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Behavioral and pharmacological investigation of anxiety and maternal responsiveness of postpartum female rats in a pup elevated plus maze

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Highlights

- Under the no pup condition, dams entered the open arms more than nulliparous rats.
- With pups, dams retrieved pups, entered the open arms more and had a higher speed.
- Haloperidol and fluoxetine decreased the time spent in the open arms and velocity.
- Diazepam did not affect pup retrieval, open arm time or entry in lactating rats.

Abstract

The present study investigated the validity of a novel pup-based repeated elevated plus maze task to detect reduced anxiety and increased maternal responsiveness in postpartum female rats and explored the roles of dopamine D2, serotonin transporter and GABA/benzodiazepine receptors in the mediation of these processes. Sprague–Dawley postpartum or nulliparous female rats were tested 4 times every other day on postpartum days 4, 6 and 8 in an elevated plus maze with 4 pups or 4 pup-size erasers placed on each end of the two open arms. When tested with erasers, untreated postpartum mother rats entered the open arms proportionally more than nulliparous rats. They also tended to spend more time in the open arms, indicating reduced anxiety. When tested with pups, postpartum rats retrieved pups into the closed arms, entered the open arms and closed arms more and had a higher moving speed than nulliparous rats, indicating increased maternal responsiveness. Both haloperidol (0.1 or 0.2 mg/kg, sc) and fluoxetine (5 or 10 mg/kg, ip) dose- and time-dependently decreased the percentage of time spent in the open arms and speed, but did not affect the percentage of open arm entries. Diazepam (1.0 or 2.0 mg/kg, ip) did not affect pup retrieval, open arm time/entry in lactating rats. Thus, the percentage of open arm entries appears to be the most sensitive measure of anxiety in postpartum female rats, while speed could be used to index maternal responsiveness to pups, which are likely mediated by the dopamine D2 and serotonin transporter systems.

Keywords: Postpartum anxiety, Maternal responsiveness, Elevated plus maze, Haloperidol, Fluoxetine, Diazepam

1. Introduction

Pregnancy, parturition and lactation bring about numerous changes in the female's brain, body and behavior which are essential for the survival and health of the offspring and necessary for the female to successfully respond to the new demands of her changed environment [1]. Animal work has identified several adaptive changes from molecules to behaviors, including decreased responsiveness of the hypothalamic pituitary adrenal (HPA) axis to stressors, decreased corticotropin-releas-

ing hormone (CRH, a stress hormone) mRNA and binding in the hypothalamic paraventricular nucleus (PVN), increased oxytocin and its receptor mRNA expressions in the PVN, reduced sensorimotor gating (an attentional filtering function) as measured in prepulse inhibition (PPI), reduced acoustic startle response, increased pup-directed maternal responses (pup retrieval and nursing), indicative of increased maternal motivation, and decreased anxiety and enhanced memory functions [1–6]. Research also suggests that disruption of these normal adaptations could lead to postpartum mood, anxiety

and memory disorders [7]. It is estimated that approximately 5–12% of mothers worldwide display postpartum anxiety [8], 5–25% postpartum depression [9], and 0.1% postpartum psychosis [10]. Some individuals also show impairments in prospective memory [11]. Therefore, pharmacological interventions are often required to manage these mental disorders.

In laboratory rats, reduced anxiety and increased maternal responsiveness to pup cues in postpartum females are often examined using an elevated plus maze (EPM) [12] and pup retrieval test, respectively [13]. The EPM is a canonical rodent test of anxiety-like behavior [14]. The percentage of testing time subjects spend in the open arms of the maze and number of entries to the open arms are thought to be inversely correlated with level of anxiety. Acute anxiolytic treatments targeting the GABA/benzodiazepine receptor complex are known to increase the number of entries and time spent in the open arms as compared to the closed arms [15]. The pup retrieval test, typically conducted in the home cage, provides two important indices of maternal responsiveness: the number of pup retrieved and pup retrieval latency. Drugs targeting dopamine D_2 and serotonin 5-HT $_2$ A and 5-HT $_2$ C receptors are shown to decrease the number of pup retrievals and increase the pup retrieval latency, implicating their involvements in maternal motivation [16–21].

Although there are some conflicting findings, the majority of studies in the literature report that postpartum rats are less anxious than virgins in behavioral tests of anxiety [7,22]. However, some important issues have not been completely resolved. How pup presence affects anxiety-like behavior [22,23], and whether physical contact with pups is essential [24] need to be further examined. Also, which measure of open-arm behaviors is more sensitive to reveal the reduced anxiety in postpartum rats has not been settled [25]. Because the anxiety-like behavior of lactating rats is modulated by the presence of pups, which inevitably activates the motivational system, it seems reasonable to assume that anxiety-like behavior, as tested in a variety of behavioral paradigms, is often influenced by the motivational level of mother rats [22]. A reciprocal interaction between emotion regulation and maternal responsiveness likely exists, that is, reduced anxiety in mother rats could facilitate their motivation to care for their offspring, and increased maternal responsiveness could also mitigate mother rats' natural fearfulness. Unfortunately, studies designed to study emotion regulation and maternal responsiveness in a single paradigm and examine their interaction are rare. The underlying neurochemical bases of their interaction are also not well understood.

The present study introduced a novel pup-based elevated plus maze (EPM) paradigm. In this task, 4 pups or pup-size erasers were placed at each end of two open arms. Each rat (postpartum or nulliparous female) was repeatedly tested 4 times every other day for 3 days in order to track changes in anxiety and motivation over time. We first identified two distinct sets of behavioral indices for maternal anxiety (Experiment 1) and maternal responsiveness (Experiment 2) by comparing primiparous females with nulliparous ones on the basis of their performances on the EPM and their responses to pups. We then explored the roles of dopamine D_2 , serotonin transporter and GABA/benzodiazepine receptors in the regulation of maternal anxiety and responsiveness by examining the effects of haloperidol (a dopamine D_2 receptor antagonist), fluoxetine (a selective serotonin reuptake inhibitor), and diazepam (a GABA/benzodiazepine agonist) treatment on various measures of maternal anxiety and responsiveness (Experiment 3).

Because these drugs are also psychotherapeutic drugs used to treatment psychosis, depression and anxiety, respectively, this study also possesses clinical implications for the understanding of behavioral mechanisms of action of these drugs.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats, approximately 200–250 g in weight (8–10 weeks old), purchased as virgins from the Experiment Animal Center (Chongqing Medical University, China) were used in this study. After arrival, they were housed in transparent polycarbonate cages (two per cage) under 12-h light/dark conditions (light on between 0800 am and 2000 pm). Room temperature was maintained at $22 \pm 2^\circ\text{C}$ with a relative humidity of 45–75%. Standard laboratory rat chow and water were available ad libitum. After a 7-day acclimation period, some virgin female rats were placed into the cage of a proven stud male for ten days to ensure pregnancy. Following the mating procedure, pregnant females were singly housed until parturition after which they were housed together with their litters for the remainder of the experiment. Non-mated virgin females ($n = 6$, 4 used in Experiment 1 and 2 in Experiment 2) and non-pregnant females (mated but not pregnant, $n = 10$, 4 used in Experiment 1 and 6 in Experiment 2), combined as the control nulliparous group, were also singly housed during the same period. We combined them as a single control group because they both did not go through the gestation, parturition and lactating processes, and did not have any maternal experience with pups. In addition, they did not differ significantly in any of the EPM measures. Their estrous cycles were not monitored as they were tested on 3 days over a 5-day period, presumably covering all the phases of the cycle. Experiments were conducted during the light cycle (between 0830 am and 1800 pm). A total of 94 postpartum female rats were tested. Data from 14 dams were not included in the final analysis because they failed to retrieve 8 pups at the baseline test on postpartum day (PP) 4 or their tests were not recorded due to malfunction of the camcorder. All animal procedures were approved by the animal care and use committee at Southwest University, China. Every effort was made to minimize the number of animals used and the suffering of the animals.

2.2. Drugs

Haloperidol (HAL, 5.0 mg/ml Ampoules, Hunan Dongting Pharmaceutical Co., Ltd., Hunan, China), fluoxetine (FLU, Sigma–Aldrich, St. Louis, MO, USA) and diazepam (DZ, 5.0 mg/ml Ampoules, Tianjin Jinyao Anjisuan Pharmaceutical Co., Ltd., Tianjin, China) were obtained by mixing the drugs with sterile water. HAL was administered subcutaneously (sc), whereas FLU and DZ were administered intraperitoneally (ip). Based on our previous work [18–20] and published work on FLU and DZ [26–28], we chose to test HAL at doses of 0.1 and 0.2 mg/kg, FLU at doses of 5 and 10 mg/kg, and DZ at doses of 1 and 2 mg/kg.

2.3. Elevated plus-maze apparatus

The EPM consisted of two open arms (50cm×10 cm), two enclosed arms (50cm×10 cm) and a central platform (10cm×10 cm) made of black Plexiglas. Each arm was supported by a sturdy plastic leg and was elevated 70cm above the floor. The two enclosed arms had high walls (40cm in height), while the two open arms had raised edges (1.0cm in height) along each side and end to decrease the possibility of falling during drug testing.

