



Role of oxytocin in the medial preoptic area (MPOA) in the modulation of paternal behavior in mandarin voles

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ABSTRACT

Parental care plays an important role in individual survival and development in mammals. Many studies have focused on the mechanisms underlying maternal behavior. However, the underlying neural mechanisms of paternal behavior are less understood. Using monogamous mandarin voles (*Microtus mandarinus*), the present study found that fathers initiated more paternal behavior and the virgin male showed more infanticide. Moreover fathers had shorter latency to approach a pup at the postnatal day (PND) 10 than PND1, PND20 than nonfathers. Fathers had a shorter latency to take care of unfamiliar pups than nonfathers. They had higher levels of paternal behavior at PND 10 than PND1 and PND20 toward the mandarin vole pups. Fathers had a significantly higher serum concentration of oxytocin (OT) than virgin males. Both RT-PCR and Western blot results indicated that the levels of the oxytocin receptor (OTR) in the medial preoptic area (MPOA) of fathers were significantly higher than in virgin males, but the levels of vasopressin 1a receptor (V1AR) mRNA and protein expression in the MPOA did not show significant differences. Microinjection of an oxytocin receptor antagonist into the MPOA significantly reduced the total duration of paternal behavior and increased the latency to approach the pup and initiate paternal behavior. Our results indicated that OT plays a key role in the modulation of paternal behavior via the MPOA.

1. Introduction

Parenting is an energetically costly and dynamic behavior. Parental care plays an important role in the survival, ontogeny and reproduction of offspring. In mammals, mothers usually play the largest or only role in rearing offspring. Transition to motherhood is accompanied by changes in hormones during pregnancy, parturition, and lactation (Bridges, 1996; Numan et al., 1994; Stern, 1989). Thus, the neuroendocrine mechanisms underlying maternal behaviors have been studied deeply and extensively. In only 5–6% of mammalian species, males are also heavily involved in parental care (Kleiman and Malcolm, 1981). Paternal care also profoundly affects the brain and behavior of the offspring (Jia et al., 2009, 2011; Yu et al., 2015). It has been suggested that hormones such as testosterone (T) and prolactin (PRL) may be involved in paternal care because fathers and nonfathers have different levels in some biparental rodents, such as prairie voles (*Microtus ochrogaster*), California mice (*Peromyscus californicus*), mandarin voles (*Microtus mandarinus*), and Djungarian hamsters (*Phodopus campbelli*) (Brown, 1993; Lonstein and De Vries, 2000; Wynne-Edwards and

Timonin, 2007). On the other hand, our recent study found that fathering experience can alter levels of central oxytocin (OT) and dopamine receptor expression in the nucleus accumbens (NAcc) and medial amygdaloid nucleus (MeA), displaying a possible association between OT and the dopamine system and paternal care (Wang et al., 2015, 2018b). However, whether these chemicals in other brain regions are also involved in paternal behavior was less investigated.

The medial preoptic area (MPOA) has been shown to be critically involved and a node for the positive control of parental behavior in rodents. Previous studies have established the critical importance of the MPOA for maternal behavior (Numan, 1974). Lesions or destruction of the MPOA impacted the onset and establishment of parental behavior (Akther et al., 2013; Lee and Brown, 2002, 2007; Numan, 1974; Numan et al., 1977; Pereira and Morrell, 2009; Sturgis and Bridges, 1997). In contrast, electrical stimulation of the MPOA facilitated parental behavior (Morgan et al., 1997; Morgan et al., 1999). Using the expression of Fos as a marker of neuronal activation (Hoffman and Lyo, 2002), it was found that fathers, after exposure to the pups, had a similar activation pattern in the MPOA as mothers; in particular, levels of c-Fos expression

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in the MPOA were significantly increased (de Jong et al., 2009; Stack and Numan, 2000; Yuan et al., 2018). Thus, we predict that the MPOA may be involved in paternal behavior.

The MPOA has a high density of oxytocin receptors (OTR) with which OT binds and exerts its effects on behavior (Yoshida et al., 2009). OT, synthesized in and centrally released from the hypothalamic paraventricular nuclei (PVN) and supraoptic nuclei (SON) (Ludwig, 1998; Zimmerman et al., 1984), plays an essential role in the onset and maintenance of maternal behavior in the early postpartum period in both human (Galbally et al., 2011; Kim et al., 2014) and rodent models (Fahrbach et al., 1984; Pedersen et al., 1994; Williams et al., 1994). OT was also found to be associated with paternal behavior in various species, including California mice (de Jong et al., 2009; Lambert et al., 2011), prairie voles (Kenkel et al., 2012; Kenkel et al., 2014), and mandarin voles (Song et al., 2010). For example, male mandarin voles with high levels of paternal behavior have higher levels of OT-immunoreactivity (OT-IR) in the PVN and SON (Li et al., 2015). A study in adult marmosets demonstrated that intranasal OT decreased latencies to respond to infant stimuli in males (Taylor and French, 2015). These studies suggest that OT may mediate paternal care. However, whether OT mediates paternal behavior via binding with OTRs in the MPOA remains unclear.

The mandarin vole is a socially monogamous rodent. Both male and female mandarin voles display high levels of social interaction, and family members communicate and interact with each other frequently. Furthermore, male mandarin voles display high levels of paternal behavior (Jia et al., 2009; Yuan et al., 2018). The male voles display high levels of paternal responses to the pups (licking, grooming, crouching, and even performing the kyphotic nursing posture) similar to female voles (Song et al., 2010; Wang et al., 2015). Therefore, the male mandarin voles could be a suitable model to explore the underlying mechanisms of paternal behavior. Using mandarin voles, the present study investigated whether new fathers have higher levels of OTR in the MPOA than nonfathers. Provided that was true, we expected that the microinjection of oxytocin receptor antagonist (OTA) into the MPOA may reduce paternal behavior. Based on this study, we attempted to determine the involvement of OT in the MPOA in the modulation of paternal care.

