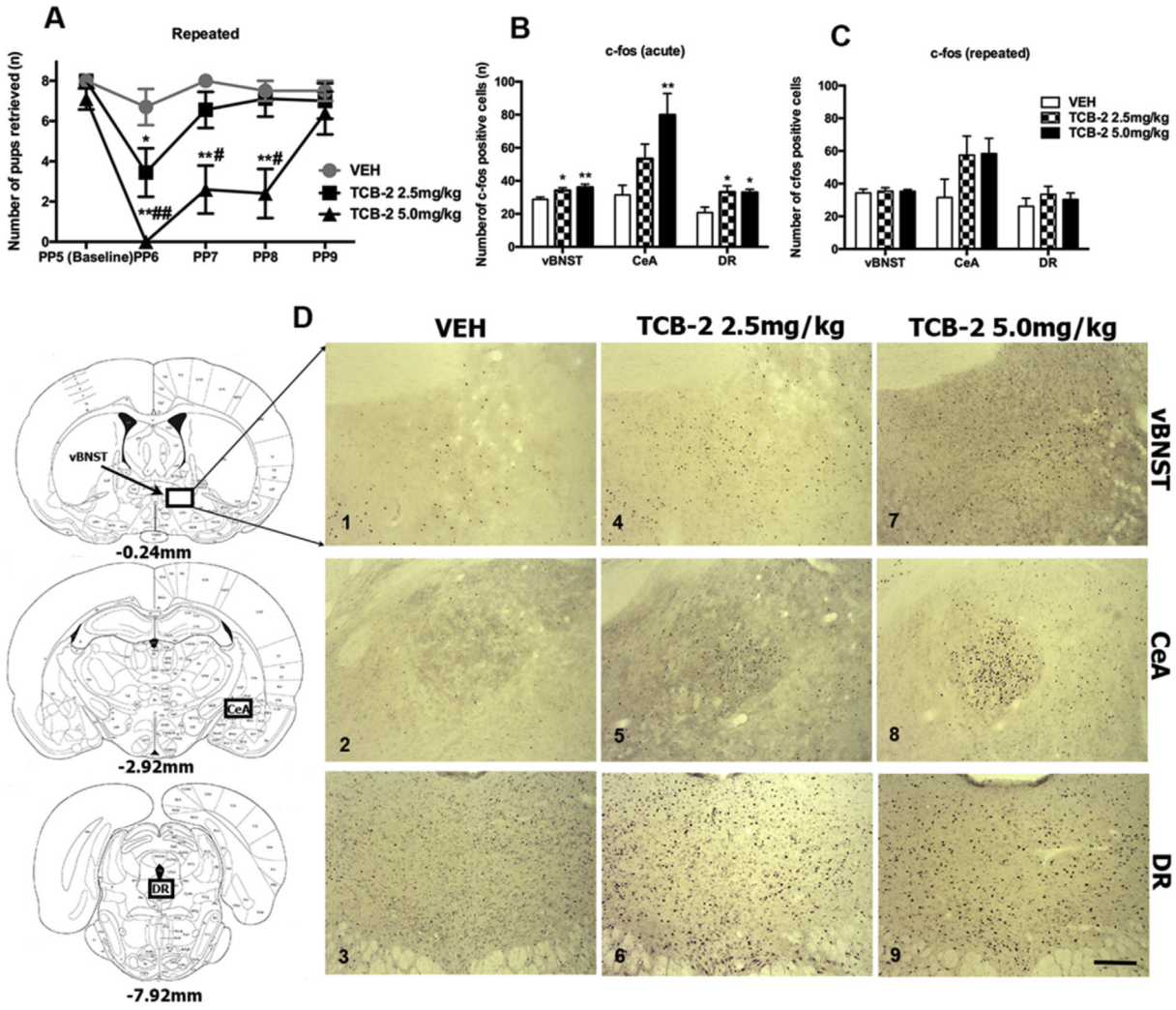

Fig. 5. (**A**) Effects of repeated TCB-2 administration on pup retrieval. Maternal behavior was tested for 10 min once daily from PP 6 to 9, starting at 30 min after the injection. Number of pups retrieved is expressed as mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different between the VEH and TCB-2. #$p < 0.05$, ##$p < 0.01$ significantly different between the two TCB-2 groups. Effects of acute (**B**) and repeated TCB-2 administration (**C**) on c-Fos immunoreactivity. On PP 10, 1 h after the drug injection, all rats were overdosed and perfused and their brains were extracted for c-Fos immunoreactivity staining. Number of c-Fos positive cells is expressed as mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different between the VEH and TCB-2. vBNST, the ventral bed nucleus of the stria terminalis; CeA, central amygdala; DR, dorsal raphe. (D), Brain atlas diagrams depict the region viewed in the representative images of these brain regions, and c-Fos staining photomicrographs in these regions. Distance from Bregma in the rostrocaudal planes is indicated. (1–3), acute VEH; (4–6), acute TCB-2, 2.5 mg/kg; (7–9), acute TCB-2 5.0 mg/kg. Scale bar = 100 μm.

et al., 1983; Bosch et al., 2010; Numan and Numan, 1995). The serotonergic neurons in the DR project to the VTA and NAc, where both 5-HT_{2A} and 5-HT_{2C} receptors have been found. Thus, the DR may regulate maternal motivation toward the young (Bridges, 2015). The CeA appears to be a crucial component for normal activation of maternal aggression circuitry and was shown to mediate the suppression of maternal care (Dulac et al., 2014). It has been shown that maternal aggression was increased after micro-injection of the 5-$HT_{2A/2C}$ receptor agonist α-Methyl-5-hydroxytryptamine maleate into the amygdala (de Almeida et al., 2006). In addition, lesion of vBNST disrupts maternal behavior, especially pup retrieval (Numan and Numan, 1996), and maternal expression is associated with increased c-Fos expression and increased binding to arginine vasopressin V1a and oxytocin receptors in the BNST (Bosch et al., 2010; Numan and Numan, 1995).

The present work has implications for our thinking about the effects of antidepressant (e.g. SSRIs) use on maternal care in depressed mothers which consist of approximately 10–20% of all mothers (Gjerdingen and Yawn, 2007; Susser et al., 2016), and more than 40% of depressed mothers are prescribed with antidepressants (Lind et al., 2017). On the one hand, SSRI treatment reduces depressive symptoms and improve certain