2.4. Experiment 1: identification of behavioral measures that are sensitive to detect reduced anxiety in lactating rats: Comparison between postpartum primiparous female rats and nulliparous rats tested on the EPM in the absence of pups

The purpose of this experiment was to examine whether postpartum female rats are generally less anxious than nulliparous rats, and if so, which measure is most sensitive to detect this difference. Eight primiparous rats were tested and compared with 8 nulliparous. For primiparous rats, each litter was culled to 8 pups (4 males and 4 females with the most visible milk bands) on PP 3.

The EPM tests were conducted on PP4, PP6, and PP8. On each test day, rats were first brought to the experimental room (~10 Lux provided by a table lamp, 6 Lux for the open arms and 5 for the closed arms) and habituated to the test environment for at least 30 min. Each rat was tested repeatedly 4 times, with the first time point at 30 min before an injection of sterile water (i.e., baseline), and the rest being carried out at 30, 100, and 240 min after the injection. During the intervals between tests, dams were with their pups. The injection procedure and time points of testing were adopted in order to match those used in Experiment 3 and allow data comparison across the three experiments. Prior to the start of each session, EPM was cleaned by 70% ethanol and dried. Four pup-sized erasers were placed at the end of each open arm. Then, each rat was placed in the central square facing a closed arm and allowed to freely explore the maze for 10 min. The 10 min test duration was chosen because Lonstein [24] showed that differences between diestrous virgins and postpartum rats in EPM are more pronounced during a 10-min test, rather than a 5-min test.

All experimental sessions were recorded by a video camera. The following parameters were later obtained using Noldus EthoVision XT 8.5 (Wageningen, The Netherlands): (1) % time in open arms (100%×time spent in the open arms/total time); (2) % open arm entries (100%×number of entries into open arms/total number of entries); (3) number of closed arm entries (as indicator of general locomotor activity) [15,24]; (4) mean speed (the total distance travelled divided by 600 s); (5) number of erasers (or pups in Experiments 2 and 3) retrieved (the number of erasers or pups which were picked up and carried back from an open arm to a close arm).

2.5. Experiment 2: identification of behavioral measures that are sensitive to detect increased maternal motivation in lactating rats: Comparison between postpartum primiparous female rats and nulliparous rats tested on the EPM with pups

The goal of this experiment was to investigate whether the EPM could also be used to assess maternal responsiveness. The basic procedure was similar to that of Experiment 1 with the only exception that 4 pups, instead of erasers, were placed at the end of each open arm. Thus, two groups were tested: the primiparous group tested with pups ($n = 8$) and nulliparous group tested with pups ($n = 8$).

2.6. Experiment 3: identification of the neurochemical basis of reduced anxiety and increased maternal motivation in lactating rats: effects of haloperidol, fluoxetine and diazepam treatment on primiparous female rats tested on the EPM with pups

This experiment was conducted to explore the roles of dopamine D₂ receptor, serotonin transporter and GABA/benzodiazepine receptor in the mediation of maternal anxiety and maternal motivation. The basic procedure was similar to that of Experiment 2 and that used in our previous study [18]. All subjects were primiparous rats tested on PP4, PP6 and PP8 after HAL (0.1 or 0.2 mg/kg), FLU (5 or 10 mg/kg), or DZ (1.0 or 2.0 mg/kg) injections (a total of 6 drug groups, $n = 8$ /group). Data from the primiparous group in Experiment 2 (in-

jected with vehicle, as the VEH group) were included in the analysis and compared with the drug-treated group.

2.4. Statistical analysis

Data from Experiments 1 and 2 were analyzed with mixed design three-way analyses of variance (ANOVAs) using reproductive state as a between-subjects factor and time point (i.e., baseline, 30, 100 and 240 min) and test days (PP4, PP6, PP8) as within-subjects factors. Data from Experiment 3 were analyzed separately for each drug condition using the same mixed design ANOVA (drug dose as a between-subjects factor, time point and days as within-subjects factors), as we were less interested in between-drug comparisons. The two drug dosage groups were compared with the primiparous group from Experiment 2 (injected with sterile water and tested with pups). In cases of the significant main effects ($p < 0.05$), pairwise post-hoc comparisons were performed using Fisher's LSD tests with significant differences indicated by $p < 0.05$.

3. Results

3.1. Experiment 1: identification of behavioral measures that are sensitive to detect reduced anxiety in lactating rats: comparison between postpartum primiparous female rats and nulliparous rats tested on the EPM in the absence of pups

Fig. 1 shows the % time in open arms (A); % open arm entries (B), mean speed (C) and number of closed arm entries (D) between the primiparous group and nulliparous group at the 4 test time points on PP4, PP6 and PP8. A mixed design three-way ANOVA on the % time in open arms revealed a main effect of day, $F(2, 28) = 11.595$, $p < 0.001$, time, $F(3, 42) = 3.867$, $p = 0.016$, and a significant interaction of day×group, $F(2, 28) = 5.654$, $p = 0.009$. Analysis of the % open arm entries yielded the same results. There was a main effect of day, $F(2, 28) = 9.422$, $p = 0.001$, time, $F(3, 42) = 3.475$, $p = 0.024$, and a significant interaction of day×group, $F(2, 28) = 11.960$, $p < 0.001$. Inspection of Fig. 1A and B suggests that the primiparous group had a relatively stable level of % open arm time and % open arm entries throughout the 3 test days, while the nulliparous group increased both time and entries with the repeated testing. Therefore, although the primiparous group had higher % time and higher % entries in the open arms than the nulliparous group on the 1st day of testing (PP4), this effect was reversed on the last test day (PP8). In order to determine the group difference at specific time points and days, one-way ANOVAs were conducted for each test day separately. The main effect of group on % open arm entries only was found on PP4, $F(1, 14) = 11.004$, $p = 0.005$. Primiparous dams were more likely to enter the open arms than the nulliparous females on the first day of testing. Two group comparisons at each time point identified that the significant difference appeared at the baseline, $t(14) = 4.687$, $p < 0.001$ and at the 240 min time point, $t(14) = 4.687$, $p = 0.039$.

On the measure of speed, there was only a main effect of time, $F(3, 42) = 3.026$, $p = 0.040$, and a significant interaction of day×time, $F(6, 84) = 4.635$, $p < 0.001$. It appears that there was a gradual decrease in the locomotor speed over the 4 test points on PP4 and PP8, but not much change occurred on PP6. The number of entries into closed arms, the most reliable indicator of general locomotor activity, did not differ between groups, $F(1, 14) = 0.684$, $p = 0.422$, but there was a significant group×day interaction, $F(2, 28) = 3.473$, $p = 0.045$, and group×time interaction, $F(3, 42) = 3.296$, $p = 0.030$. The primiparous group had lower closed arm entries on the 1st day of testing, but this group difference disappeared on PP6 and