2. Materials and methods

2.1. Subjects

The animals used in this study were a laboratory-reared generation derived from a wild population of mandarin voles in Henan, China. Animals were maintained on a 12:12 light-dark cycle (light on at 20:00, Beijing time) at an ambient temperature of approximately 23 °C and 65% humidity. All animals were allowed free access to food (carrots and rabbit chow) and water and cotton nesting material in polycarbonate cages (44 cm × 22 cm × 16 cm). The males and females were paired at 70 days of age. The gestation was 21 days. The virgin males had no reproductive experience and were housed in a group of 3 individuals per cage. The new father was defined as the male that was paired and copulated with a female vole and became a first time father. Twelve male virgin (90 days of age) and 41 new father mandarin voles (90 days of age) were used in this study. Some animal in which cannula tips were not successfully located in the MPOA or were died during the experiments, are not included in these numbers. The experimental procedures were approved by the Animal Care and Use Committee of Shaanxi Normal University and were in accordance with the Guide for the Care and Use of Laboratory Animals of China. All efforts were made to minimize the number of animals used and their suffering during the experiments.

2.2. Experiment 1

2.2.1. Paternal behavior

Ten virgin male voles were paired with female voles at approximately 70 days of age, and the pups were born approximately 20 days later. The litters were culled to 2–3 pups. Separate sets of voles were used for two experiments. First, to characterize paternal behaviors, after delivery, new fathers were exposed to their own pups repeatedly on PND1 (n = 9), PND10 (n = 9) and PND20 (n = 9), and the paternal behaviors of new fathers were recorded and scored. Second, to compare the behavioral responses of new fathers and virgin males to unfamiliar pups, the new fathers at PND1 (n = 6), PND10 (n = 6) and PND20 (n = 6), and virgin males (n = 6) were exposed to an unfamiliar pup. In tests of these four groups, a one-day-old unfamiliar pup was exposed to new father when his own pups were one day old (PND1); a 10-day-old unfamiliar pup was exposed to father whose own pups were 10 days old (PND10), 20-day-old unfamiliar pup was exposed to father whose own pups were 20 days old (PND20), and a one-day-old unfamiliar pup was exposed to a virgin male. When assessed paternal behavior, the males were introduced into a new test cage (it was the same size as the home cage) for a 1 h habituation period, and then one unfamiliar pup was brought to the farthest corner from the fathers or virgins. All the pups were held outside the cage for 10 min in an incubator at 32 °C before the test. During the test of paternal behaviors of new fathers, mothers stayed in breeding cages with the remaining pups. Latency to approach the pups and latency to engage in paternal care were recorded and analyzed. If the subjects attacked the pup and infanticide occurred, the tests were terminated immediately, and the pups were euthanized. Paternal behavior was recorded for further analysis. All paternal behaviors were recorded for 10 min between 8:00 am and 12:00 pm.

The paternal behaviors that were scored and analyzed included total duration of sniffing, retrieving, licking and grooming, crouching, resting, and walking; the latency to approach and initiate paternal behavior toward the pup was recorded. Sniffing was defined as sniffing the body of the pup. Licking and grooming were defined as licking the body of the pup and grooming the pup's fur. Retrieving was defined as pups being transported to opposite side of the cage. Crouching was defined as high or low kyphosis (i.e., an upright crouch) characterized by placing all limbs on the ground in a splayed and rigid manner, depression of the head, and pronounced dorsal arch, which was strikingly similar to that observed in lactating rats (Wang et al., 2018b). Resting was defined as the voles showing no movement. Walking was defined as the voles walking in the test cage. The cumulative duration of paternal behavior was defined as the total time spent crouching, licking and grooming. The latency of the father vole to approach a pup was defined as the time from pup exposure to the first time to sniff or contact the pups. The latency of the father vole to initiate paternal behavior was defined as the time from pup exposure to the first time initiating crouching or licking and grooming behavior. The definitions of the assessed paternal behaviors are presented in Table 1. Behavioral data from videotapes were analyzed using J-Watcher v1.0 (Macquarie University and UCLA; <http://www.jwatcher.ucla.edu/>) (Blumstein and Daniel, 2007) by a trained experimenter blind to the experimental conditions.

2.3. Experiment 2

To compare serum OT levels and OTR and vasopressin 1a receptor (V1AR) protein and mRNA expression levels in the MPOA between virgin males (n = 6) and new fathers (n = 6), a separate set of animals was used in Experiment 2. Virgin males and new fathers (at approximately 100 days of age) were exposed to an unfamiliar 10-day-old pup for 10 min. The exposure procedure was same as described in the experiment 1. If the virgin males attacked the pups and infanticide occurred, the tests were immediately terminated, and the pups were overanesthetized for euthanasia. Immediately following the tests, all

Table 1
The definitions of paternal behaviors.

Paternal behavior	Definition
Sniffing	Sniffing the body of the pups.
Licking and grooming	Licking the body of the pup and grooming the pup's fur.
Retrieval	Transporting pups to the opposite side of the cage.
Crouching	High or low kyphosis (i.e., an upright crouch) characterized by placing all limbs on the ground in a splayed and rigid manner, depression of the head, and pronounced dorsal arch, which was strikingly similar to that observed in lactating rats.
Inactivity	Resting with no movement.
Walking	Walking in the testing cage.
Total time of paternal behavior	Crouching, licking and grooming the pup.
Latency to approach pups	The time from pup exposure to the first time to sniff or contact the pups.
Latency to initiate paternal behavior	The time from pup exposure to first time to initiate crouching or licking and grooming behavior.

subjects were anesthetized for sample collection.