functions needed for adequate maternal care, such as the overall functioning and maternal role functioning (e.g. gratification in the maternal role) (Logsdon et al., 2009, 2011). Thus, SSRI use is beneficial for improving maternal care. On the other hand, chronic SSRI treatment is known to induce an up-regulation of the 5-HT_{2A} receptors in the frontal cortex (Hamon and Blier, 2013; Massou et al., 1997). As our findings indicate that enhanced frontal 5-HT_{2A} receptors might be detrimental to maternal behavior, chronic SSRI treatment itself might cause a negative impact on the quality of maternal care. Animal research using postpartum depression models are important in untangling the effects of depression and SSRI treatment on maternal behavior.

Taken together, the 5-HT_{2A} receptor plays an important role in the modulation of maternal behavior in rats. We suggest three neural systems where 5-HT_{2A} receptors may achieve this effect. First, the 5-HT_{2A} receptors in the VTA and NAc may modulate the

neuronal activity of dopamine neurons and dopamine release to affect incentive aspects of maternal care (Li and Fleming, 2003; Numan, 2007). Second, the 5-HT_{2A} receptors in the mPFC could modulate the glutamatergic and GABAergic neurotransmission and regulate impulsivity and motivation. Finally, the 5-HT_{2A} receptors in the vBNST, CeA and DR may interact with other subcortical structures (e.g. medial preoptic area) to alter maternal behavior. Clinically, our finding that the 5-HT_{2A} receptor is critically important for maternal behavior in rats may have revealed that one possible cause of postpartum mental disorders (e.g. depression and psychosis) is the functional disruption of this receptor system (Guiard and Di Giovanni, 2015; Messa et al., 2003). This idea is supported by the observations that one mechanism of major depression and the antidepressant action of selective serotonin reuptake inhibitors (SSRIs) are mediated by the 5-HT_{2A} receptors (Hamon and Blier, 2013). Future research needs to be conducted to elucidate the exact central mechanisms of 5-HT_{2A} receptor in maternal behavior. This research will shed light on the possible functional dysregulation of the 5-HT_{2A} receptor in postpartum mental disorders. Such knowledge may help inform the development of effective interventions to promote and facilitate maternal care.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuropharm.2017.09.037.

Conflict of interest

None.

Disclosures

All authors declare no conflict of interest.

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