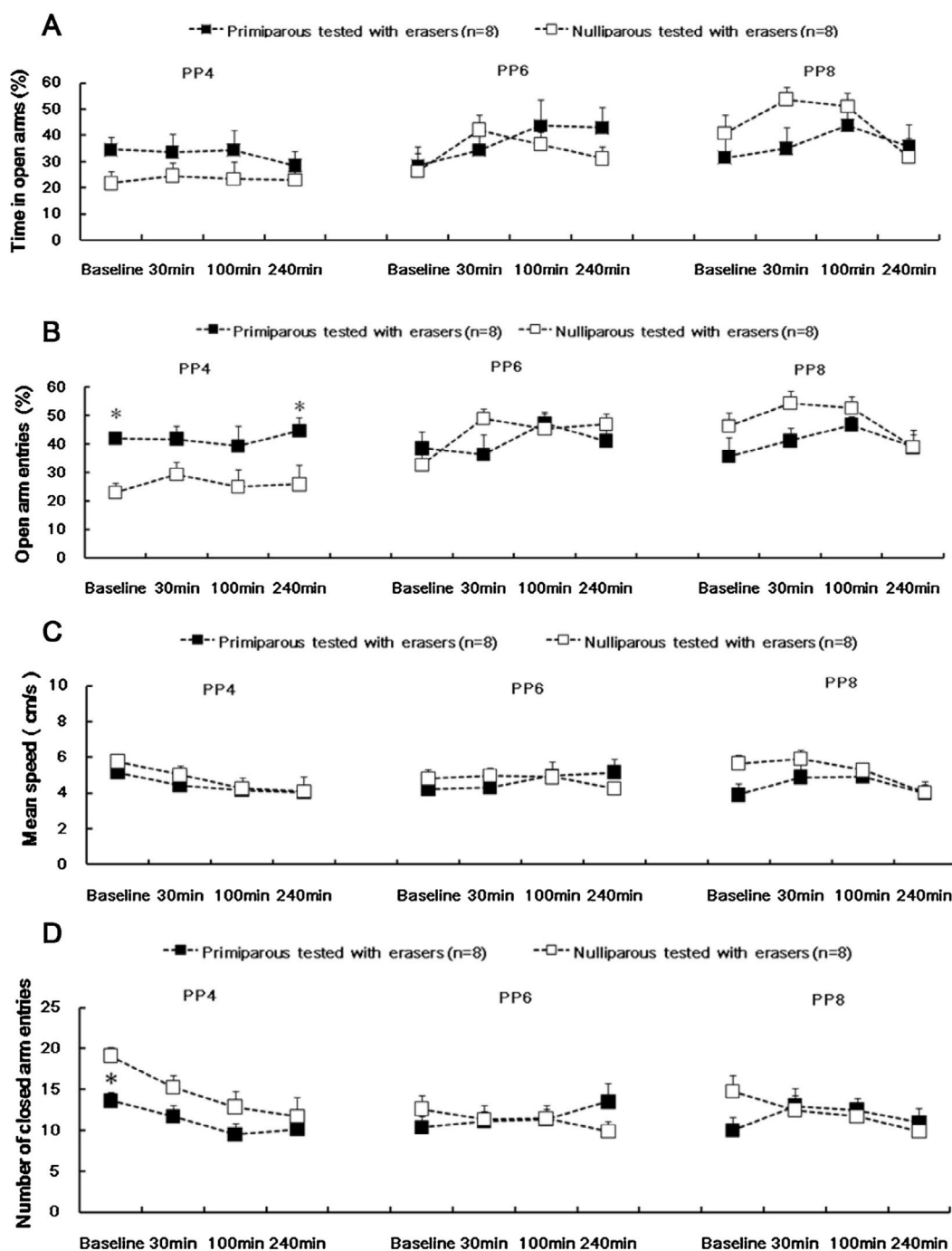


Fig. 1. Percentage of time spent in the open arms (A), percentage of entries to the open arms (B), speed (C) and number of entries into the closed arm (D) by primiparous lactating and nulliparous female rats tested with erasers at 4 time points on days 4, 6 and 8 postpartum (for lactating rats). Asterisk (*) indicates significant difference between the two groups, $p < 0.05$.

PP8, suggesting an initially lower motor activity of lactating mothers.

On the eraser retrieval (Fig. S1), we did not find any significant effect of group, day, time or their interactions, all $p > 0.05$.

Assuming that primiparous rats are indeed less anxious than nulliparous rats [7], and both the % time in open arms and % open arm entries are commonly used behavioral measures of anxiety, results from this experiment suggest that the % open arm entries is more sensitive than % open arm time in measuring anxiety and differentiating postpartum from nulliparous rats in this setup.

3.2. Experiment 2: identification of behavioral measures that are sensitive to detect increased maternal motivation in lactating rats: comparison between postpartum primiparous female rats and nulliparous rats tested on the EPM with pups

Fig. 2 shows the % time in open arms (A); % open arm entries (B), mean speed (C) and number of closed arm entries (D) between the primiparous group and nulliparous group with pups on PP4, PP6 and PP8. Similar to what was observed in Experiment 1, the primiparous group had a relatively stable level of

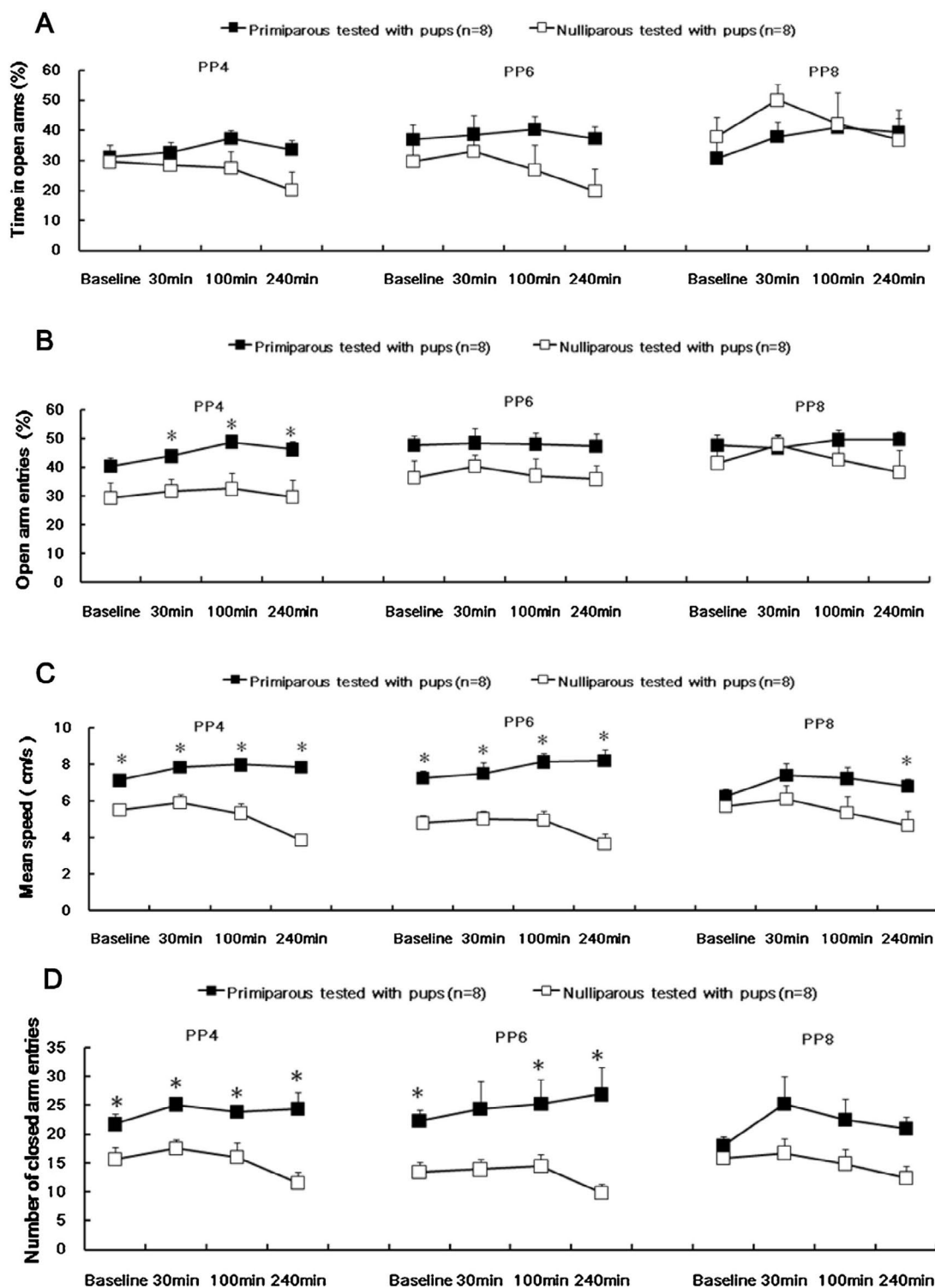


Fig. 2. Percentage of time spent in the open arms (A), percentage of entries to the open arms (B), speed (C) and number of entries into the closed arm (D) by primiparous lactating and nulliparous female rats tested with pups at 4 time points on days 4, 6 and 8 postpartum (for lactating rats). Asterisk (*) indicates significant difference between the two groups, $p < 0.05$.

% time spent on the open arms over the 3 test days, while the nulliparous group increased its time, as indicated by the significant group \times day interaction, $F(2, 28) = 5.008$, $p = 0.014$. Primiparous dams were also more likely to enter the open arms than the nulliparous rats, especially on the first test day. One-way ANOVA revealed a main effect of group for % open arm entries on PP4, $F(1, 14) = 7.639$, $p = 0.015$, but not on PP6 or PP8, $p > 0.074$. Independent-samples T test found that the pri-

parous rats entered the open arms more than the nulliparous rats at the 30, 100, 240 min test time, $p = 0.025$, $p = 0.011$ and $p = 0.028$, respectively.

Across the 3 test days, the primiparous group had a higher speed than the nulliparous group. Within each test day, the primiparous group maintained the higher speed, while the nulliparous group showed a gradual decrease. The mixed design three-way ANOVA found a main effect of group, $F(1, 14) =$

19.107, $p = 0.001$; time, $F(3, 42) = 5.843$, $p = 0.002$; time \times group interaction, $F(3, 42) = 8.750$, $p < 0.001$; and day \times group interaction, $F(2, 28) = 5.532$, $p = 0.009$. The primiparous group consistently entered the closed arms more than the nulliparous group over the repeated testing period (a main effect of group, $F(1, 14) = 12.196$, $p = 0.004$, but no other main effects or interactions), due to the deposition of pups.

On the pup retrieval (Fig. S2), only primiparous rats consistently retrieved pups from the open arms to the closed arms at each test point, although some nulliparous rats started to show pup retrieval towards the end of repeated testing days, possibly reflecting a "pup sensitization" effect [29]. There was a main effect of group, $F(1, 14) = 30.915$, $p < 0.001$; day, $F(2, 28) = 72.512$, $p < 0.001$; and time, $F(3, 42) = 8.668$, $p < 0.001$. There were also significant interactions between group and day, $F(2, 28) = 20.398$, $p < 0.001$; group \times time, $F(3, 42) = 72.512$, $p = 0.006$; day \times time, $F(6, 84) = 4.989$, $p < 0.001$; and group \times time \times day, $F(6, 84) = 3.533$, $p = 0.004$.

Thus, under the pup presence condition, the speed measure seems to capture the intensity of sensitivity to pup cues better than other measures. The number of pup retrieved and related number of closed arm entries are additional indices of maternal responsiveness. Once again, the reduced anxiety in postpartum rats is confirmed in this experiment by the increased % open arm entries in the primiparous group.