2.3.1. Serum oxytocin assay

Blood was collected directly in a microcentrifuge tube from the heart. After clotting, blood was centrifuged at 3000 rpm for 15 min at 4 °C, and the supernatant was collected. Serum OT levels were measured by a vole OT enzyme-linked immunosorbent assay (catalog number: F18540, Xitang Biotechnology, Shanghai, China) according to the manufacturers' instructions. Absorbance was measured using a microplate reader (BioTek Instruments, Winooski, USA) at 450 nm. The reading was zeroed using a blank well.

2.3.2. Total mRNA extraction

While collecting the serum, brains were also quickly removed and fresh frozen in liquid nitrogen. Brains were sectioned (100 μm) on a cryostat and mounted onto slides. The MPOA (bregma 0.26 to –0.58) was dissected out using a tissue punch (1 mm diameter). Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol. Briefly, the sample was punched into an RNase-free Eppendorf tube, and 500 μL RNAiso reagent was added. Then, 100 μL chloroform was added, and the tube was vigorously shaken by hand for 15 s. The sample was centrifuged at 12,000 ×g for 15 min at 4 °C. The aqueous phase of the sample was removed into a new RNase-free Eppendorf tube, and the lower red phenol-chloroform phase was used for follow-up protein extraction. Equal volumes of isopropanol were added to the aqueous phase supernatant and incubated at room temperature for 10 min and then centrifuged at 12,000 ×g for 10 min at 4 °C. The supernatant was removed from the tube, and the RNA pellet was washed twice with 1000 μL of 75% ethanol DEPC water. The RNA pellet was air dried for 10 min and then resuspended in 20 μL of DEPC water. A NanoDrop spectrophotometer (Thermo Scientific, USA) was used to determine the quality (260/280 > 1.8) and quantity of the RNA. Total RNA (150 ng) was reverse transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's protocol.

2.3.3. Quantitative PCR

Quantitative real-time PCR was performed by the CFX Real-Time PCR detection system (Bio-Rad, USA). The following genes were detected using the Light Cycler System with SYBR Green II (TaKaRa, Dalian, China): OTR (XM_005364985.1), V1AR (NM_016847.2), and the reference gene β-actin (XM_005368002.2) according to previous studies (Du et al., 2017). Oligonucleotide primers for OTR, V1AR, and β-actin were designed and synthesized by Sangon Biotech (Shanghai, China). Primer sequences and the lengths of OTR, V1AR, and the reference gene β-actin are presented in Table 2. All real-time PCRs were performed in triplicate. Each PCR was performed in a total volume of 20 μL containing diluted (1/5) cDNA template (2 μL), forward and reverse primers (1 μM each), 10 μL of SYBR Green II master mix and water as needed to reach the total volume. After an initial Taq activation at 95 °C for 1 min, light-cycle PCR was performed using 40 cycles, and the

cycling conditions are described in Table 2. To verify the purity of the products, a melting curve was produced after each run by first holding the reaction mixtures at 55 °C for 10 s and then increasing their temperature to 95 °C at a rate of 0.1 °C/s. Threshold (Ct) values were determined using the Light Cycler software (Priego et al., 2008). The results after calibration with β-actin expression were calculated using the $\Delta\Delta$ CT method (Pfaffl, 2001) and are presented as the fold increase relative to virgin males as a control.

2.3.4. Total protein extraction

The MPOA was dissected as described above in the Total mRNA extraction section. Briefly, 750 μL isopropanol was added to the lower red phenol-chloroform phase supernatant as described above. Samples were incubated for 10 min at room temperature and then centrifuged at 12,000 ×g for 10 min at 4 °C to pellet the protein. The supernatant was discarded, and the protein pellet was washed with 1000 μL 0.3 M guanidine hydrochloride in 95% ethanol, incubated for 20 min at room temperature, and then centrifuged at 7500 ×g for 5 min at 4 °C. The wash procedure was repeated twice more. Then, 1000 μL of 100% ethanol was added to wash the protein pellet and centrifuged at 7500 ×g for 5 min at 4 °C. The supernatant was discarded, and the protein pellet was air dried for 10 min. Total protein was resuspended in RIPA lysis buffer (R0010, Solarbio Biotechnology, China) containing a protease inhibitor (Sigma). The homogenate was sonicated on ice for 60 s (paused for 5 s after every 5 s of sonication to avoid sample overheating), left on ice for 30 min and then centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was harvested and quantified by BCA assay (PA115-01, Tiangen, China) and diluted to a final concentration of 2 μg/μL.

2.3.5. Western blotting

Protein samples (20 μg/well) and molecular weight ladders (161-0374, Bio-Rad, Hercules, CA) were separated by 10% SDS-polyacrylamide gels for approximately 30 min at 80 V followed by 100 min at 120 V. Proteins were transferred to 0.45 μm PVDF membranes (Millipore, Billerica, MA, USA) in transfer buffer at 4 °C for 120 min at 200 mA. The membranes were blocked with BSA for 2 h. The membranes were incubated with the following diluted primary antibodies at 4 °C overnight: OTR (ab181077, 1:5000, Abcam, UK), V1AR (GTX89114, 1:6000, GeneTex, USA), and β-tubulin (CW0098M, 1:4000, CoWin Biosciences, China). The next day, the membranes were washed 3 × 10 min with 1 × TBST, incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000, ZhongShan Goldenbridge Biotechnology, China) for 2 h at room temperature and then washed 3 × 5 min with 1 × TBST. Membranes were revealed with ECL (WBKLS0500, Millipore) exposure using a Luminescent Imaging workstation (Tanon 6200 Luminescent Imaging Workstation, Tanon, China). The integral optical density values of the blots in the pictures were measured with Image-Pro Plus 6 software. β-tubulin was used as an internal control to verify consistency in the amount of protein in all samples. The membrane optical density values were compared, and the

Table 2
Nucleotide sequences of primers and cycling conditions used for RT-PCR amplification.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Cycling conditions
OTR	CGTGCAGATGTGGAGCGTC	GAGCAGAGCAGCAGAGGAAG	95 °C for 10 s 60 °C for 30 s
V1AR	GCGTGCTTCTGGCTTTGC	TGTAGCGGTCGGCAGTCA	95 °C for 10 s 60 °C for 30 s
β-actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTAA	95 °C for 10 s 60 °C for 30 s

ratio relative to levels obtained in the virgin males, which were used as a reference.