Results from the above experiments suggest that the % open arm entries appears to be the most sensitive measure of anxiety in postpartum female rats, while locomotor speed is best used to index maternal responsiveness to pup cues. To further examine whether this conclusion holds, we compared each pair of primiparous groups and nulliparous groups separately. If the speed indeed measures the sensitivity to pup cues, we would expect that the primiparous group tested with pups should have higher speed than the primiparous group tested with erasers, but they should not differ from each other on the % open arm time or % open arm entries (assuming that they all had similarly reduced anxiety). On the other hand, the two nulliparous groups should not differ in the speed, nor on the % open arm time or % open arm entries. Results were indeed consistent with this conclusion (Figs. S3 and S4). For the two primiparous groups, repeated measures ANOVA found a significant group effect only on the speed, $F(1, 14) = 34.023$, $p < 0.001$, but not on the % open arm time, $F(1, 14) = 0.020$, $p = 0.890$; or % open arm entries, $F(1, 14) = 1.788$, $p = 0.202$. For the two nulliparous groups, no group effect was found on any of these measures, all $p > 0.639$ (Fig. S4).

To examine how repeated EPM testing affected measurements of anxiety, motor activity and maternal responsiveness in primiparous and nulliparous rats differently, we averaged the % open arm time, % open arm entries, speed and number of closed arm entries at 4 time points on each test day, and plotted the data for each group. As can be seen in Fig. 3A and B, only the two nulliparous groups increased their % time and entries in the open arms, indicating a reduction in anxiety with repeated exposure to EPM. The primiparous rats showed a stable level of anxiety-like behaviors over time and were not affected by the repeated testing. The mixed design three-way ANOVA on the averaged % open arm time showed a main effect of day, $F(2, 56) = 18.088$, $p < 0.001$, and day \times group interaction, $F(6, 56) = 3.878$, $p = 0.003$, but no main effect of group, $F(3, 28) = 0.181$, $p = 0.908$. Analysis of the averaged % open arm entries yielded the same results: a main effect of day, $F(2, 56) = 16.413$, $p < 0.001$, and day \times group interaction, $F(6, 56) = 5.544$, $p < 0.001$, but no main effect of group, $F(3, 28) = 1.825$, $p = 0.166$. One-way ANOVA only found the main effect of day in the two nulliparous groups, all $p < 0.009$. Paired-samples T

tests showed nulliparous rats, regardless of their testing condition (i.e., with pups or erasers), tended to spend more time in the open arms on PP8 than on PP4, $p < 0.05$. They also made more entries into the open arms on PP8 than on PP4, $p < 0.05$.

On the averaged speed measure, the primiparous group tested with pups had a significantly higher speed than other groups (Figure 3C). The mixed design three-way ANOVA showed a main effect of group, $F(3, 28) = 11.567$, $p < 0.001$ and a significant day \times group interaction, $F(6, 56) = 2.945$, $p = 0.014$, but no main effect of day, $F(2, 56) = 0.120$, $p = 0.888$. Individual ANOVA found that only the primiparous group tested with pups had a main effect of day, $F(2, 14) = 5.926$, $p = 0.014$. Paired-samples T tests showed a reduction of speed from PP4 to PP8, $p < 0.05$. Similarly, on the averaged closed arm entries (Fig. 3D), there was only a main effect of group, $F(3, 28) = 12.745$, $p < 0.001$, with the primiparous group tested with pups showing a significantly higher number of closed arm entries than the other 3 groups, further demonstrating higher maternal responsiveness of this group. The main effect of day or day \times group interaction was not significant, all $p > 0.174$.

3.3. Experiment 3: identification of the neurochemical basis of reduced anxiety and increased maternal motivation in lactating rats: effects of haloperidol, fluoxetine and diazepam treatment on primiparous female rats tested on the EPM with pups

Fig. 4 shows the treatment effect of HAL on the number of pups retrieved (A), % time in open arms (B); % open arm entries (C), mean speed (D) and number of closed arm entries (E) in postpartum female rats tested with pups on PP4, PP6 and PP8. In comparison to the vehicle treatment, HAL decreased pup retrieval, % open arm time, mean speed and number of closed arm entries in a dose-dependent and time-dependent fashion, but did not affect the % open arm entries. The mixed design three-way ANOVA on pup retrieval showed a main effect of dose, $F(2, 21) = 6.745$, $p = 0.005$; time, $F(3, 63) = 7.607$, $p < 0.001$, and time \times dose interaction, $F(6, 63) = 3.220$, $p = 0.008$; and time \times dose \times day interaction, $F(12, 126) = 2.197$, $p = 0.015$. Specific group differences at each time point on the 3 tests are indicated in Fig. 4A. It is apparent that HAL at 0.2 mg/kg decreased pup retrieval mainly at the 30 min and 100 min time points.

Primiparous rats treated with both doses of HAL consistently spent less time in the open arms over the 3 test days. There was a main effect of drug dose, $F(2, 21) = 13.401$, $p < 0.001$; time, $F(3, 63) = 8.263$, $p < 0.001$, and time \times dose interaction, $F(6, 63) = 5.564$, $p < 0.001$. Inspection of Fig. 4B suggests that both doses of HAL decreased the % open arm time at 30min, 100 min and 240 min. In contrast, HAL treatment did not affect the % open arm entries (Fig. 4C). We did not find a main effect of drug dose, $F(2, 21) = 0.020$, $p = 0.980$, nor its interaction with other factors.

HAL decreased the mean speed in the same way as it decreased pup retrieval (Fig. 4D). The mixed design three-way ANOVA showed a main effect of drug dose, $F(2, 21) = 56.738$, $p < 0.001$; day, $F(2, 42) = 15.654$, $p < 0.001$; time, $F(3, 63) = 71.486$, $p < 0.001$; time \times dose interaction, $F(6, 63) = 34.762$, $p < 0.001$; day \times time interaction, $F(6, 126) = 2.627$, $p = 0.020$; and time \times dose \times day interaction, $F(12, 126) = 2.198$, $p = 0.015$. Similarly, HAL decreased the number of closed arm entries over the 3 test days (Fig. 4E). The main effects of drug dose, $F(2, 21) = 28.990$, $p < 0.001$; day, $F(2, 42) = 3.246$, $p = 0.049$; time, $F(3, 63) = 13.048$, $p < 0.001$; time \times dose interaction, $F(6, 63) = 9.228$, $p < 0.001$ were all significant.

Fig. 5 shows the treatment effect of FLU on the number of pups retrieved (A), % time in open arms (B); % open arm entries

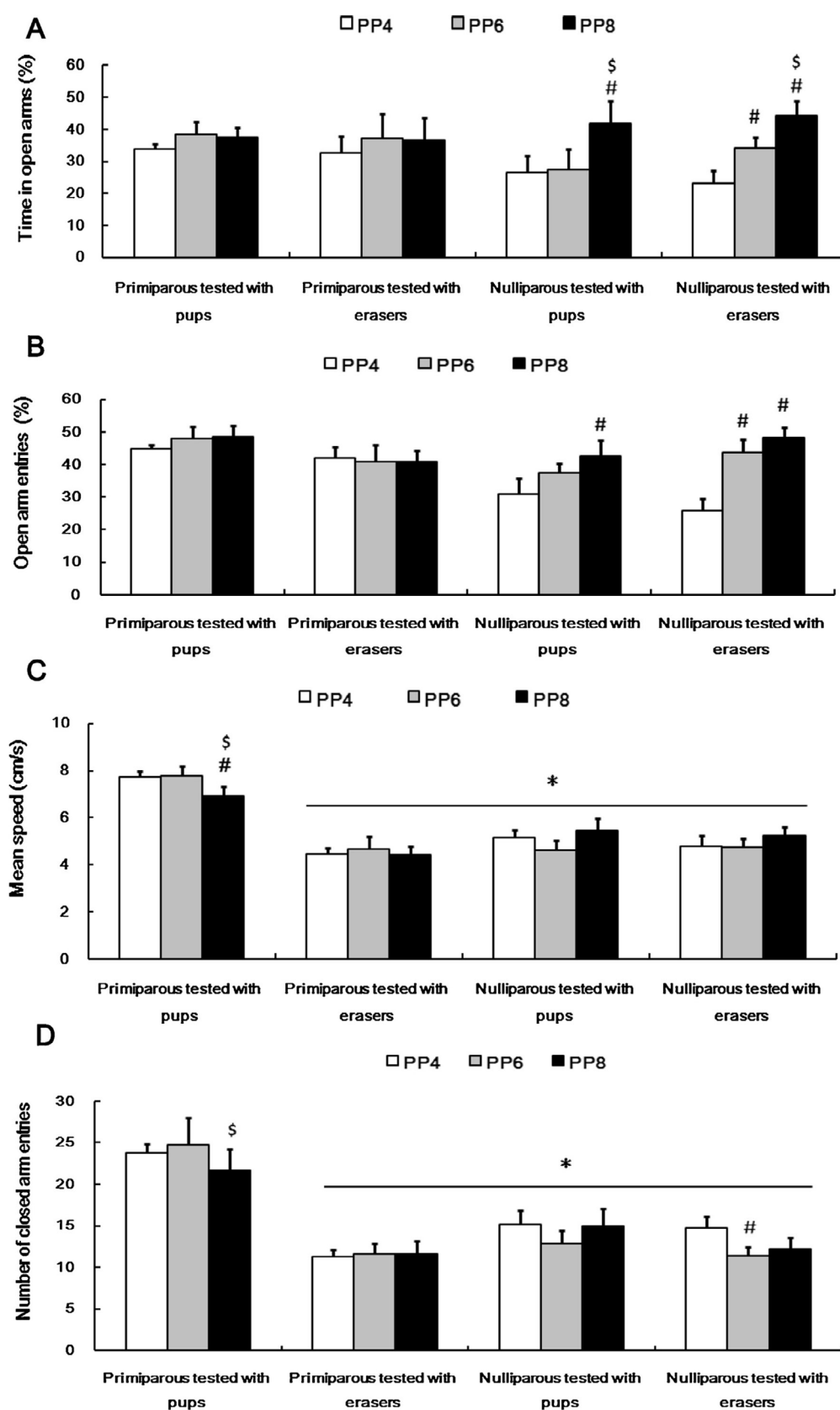


Fig. 3. Averaged daily percentage of time spent in the open arms (A), percentage of open arm entries (B), locomotor speed (C) and number of entries into the closed arm (D) by the 4 groups tested under the vehicle condition over the 3 days.

* indicates significant difference relative to the primiparous group with pups, $p < 0.05$;

indicates significant difference between PP8 and PP6 relative to PP4, $p < 0.05$;

\$ indicates significant difference between PP8 relative to PP6, $p < 0.05$.

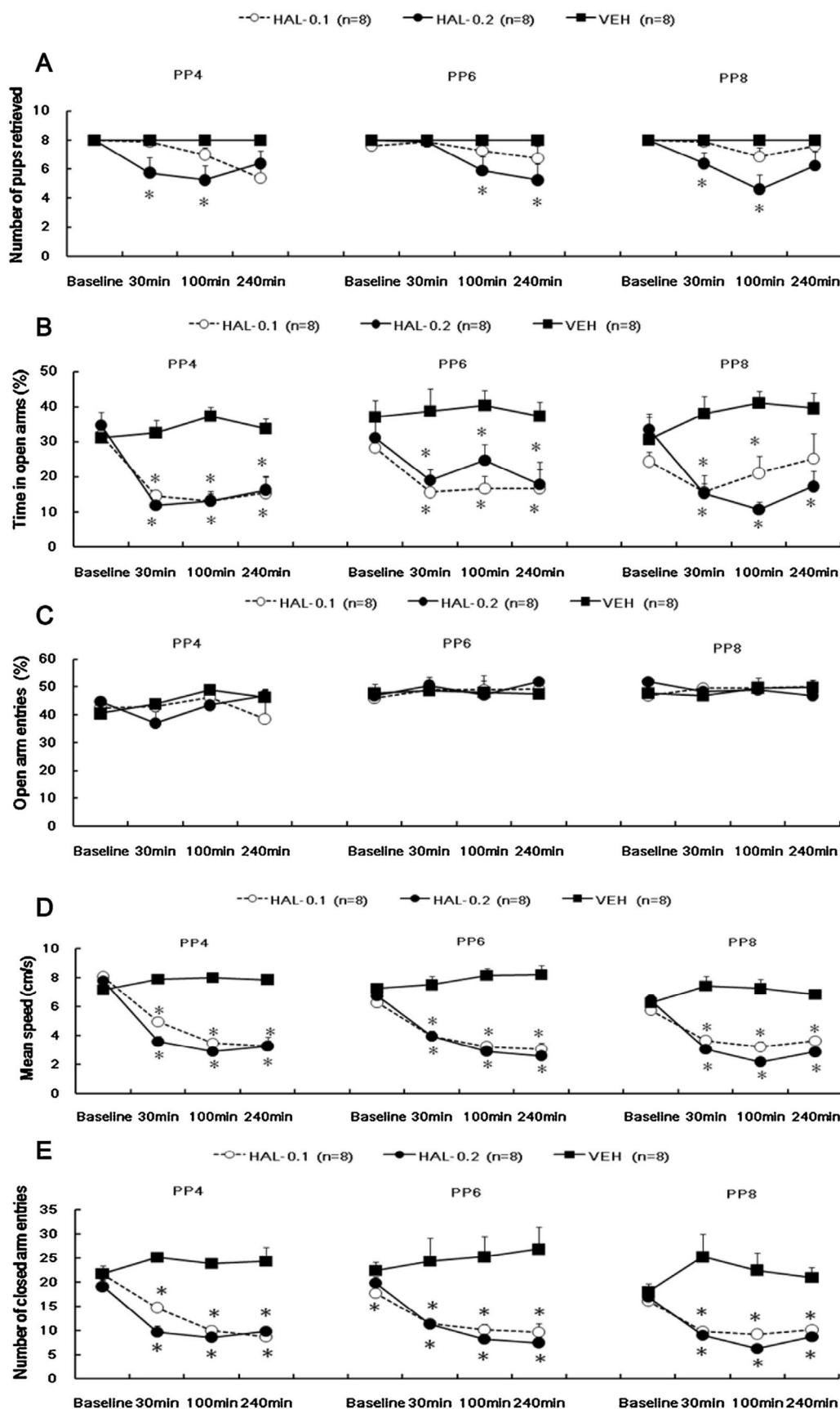


Fig. 4. Number of pups retrieved (A), percentage of time spent in the open arms (B), percentage of entries to the open arms (C), locomotor speed (D) and number of entries into the closed arm (E) by primiparous lactating female rats tested under saline or haloperidol (0.1 and 0.2 mg/kg) treatment at 4 time points on days 4, 6 and 8 postpartum. Asterisk (*) indicates significant difference relative to the vehicle group, $p < 0.05$.

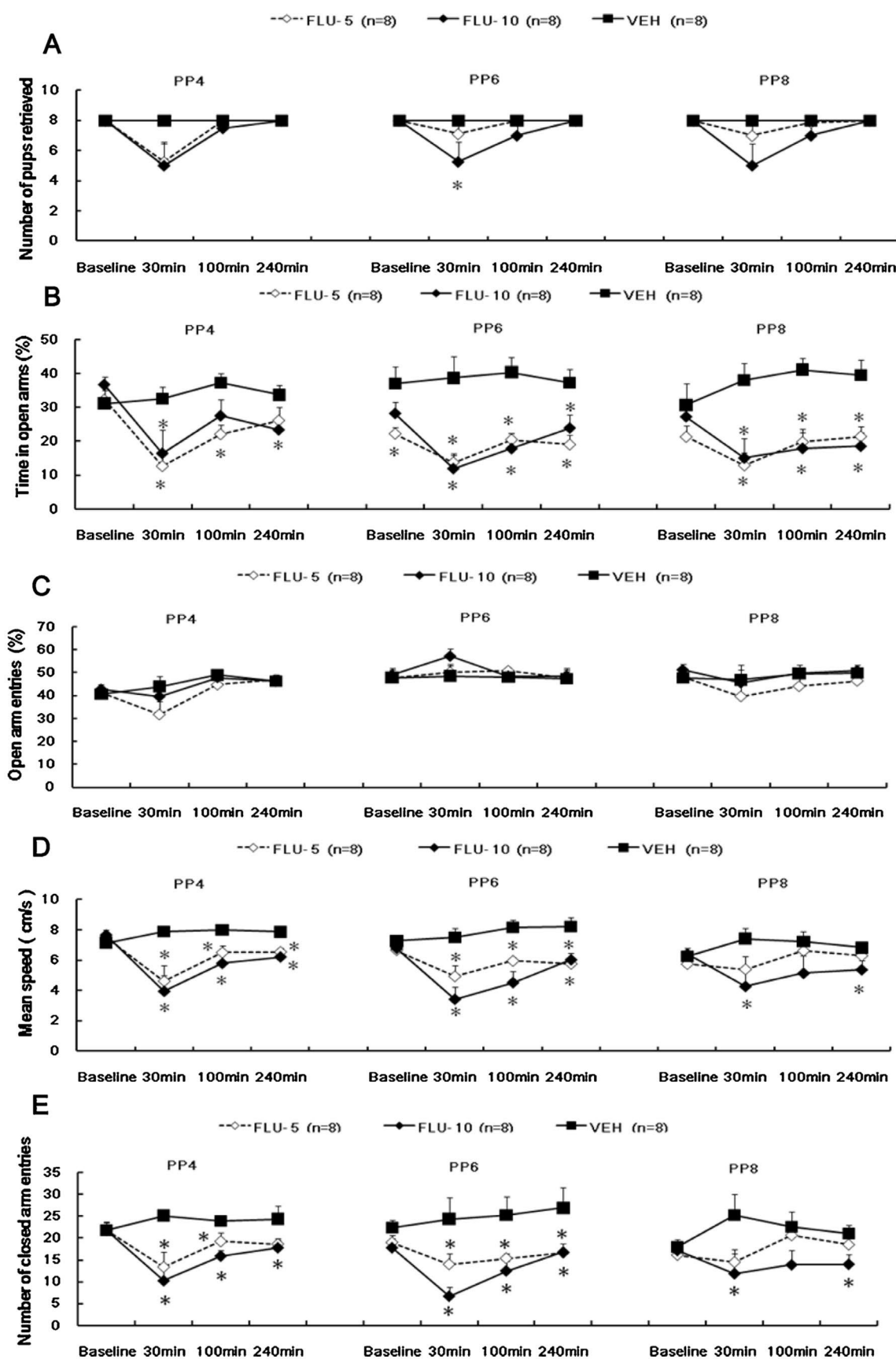


Fig. 5. Number of pups retrieved (A), percentage of time spent in the open arms (B), percentage of entries to the open arms (C), locomotor speed (D) and number of entries into the closed arm (E) by primiparous lactating female rats tested under saline or fluoxetine (5.0 and 10.0 mg/kg) treatment at 4 time points on days 4, 6 and 8 postpartum. Asterisk (*) indicates significant difference relative to the vehicle group, $p < 0.05$.