2.4. Experiment 3

2.4.1. Stereotaxic surgery and microinjections of OTA

A separate set of animals was used for the pharmacological experiments. Stereotaxic surgery was carried out on the 6th day after the fathers' litters were born. Subjects were inhaled-anesthetized using isoflurane and stereotaxically implanted with a 0.25 mm diameter bilateral guide cannula (RWD Life Science, Shenzhen, China) positioned in the MPOA (AP + 0.14 mm, ± ML 0.5 mm, and DV −5.2 mm to the bregma brain surface). The cannulas were secured to the skull with dental cement. The OTA ([d(CH₂)₅¹, Tyr(Me)², Thr⁴, Orn⁸, des-Gly-NH₂⁹]-Vasotocin trifluoroacetate salt, Bachem) was dissolved in saline (1 ng, 10 ng/side) (Yu et al., 2016). After 3 days of recovery, subjects that returned to normal activity, i.e., similar to animals without surgery, received microinjections of either vehicle (*n* = 7), 1 ng OTA (*n* = 7), or 10 ng OTA (*n* = 6). Different groups of animals received different doses of the microinjections. The speed of the microinjections was 0.1 μL/min and required approximately 2 min. Subjects were exposed to their own pups at the age of 10 days old, and paternal behaviors were recorded and scored as described above for 10 min beginning 20 min following drug microinjection. After the behavioral tests were completed, all subjects were anesthetized and the brains were harvested. Then, the brains were cut into 40 μm sections on a cryostat to histologically verify the injection sites. The experimental data from the animals in which the cannula tips were successfully located in the MPOA were analyzed.

2.5. Statistical analysis

Statistical analyses were conducted using SPSS 22.0 (SPSS Inc., Chicago, USA), and all the data were checked for normality using a sample Kolmogorov-Smirnov test. The behavior duration and latency in the parental behavior test among the groups were compared using one-way ANOVA followed by Bonferroni post hoc tests. The OTR and V1AR mRNA differences and protein optical density ratio were compared between virgin males and fathers using t-tests. Effect sizes were calculated and reported as follows: eta-squared was calculated using $\eta^2 = SS_{\text{effect}} / SS_{\text{total}}$ for one-way ANOVA. Cohen's *d* was calculated using $d = M_1 - M_2 / \sigma_{\text{pooled}}$ for pairwise comparisons. All data are presented as the mean ± SE, and significance was established at *P* < 0.05.

3. Results

3.1. Paternal behaviors

One-way ANOVA revealed that fathers had different latencies to approach ($F_{2,24} = 6.571$, *P* < 0.01, $\eta^2 = 0.354$) (Fig. 1A) or care for pups of different ages ($F_{2,24} = 10.270$, *P* < 0.01, $\eta^2 = 0.461$) (Fig. 1B). In addition, virgin males exposed to unfamiliar pups and fathers exposed to different ages of unfamiliar pups also showed a significant difference in latency to approach to pups ($F_{3,20} = 4.425$, *P* < 0.05,

$\eta^2 = 0.399$) (Fig. 1C) and to engage paternal care to unfamiliar pups ($F_{3,20} = 111.885$, *P* < 0.01, $\eta^2 = 0.944$) (Fig. 1D). When virgin males were exposed to pups, they showed either infanticide (6 virgin males) or negligence (2 virgin males) to pups, which was different from new fathers. Post hoc comparisons indicated that fathers had a shorter latency to approach their own pups at 10 days of age than one day of age and 20 days of age (10 day vs. 1 day, *P* < 0.05, *d* = 2.324; 10 day vs. 20 day, *P* < 0.05, *d* = 1.432). They also had shorter latency to take care of pups at 10 days than pups at one day and 20 days (10 day vs. 1 day, *P* < 0.05, *d* = 2.714; 10 day vs. 20 day, *P* < 0.01, *d* = 0.687). When exposed to unfamiliar pups, fathers had a shorter latency to take care of pups of any age than virgin males (virgin-1 day vs. father-1 day, *P* < 0.01, *d* = 29.251; virgin-1 day vs. father-10 day, *P* < 0.01, *d* = 83.849; virgin-1 day vs. father-20 day, *P* < 0.01, *d* = 4.479). Fathers approached an unfamiliar pup at 10 days of age more quickly than pups of one day and 20 days of age (father-10 day vs. father-1 day, *P* < 0.05, *d* = 0.617; father-10 day vs. father-20 day, *P* < 0.05, *d* = 5.604). In addition, the behavioral responses of father voles to their own pups of 1, 10 and 20 days of age were observed for 10 min. One-way ANOVA revealed significant differences in sniffing ($F_{2,24} = 11.370$, *P* < 0.01, $\eta^2 = 0.487$), crouching ($F_{2,24} = 14.056$, *P* < 0.01, $\eta^2 = 0.540$), licking and grooming ($F_{2,24} = 10.100$, *P* < 0.01, $\eta^2 = 0.457$), walking ($F_{2,24} = 8.543$, *P* < 0.01, $\eta^2 = 0.413$), inactivity ($F_{2,24} = 19.929$, *P* < 0.01, $\eta^2 = 0.624$) and paternal behavior ($F_{2,24} = 19.391$, *P* < 0.01, $\eta^2 = 0.618$) to different ages of pups (Fig. 2). Fathers engaged in more licking and grooming (10 day vs. 1 day, *P* < 0.05, *d* = 1.148; 10 day vs. 20 day, *P* < 0.01, *d* = 1.866) and sniffing (10 day vs. 1 day, *P* < 0.05, *d* = 1.498; 10 day vs. 20 day, *P* < 0.05, *d* = 2.415) to 10-day-old pups than 1- and 20-day old pups. We found that fathers spent less time in inactivity (1 day vs. 20 day, *P* < 0.01, *d* = 2.730; 10 day vs. 20 day, *P* < 0.01, *d* = 2.239) and more time in crouching (1 day vs. 20 day, *P* < 0.05, *d* = 1.933; 10 day vs. 20 day, *P* < 0.01, *d* = 0.174), walking (1 day vs. 20 day, *P* < 0.05, *d* = 1.139; 10 day vs. 20 day, *P* < 0.01, *d* = 2.313) and paternal behavior (1 day vs. 20 day, *P* < 0.01, *d* = 1.914; 10 day vs. 20 day, *P* < 0.01, *d* = 3.673) when pups were one day and 10 days of age than pups of 20 days of age. We found no significant difference in retrieving ($F_{2,24} = 0.281$, *P* > 0.05, $\eta^2 = 0.023$) when pups were different ages.