(C), and mean speed (D) and number of closed arm entries (E) in postpartum female rats tested with pups on PP4, PP6 and PP8. In comparison to the vehicle treatment, FLU only slightly decreased pup retrieval, but significantly decreased % time spent on the open arms and mean speed. Data analyses on pup retrieval showed a main effect of drug dose, $F(2, 21) = 3.623$, $p = 0.044$; time, $F(3, 63) = 10.581$, $p < 0.001$; and time \times dose interaction, $F(6, 63) = 3.433$, $p = 0.005$. FLU transiently decreased pup retrieval only at 30 min time point (Fig. 5A).

Primiparous rats treated with both doses of FLU also spent less time in the open arms over the 3 test days. There was a main effect of drug dose, $F(2, 21) = 12.249$, $p < 0.001$; time, $F(3, 63) = 8.310$, $p < 0.001$, and time \times dose interaction, $F(6, 63) = 4.611$, $p = 0.001$. Both doses of FLU decreased the % open arm time at the 3 test points after injection. In contrast, FLU treatment did not affect the % open arm entries. The same ANOVA did not find a main effect of dose, $F(2, 21) = 0.556$, $p = 0.581$, nor its interaction with other factors.

Like HAL, FLU treatment decreased the speed and number of closed arm entries in a time- and dose-dependent manner. The mixed design three-way ANOVA showed a main effect of drug dose, $F(2, 21) = 7.174$, $p = 0.004$; time, $F(3, 63) = 11.844$, $p < 0.001$, time \times dose interaction, $F(6, 63) = 7.745$, $p < 0.001$ for the speed; and a main effect of dose, $F(2, 21) = 7.363$, $p = 0.004$; time, $F(3, 63) = 3.919$, $p = 0.012$, time \times dose interaction, $F(6, 63) = 4.407$, $p = 0.001$ for the number of closed arm entries.

In comparison to the vehicle treatment, DZ appeared to decrease the % open arm time without affecting other measurements (Fig. 6). The mixed design three-way ANOVAs on pup retrieval, % open arm entries, mean speed and number of closed arm entries failed to find a main effect of group, nor its interaction with time and day, all $p > 0.05$. Even on the % time on open arms, the main effect of group approached significance, $F(2, 21) = 3.324$, $p = 0.056$. Its interaction with day also approached significance, $F(4, 42) = 2.438$, $p = 0.062$. Taken together, HAL and FLU had a similar effect on % time spent on the open arms and mean speed, but differed in pup retrieval and number of closed arm entries. DZ differed from HAL and FLU for its lack of effect on maternal performance (e.g., pup retrieval and mean speed) and anxiety-like behaviors (e.g., % time, % entries on open arms and number of closed arm entries).

4. Discussion

The present study provides an initial evaluation of utility of EPM to simultaneously study emotion regulation and maternal responsiveness and associated neurochemical basis in postpartum female rats. Our results revealed a stable and reduced anxiety level in lactating rats during early to mid-lactation period, and suggest that the % open arm entries is a sensitive measure of this postpartum behavioral change (not unduly influenced by the general motor activity as measured in the closed arm entries under the eraser condition). We also identified the locomotor speed in the presence of pups as a sensitive measure of maternal responsiveness, in addition to number of pups retrieved and related number of closed arm entries. Armed with these findings, we took a pharmacological approach and examined the roles of dopamine D₂, serotonin transporter and GABA/benzodiazepine receptors in mother rats' maternal responsiveness and anxiety-like behaviors in this task. We found that HAL and FLU dose- and time-dependently decreased mean speed, suggesting that their impairment effects on maternal behavior are achieved by their actions on the maternal motivational process. This conclusion was additionally supported by the observations that both drugs suppressed pup retrieval and closed arm entries on the EPM. DZ

treatment did not affect pup retrieval, mean speed, number of closed arm entries, % open arm time or open arm entries in lactating rats. These findings imply that the dopamine D₂ receptor and 5-HT transporter are likely involved in the mediation of maternal motivation, while the role of GABA/benzodiazepine receptors in this process could not be determined, given its marginal effect on the % time on open arms.

The EPM has been widely used to study postpartum anxiety [24]. A large body of literature demonstrates that postpartum rats are often less anxious than virgin females, as indicated by group differences in open-arm behavior [7,30]. This notion has also received support from studies using other behavioral tests of anxiety, such as the open field test and light-dark box [31,32]. The present study used a repeated testing procedure and confirmed this observation. EPM is typically used only once, as there is a concern of anxiety reduction with repeated testing. For the purpose of drug screening, this concern is legitimate because any potential floor effect resulted from anxiety reduction could make detection of an anxiolytic effect impossible. However, for the behavioral studies of the psychological processes involved in the regulation of maternal behavior, repeated testing can be used to examine the changes in emotion regulation (e.g., anxiety reduction) and maternal responsiveness over time. We reported a stably reduced anxiety-level in lactating rats relative to nulliparous females in the early stage of postpartum period. Nulliparous rats did appear to become less anxious with repeated testing, possibly due to their habituation of the testing situation and to the effect of "maternal sensitization" resulted from repeated pup exposure [33], both of which may have led to reduced anxiety (but see Ferreira et al. [34]). We want to point out that although the unique hormone state and constant contact with pups play a major role in lowered anxiety in lactating females [7], the single housing condition and the aversive effect of pups to nulliparous females [35] may have increased anxiety in nulliparous females and contributed to the primiparous-nulliparous differences. In our setup, the % open arm entries appears to be more sensitive than the % time in the open arms to detect the primiparous-nulliparous group difference, as this group difference was statistically significant in the former but not the latter.

Another innovative use of the EPM was that we placed rat pups on each end of the open arms, which allowed us to examine maternal responsiveness concurrently with anxiety. This became possible because the two sets of measurement putatively reflecting maternal responsiveness (number of pup retrieved, speed and number of closed arm entries) did not overlap with those reflecting anxiety (percentage of open arm time and entries). In addition, primiparous dams only retrieved pups, not erasers, and only made more closed arm entries when they were exposed to pups. These findings highlight that this task is truly maternally motivated and rats do not just retrieve any object placed on the maze. We suggest that the mean speed is a sensitive measure of maternal motivation based on the following observations. First, primiparous rats tested under the pup presence condition (presumably possessing a higher maternal motivation than nulliparous rats) were faster than nulliparous rats. Interestingly, when tested with erasers, primiparous rats (presumably not exhibiting a higher maternal motivation) did not differ from nulliparous rats. Second, primiparous rats tested with pups had a higher mean speed than primiparous rats tested without pups. Third, the two nulliparous groups did not differ from each other on the mean speed, consistent with their equal and lower maternal responsiveness, as they are typically not spontaneously maternal. To our knowledge, there are two studies that used a similar procedure as ours and the results are comparable. Scanlan et al. [36] placed 3 rat pups on

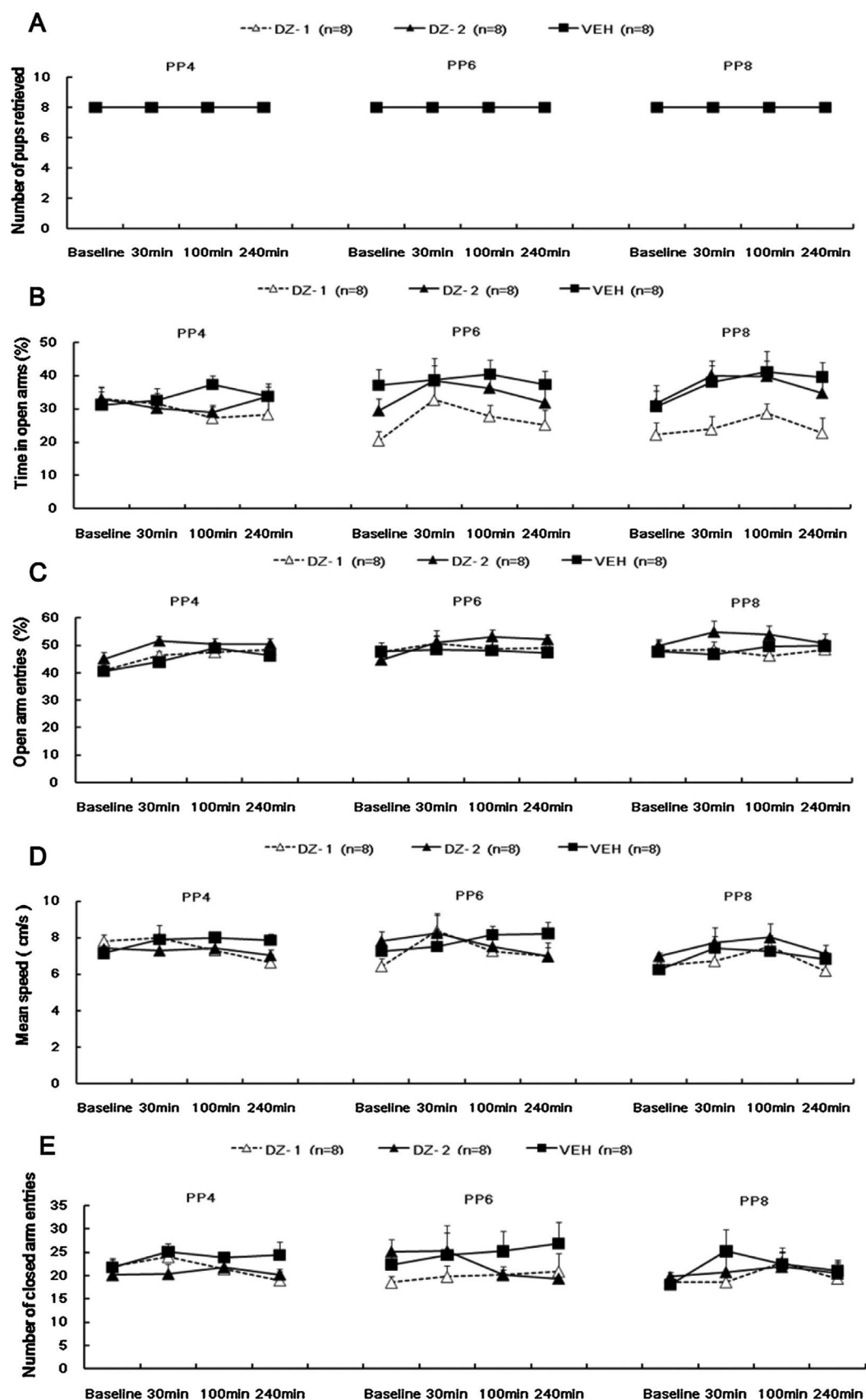


Fig. 6. Number of pups retrieved (A), percentage of time spent in the open arms (B), percentage of entries to the open arms (C), locomotor speed (D) and number of entries into the closed arm (E) by primiparous lactating female rats tested under saline or diazepam (1.0 and 2.0 mg/kg) treatment at 4 time points on days 4, 6 and 8 postpartum.

a platform 15cm away from the end of one open arm, only allowing rats to be exposed to pup cues during the test but not to physically contact them. They found that primiparous females tested in the presence of pups traveled a greater distance and spent more time on the open arms than did nulliparous females and primiparous rats tested without pups. Similarly, Pereira et al. [22] placed 3 pups in a mesh bag attached at the end of each open arm and reported that pup presence significantly increased the percentage of time spent in and entries into the open arms in both lactating and pup-induced maternal virgin rats. Neither one of these studies clearly distinguished behavioral measures of anxiety from those of maternal responsiveness. In our setup, when subject rats were allowed to physically interact with pups, primiparous rats had a significantly higher moving speed, but not higher percentage of time spent in the open arms than those tested without pups. Therefore, our paradigm has an advantage of separating behavioral measures of anxiety from those of maternal motivation.

This advantage allowed us to examine the roles of dopamine D_2 , serotonin transporter and GABA/benzodiazepine receptors in emotion regulation and maternal responsiveness, two psychological building blocks important for the onset and maintenance of maternal behavior. Previous work has implicated dopamine D_2 receptor in the motivational and motoric aspect of maternal behavior, as HAL treatment disrupts active maternal behaviors (e.g., pup retrieval, pup licking and nest building) in the postpartum female rats [16,18,37], and this disruption could be attenuated to some extent by separating dams from their pups for 4 h before testing, a pup-separation procedure presumably increasing maternal motivation [19,20] or by making pups more demanding (the motor impairment effect of HAL may also play a role in its maternal effect). Indeed, the involvement of the mesolimbic dopamine system in the control of the motivational aspects of maternal behavior is one of the most well documented evidence in this field [13,38–43]. The present finding that HAL dose- and time-dependently selectively decreased pup retrieval and speed, but not the percentage of open arm entries is consistent with previous work and further demonstrates the validity of this paradigm for the study of maternal motivation.

We are not aware of any study that examined the acute effect of FLU treatment on maternal behavior. One study administered FLU to dam rats throughout gestation (GD 1–20) and observed maternal behavior on day 1 postpartum, several days after the last FLU treatment [27]. Only the touch/sniff of pups was significantly increased by FLU at 8.0 mg/kg. Consistent with this study, the present study also only found a transient and mild disruption of pup retrieval, which could be explained partly by the acute anxiogenic effects of FLU [44]. This finding is also consistent with earlier lesion and pharmacological studies showing that disruption of 5-HT neurotransmission only causes a transient and nonspecific deficit [45]. Our recent work, however, indicates that direct manipulation of 5-HT_{2A} or 5-HT_{2C} receptor causes more severe disruption of maternal behavior, as acute administration of a mixed 5-HT_{2A/2C} agonist 2,5-dimethoxy-4-iodo-amphetamine (DOI), a highly selective 5-HT_{2A} agonist TCB-2, a serotonin 5-HT_{1A} receptor partial agonist buspirone, or a highly selective 5-HT_{2C} agonist MK212 disrupts various maternal behavior [21,46,47] (unpublished observation). Our finding that FLU decreased the locomotor speed on the EPM suggests that FLU as well as other 5-HT drugs might suppress maternal behavior by decreasing maternal motivation, but not by affecting anxiety. More studies are needed to further test this idea. The additional finding that FLU decreased the percentage of time spent in the open arms is in agreement with Pawluski et al. [48] who reported that chronic FLU treatment during the postpartum period increased anx-

iety-related behavior as measured in an elevated zero maze.

Our lack of a robust DZ effect on anxiety behaviors is in conflict with available evidence in the literature. Fernandez-Guasti et al. [26] reported that DZ at 2.0 mg/kg increased time spent in the open arms in the EPM test that was not confounded by DZ's motor actions. Similarly, Byrnes and Bridges [28] also found that DZ at 2.0 mg/kg increased the percentage of open arm time and distance traveled. In both studies, the percentage of open arm time in the lactating group was less than 20%, while in our study, it was over 30%. It is possible that our dams already exhibited a relatively lower level of reduced anxiety, a ceiling effect which prevented the detection of the anxiolytic effect of DZ. The lack of maternal disruptive effect of DZ was in conflict with a previous report that DZ at 2.0 mg/kg disrupted pup retrieval and nest building [47]. The exact reason for this discrepancy is also not clear. More studies are needed to resolve this issue. Given that other anxiolytic drugs such as chlordiazepoxide are found to be ineffective to disrupt pup retrieval, the maternal disruptive effect of DZ might be dependent on the test situation and rat strain (Sprague-Dawley vs. Wistar).

As mentioned before, the neurobiological basis of maternal motivation is well understood, including the involvement of the mesolimbic dopamine system (the ventral tegmental area to the nucleus accumbens) and the medial preoptic area, the so-called excitatory neural basis underlying maternal behavior [49]. In contrast, the neural basis of reduced anxiety in postpartum is less clear. Research on this topic has focused on the ventrocaudal periaqueductal gray (cPAGv), the ventral bed nucleus of the stria terminalis (BSTv), and the amygdala. For example, lesion of the cPAGv decreases anxiety-related behavior in lactating rats [50], while infusion of a highly specific oxytocin receptor antagonist directly into the cPAGv increases anxiety in dams (as tested in the EPM) to the levels typically found in virgin females [51]. Infusion of bicuculline, a GABAA receptor antagonist in the cPAGv also significantly increases anxiety in dam rats [52]. It has been suggested that the pup-induced decrease in anxiety in mother rats might be mediated by the vBST and amygdala, as there was a higher Fos expression in the vBST in lower-anxiety mother rats (no pup separation) than higher-anxiety dams (whose pups were removed from them for 4 h before EPM testing) [53], and higher in the medial and cortical subregions of the amygdala in primiparous females tested with pups on the EPM than those tested without pups or to nulliparous females tested with or without pups [36], suggesting that these brain areas are involved in processing of pup cues. Because our paradigm provides sensitive behavioral measures of maternal motivation and anxiety, it would be useful not only for the identification of distinct neural systems involved in these processes, but also for the delineation of their interactions.