3.2. OT levels in serum

The serum concentrations of OT in the virgin males and new fathers are presented in Fig. 3. The t-tests revealed that the OT concentration in the father's serum was significantly higher than that in the virgin males ($t_{10} = 5.574$, *P* < 0.01, *d* = 3.218).

3.3. OTR mRNA and protein expression in the MPOA

The levels of OTR mRNA and protein expression in the MPOA were compared between virgin males and fathers (Fig. 4). T-test indicated that expression levels of OTR mRNA ($t_8 = 6.234$, *P* < 0.01, *d* = 3.943) (Fig. 4A) and protein ($t_8 = 2.791$, *P* < 0.05, *d* = 1.765) (Fig. 4B) in the MPOA were significantly higher in fathers than virgin males.

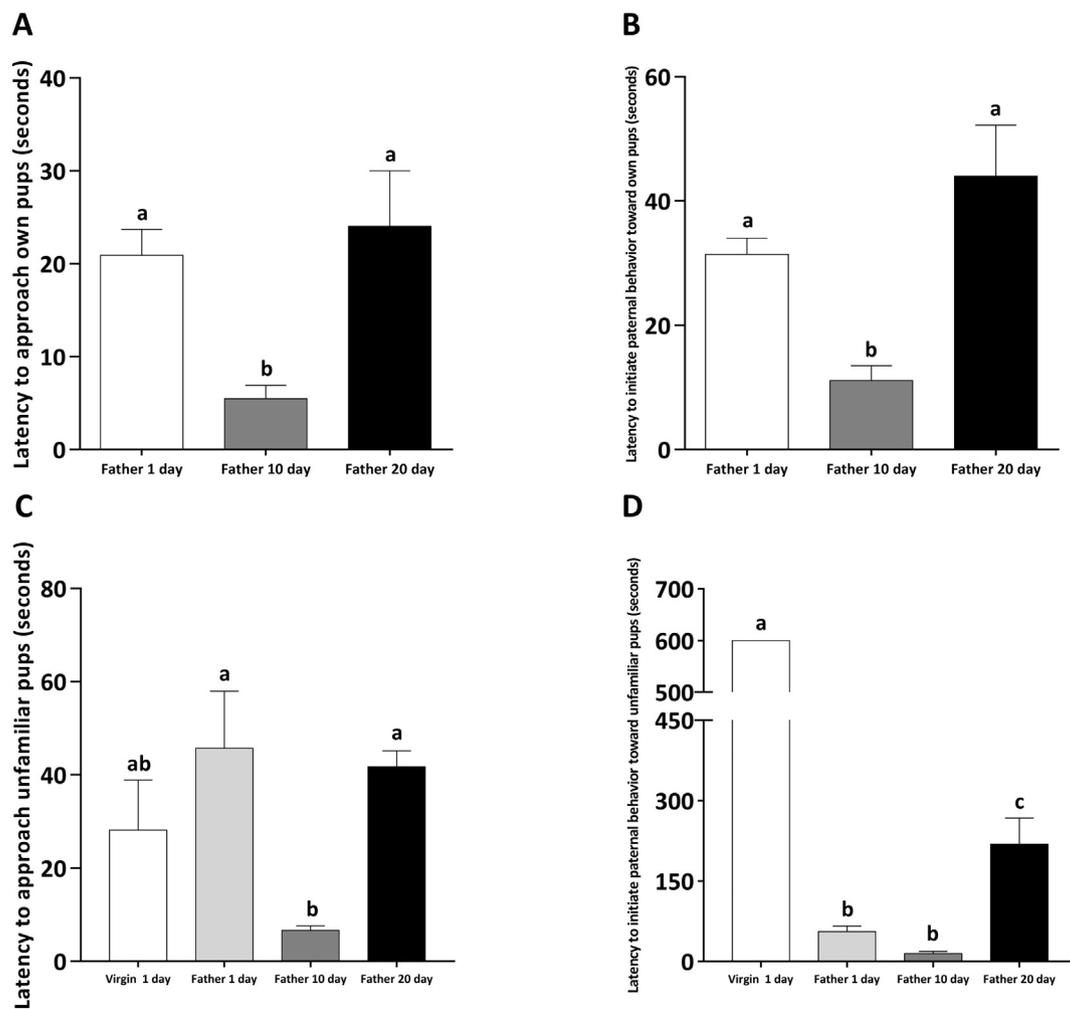


Fig. 1. Latency of mandarin voles to approach pups and initiate paternal behavior to pups. (A) Latency of father mandarin voles to approach their own pups of different ages. Fathers were exposed to one-day-old pups ($n = 9$), 10-day-old-pups ($n = 9$) and 20-day-old pups ($n = 9$) for 10 min. (B) Latency of male mandarin voles to initiate paternal behavior to their own pups at different ages. Fathers were exposed to one-day-old pups ($n = 9$), 10-day-old pups ($n = 9$) and 20-day-old pups ($n = 9$) for 10 min. (C) Latency of virgin mandarin voles and father mandarin voles to approach unfamiliar one-day-old pups ($n = 6$) and father voles were exposed to one-day-old pups ($n = 6$), 10-day-old pups ($n = 6$) and 20-day-old pups ($n = 6$) for 10 min. (D) Latency of virgin mandarin voles and father mandarin voles to initiate paternal behavior to unfamiliar pups. Virgin voles were exposed to one-day-old pups ($n = 6$), and fathers were exposed to one-day-old pups ($n = 6$), 10-day-old pups ($n = 6$) and 20-day-old pups ($n = 6$) for 10 min. Groups not sharing the same letters are significantly different ($P < 0.05$). Data are presented as the mean \pm SE.

3.4. *VIAR* mRNA and protein expression in the MPOA

The levels of *VIAR* mRNA and protein expression in the MPOA between virgin and father were also compared (Fig. 5). In contrast to the levels of *OTR* expression, no significant differences in the levels of *VIAR* mRNA ($t_8 = 0.942$, $P > 0.05$, $d = 0.596$) (Fig. 5A) and protein ($t_8 = 1.159$, $P > 0.05$, $d = 0.733$) (Fig. 5B) expression in the MPOA between the two groups were found.

3.5. Effect of OTA microinjection into the MPOA on levels of paternal behavior