The postpartum period is a time when a host of changes occur at molecular, cellular, physiological and behavioral levels to prepare female humans for the challenge of maternity. If these adaptations are prevented by certain environmental or genetic factors, the female's cognitive abilities and emotion regulation could be compromised and her risk to develop anxiety, depression, and psychosis is increased. For those who develop postpartum mental disorders, a large portion of them are prescribed antidepressants, anxiolytics and antipsychotics, which are generally safe and effective interventions for both mothers and infants [54–56]. With the symptom improvement, maternal care improves along the way. There is little information regarding whether these psychotherapeutic drugs may have subtle detrimental effects on maternal care and how they may affect the development of children in the long run [57]. If there is any, it would most likely be masked by their improvement effects on symptoms of depression, anxiety and psychosis. Therefore,

from the clinical perspective, the results from our HAL, FLU and DZ study further our understanding of behavioral mechanisms of action of these psychotherapeutic drugs and how reproductive experience alters behavioral and brain responses to these medications. Because the new paradigm introduced here is based upon a repeated drug dosing and testing procedure, which mimics clinical treatment conditions, it could be used to reveal the impact of pharmacotherapies on the psychology and brain function of postpartum women. Such knowledge may in turn inform clinical practices regarding drug choices and doses.

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Appendix A. Supplementary data — Supplementary data associated with this article can be found, following the References.

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Figure S1. Number of erasers retrieved by primiparous and nulliparous females tested at 4 time points on days 4, 6 and 8 postpartum. * indicates significant difference relative to the vehicle group, $p < 0.05$.

Figure S2. Number of pups retrieved by primiparous and nulliparous females tested at 4 time points on days 4, 6 and 8 postpartum. * indicates significant difference relative to the vehicle group, $p < 0.05$.

Figure S3. Number of pups or erasers retrieved (A), percentage of time spent in the open arms (B), percentage of entries to the open arms (C), locomotor speed (D) and number of closed arm entries (E) by the two primiparous groups tested with either pups or erasers at 4 time points on days 4, 6 and 8 postpartum. * indicates significant difference between the two groups, $p < 0.05$.

Figure S4. Percentage of time spent in the open arms (A), percentage of entries to the open arms (B), locomotor speed (C) and number of closed arm entries (D) by the two nulliparous groups tested at 4 time points on days 4, 6 and 8 postpartum.

Figure S1

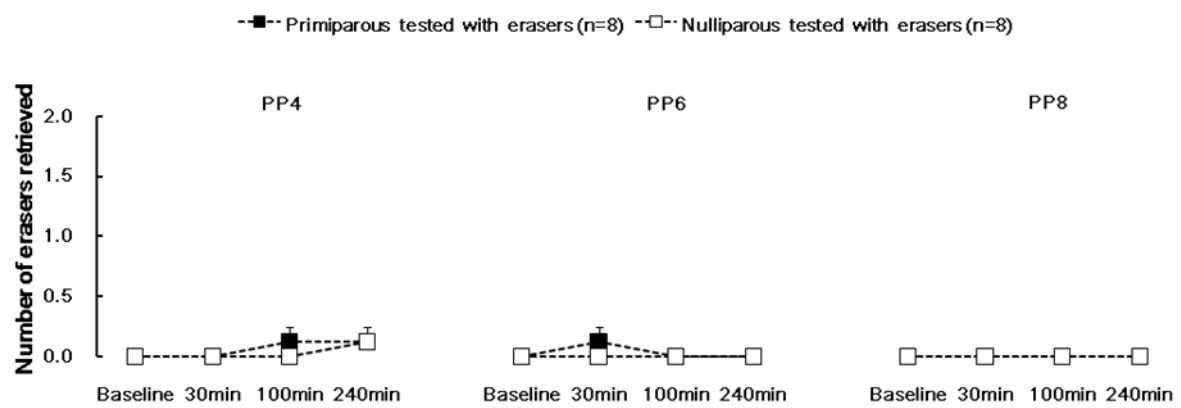


Figure S2

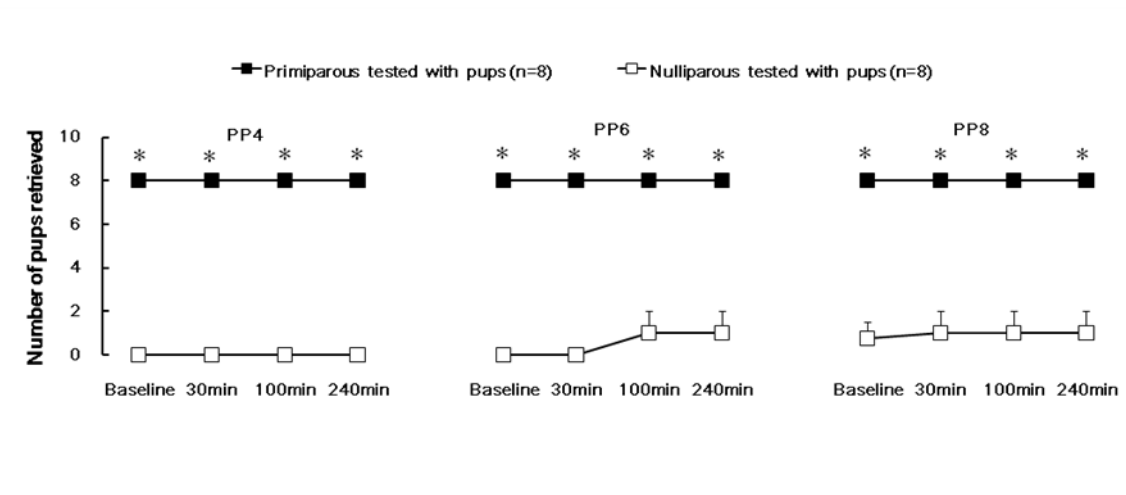


Figure S3

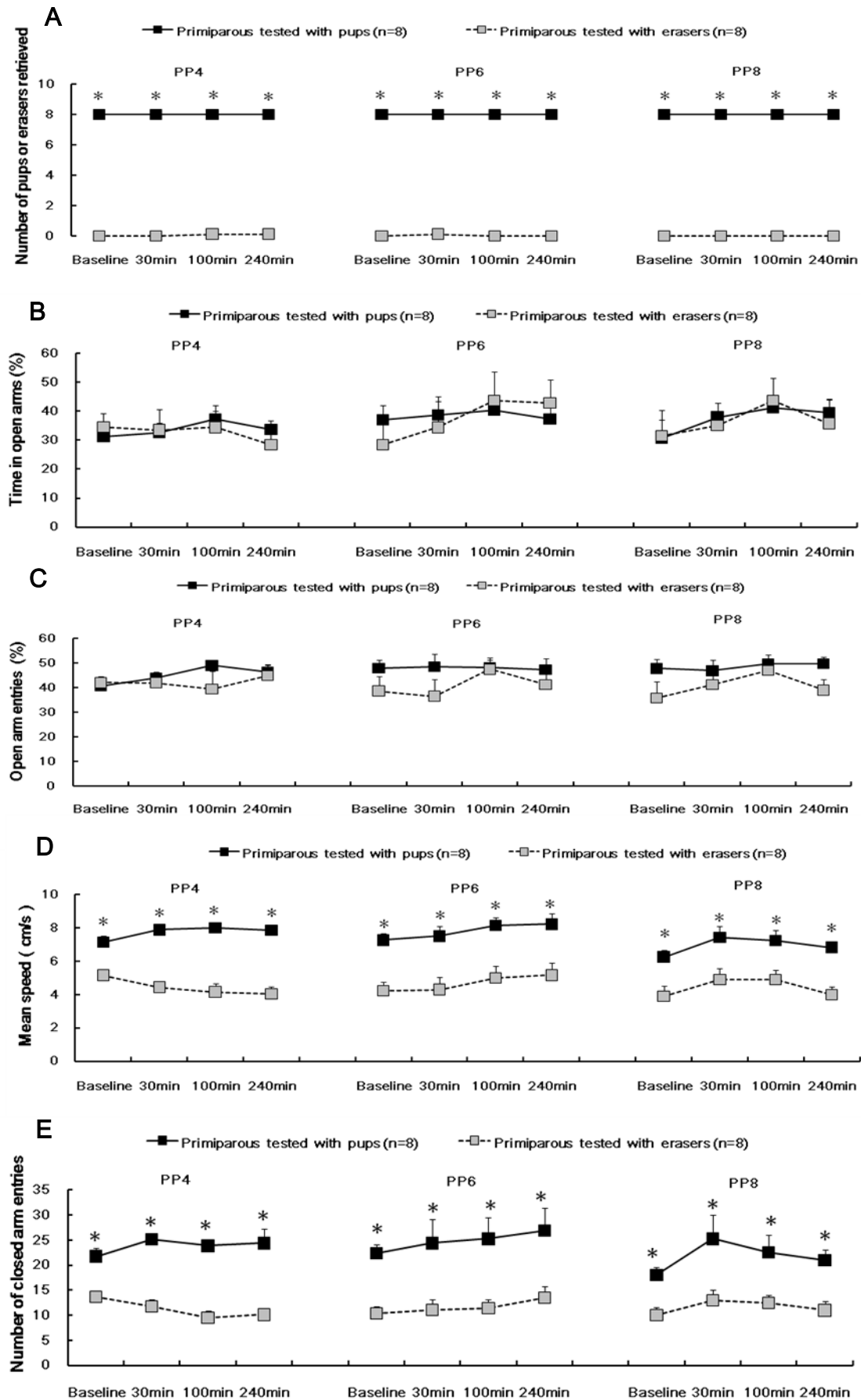


Figure S4