The effects of OTA microinjection into the MPOA on levels of paternal behaviors are presented in Figs. 6 and 7. Histological representations of the microinjection sites in the MPOA are displayed in Fig. 6A. One-way ANOVA revealed that OTA treatments significantly affected total duration of sniffing ($F_{2,17} = 4.198$, $P < 0.05$, $\eta^2 = 0.331$), licking and grooming ($F_{2,17} = 26.958$, $P < 0.01$, $\eta^2 = 0.760$), crouching ($F_{2,17} = 34.523$, $P < 0.01$, $\eta^2 = 0.802$) and inactivity ($F_{2,17} = 8.468$, $P < 0.01$, $\eta^2 = 0.499$); the latency to approach the pup ($F_{2,17} = 4.744$, $P < 0.05$, $\eta^2 = 0.358$) and latency to

take care of the pups ($F_{2,17} = 4.299$, $P < 0.05$, $\eta^2 = 0.336$) were also altered by OTA. Different doses of OTA and vehicle microinjections did not induce significant differences in retrieving ($F_{2,17} = 0.579$, $P > 0.05$, $\eta^2 = 0.064$) and walking ($F_{2,17} = 1.201$, $P > 0.05$, $\eta^2 = 0.124$). A post hoc test showed that OTA reduced the total duration of sniffing (10 ng OTA vs. vehicle, $P < 0.01$, $d = 1.379$), licking and grooming (1 ng OTA vs. vehicle, $P < 0.01$, $d = 2.383$; 10 ng OTA vs. vehicle, $P < 0.01$, $d = 3.671$), and crouching (1 ng OTA vs. vehicle, $P < 0.01$, $d = 2.977$; 10 ng OTA vs. vehicle, $P < 0.01$, $d = 4.034$); OTA also increased the duration of inactivity (1 ng OTA vs. vehicle, $P < 0.05$, $d = 1.337$; 10 ng OTA vs. vehicle, $P < 0.01$, $d = 3.041$) (Fig. 6B). Treatment with 10 ng OTA increased the latency to approach the pup (10 ng OTA vs. vehicle, $P < 0.05$, $d = 1.186$; 10 ng OTA vs. 1 ng OTA, $P < 0.05$, $d = 1.158$) (Fig. 7A) and the latency to take care of the pups (10 ng OTA vs. vehicle, $P < 0.05$, $d = 1.113$; 10 ng OTA vs. 1 ng OTA, $P < 0.05$, $d = 1.139$) (Fig. 7B), while 1 ng OTA did not produce effects on the latency to approach the pups (vehicle vs. 1 ng OTA, $P > 0.05$, $d = 0.167$) and the latency to take care of the pups (vehicle vs. 1 ng OTA, $P > 0.05$, $d = 0.057$), thereby displaying dose-dependent effects.

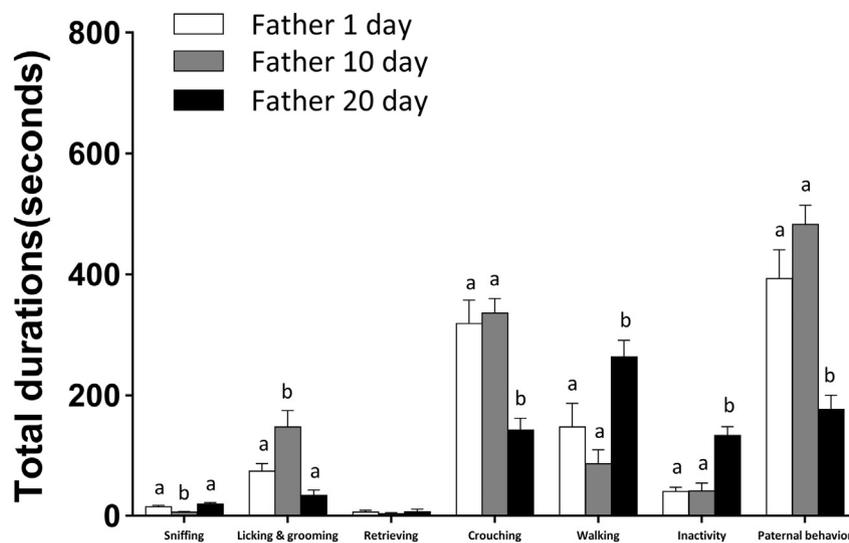


Fig. 2. Behavioral responses of male mandarin voles to their own pups over a 10-min test session. Fathers exposed to one-day-old pups ($n = 9$), 10-day-old pups ($n = 9$) and 20-day-old pups ($n = 9$) for 10 min. Groups not sharing the same letters are significantly different ($P < 0.05$). Data are presented as the mean \pm SE.

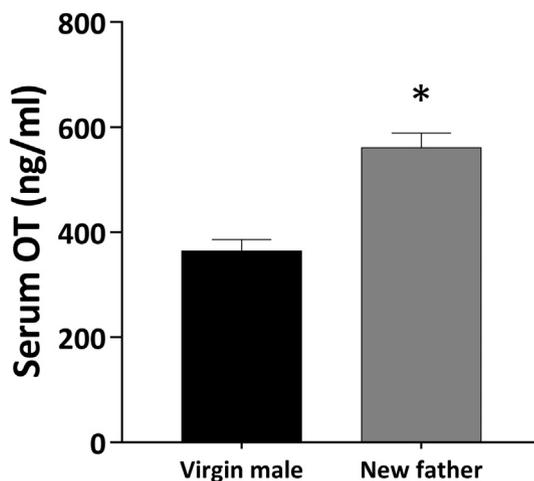


Fig. 3. Serum OT levels in virgin males and new fathers following exposure to pups. Virgin male ($n = 6$) and new father ($n = 6$). Data are presented as the mean \pm SE. Groups with * are significantly different ($P < 0.05$).

4. Discussion

The present study found that new fathers had a shorter latency to take care of pups and engaged in more paternal behavior than non-fathers (virgin males). New fathers had higher levels of OT in serum and higher levels of OTR mRNA and protein expression in the MPOA than virgin males. OTA microinjection into the MPOA reduced the levels of paternal behavior and increased the latency to approach the pup and initiate paternal behavior.

4.1. Behavioral response to pups in new fathers and virgin male

The new fathers showed a shorter latency to take care of the pups than the virgin males. In contrast, virgin males displayed more infanticide and negligence to pups, which is consistent with previous studies in California mice (de Jong et al., 2009; Gubernick and Laskin, 1994; Gubernick and Nelson, 1989). In addition, in the experiment 2, new father exposed to a 10-day-old unfamiliar pup before the brain collection also showed paternal behavior but the virgin male didn't show paternal behavior after exposed to a 10-day-old unfamiliar pup like exposure of virgin males to one day old pups. The possible reason

may be that virgin male encountered the pups first time at these two conditions and display more infanticide or negligence to unfamiliar pup whether they are one day old or ten days old. This result indicated that paternal care in the mandarin vole is not spontaneous and must be induced by mating or continuous exposure to pups. This hypothesis is consistent with previous findings that reproductive experience increased active paternal behaviors to pups (Wang et al., 2015; Wang et al., 2018a; Wang et al., 2018b). In female mice, continuous exposure to pups decreased the latency of retrieving the unfamiliar pup (Okabe et al., 2017). The previous study could explain that fathers display more paternal behavior toward unfamiliar pup than virgin males while both new fathers and virgin males were isolated for 1 h and exposed to an unfamiliar pup for 10 min. As expected, mandarin vole fathers showed different levels of paternal care, while pups were at different ages. New fathers spent less time taking care of newborn pups at postnatal day 1, had a shorter latency to approach pups and engaged in more paternal behavior when pups were 10 days old, and displayed less paternal care when pups were 20 days old. The same pattern was found in fathers taking care of the unfamiliar pups. This is because after 10 days of exposure, fathers were familiar with their pups, and 10-day-old pups have incomplete fur and need more warmth and protection from the parents. Thus, less paternal care to the 20-day-old pups may be associated with its complete fur and ability to move freely. Our data showed that the fathers had a significantly longer duration of licking and grooming the 10-day-old pups than the one- and 20-day-old pups, which is consistent with a previous study (Wang et al., 2018a; Wang et al., 2018b). Thus, continuous exposure to pups and fathering experience may alter levels of the relevant hormone and receptors (Wang et al., 2018b) and subsequently change their responses to a pup. As a consequence, new fathers display shorter latency to take care of unfamiliar pups than virgin males, and they also showed shorter latency to approach and take care of pups that were 10 days of age than pups that were one day old. The similar latencies to approach unfamiliar pups between virgin and new fathers is possibly because that one day fathering experience is not enough to inhibit fear to novel pups (Wang et al., 2018b). After approaching and sniffing the novel pups, new father begin to show paternal care that cause different latency to paternal behavior between new fathers and virgin males. Another interesting finding was that the latency to initiate paternal behavior on PND 1 and PND 10 is not different for an unfamiliar pup. The reason is possibly that fathers could recognized their own pups through sniffing and behave discriminatively toward their own and unfamiliar pups, similar to mothers (Gubernick and Klopfer, 1981; Holmes, 1990; Clutton-Brock,

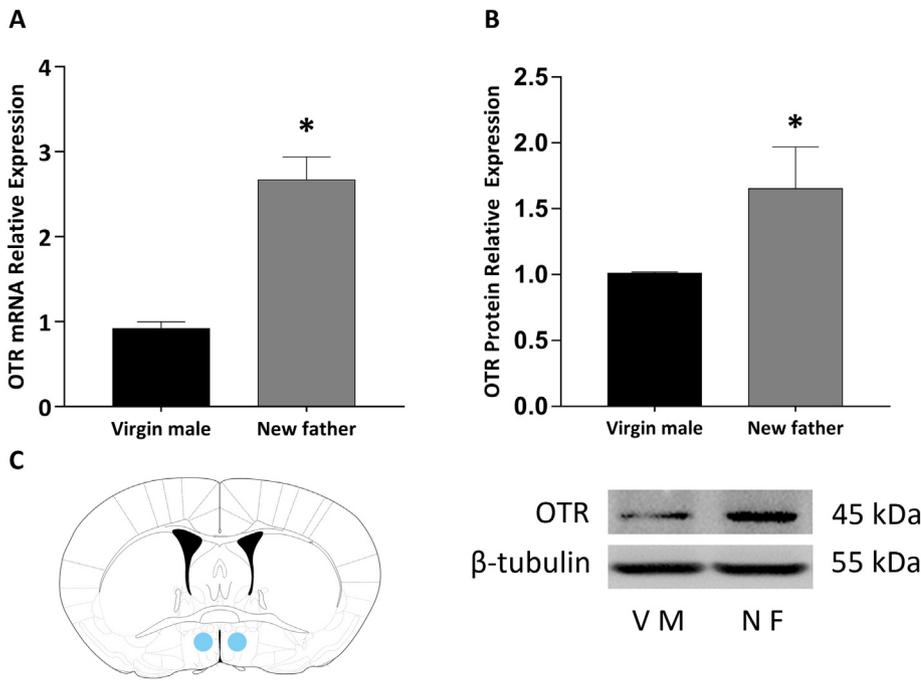


Fig. 4. OTR expression in the MPOA of virgin males and new fathers following exposure to pups. Virgin males (n = 5) and new fathers (n = 5). (A) OTR mRNA expression in the MPOA. (B) OTR protein expression in the MPOA. (C) Schematic diagrams show the location of the sampling site in the MPOA. Data are presented as the mean \pm SE. Groups with * are significantly different ($P < 0.05$).

1991). Different levels of paternal care to their own pups of 1 days age and 10 days age is possibly due to 10 days fathering experience to their own pups (Wang et al., 2018a).

4.2. Levels of OT, OTR, VIAR mRNA and protein expression in new fathers and virgin males

OT is currently known to reduce stress responses, facilitate parental and affiliative behavior (Bosch and Neumann, 2012; Ross and Young, 2009) and other functions, such as the formation of partner preference (Williams et al., 1994). Studies have demonstrated that OT facilitates the onset and maintenance of maternal behavior (Pedersen et al., 1994). These reports support our finding that the serum OT levels in new fathers were significantly higher than those in virgin males. In

humans, parent-infant contact has been associated with peripheral OT changes. This result is consistent with previous findings that plasma OT concentrations were positively correlated with levels of parental care in fathers and mothers, with higher levels of plasma OT concentrations associated with higher levels of parental care (Feldman et al., 2012; Li et al., 2015). Mothers who provided high levels of contact with the child showed an increase of OT, but such an increase was not found in mothers displaying minimal contact (Feldman et al., 2010). In addition, another study in primates demonstrated that OT administration to adult marmosets enhanced responsiveness to infants (Taylor and French, 2015). CD38 is a type II transmembrane protein that is strongly expressed in the hypothalamus and crucial for the release of OT from oxytocinergic neurons. CD38 knockout mice displayed no parental behavior, but subcutaneous injections of OT recovered this behavior

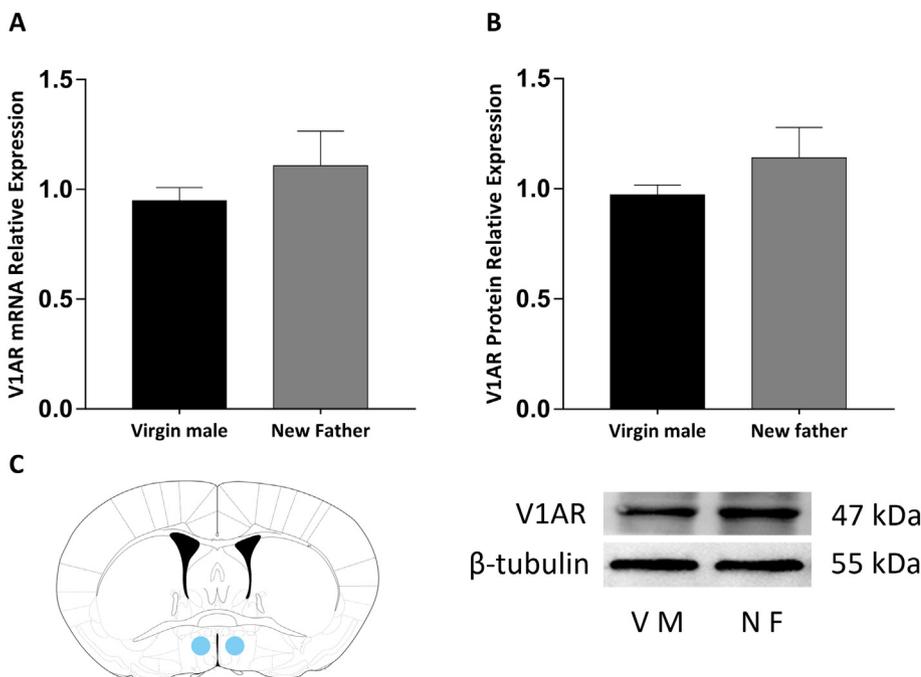


Fig. 5. VIAR expression in the MPOA of virgin males and fathers following exposure to pups. Virgin males (n = 5) and new fathers (n = 5). (A) VIAR mRNA expression in the MPOA. (B) VIAR protein expression in the MPOA. (C) Schematic diagrams showing the location of the sampling site in the MPOA. Data are presented as the mean \pm SE.

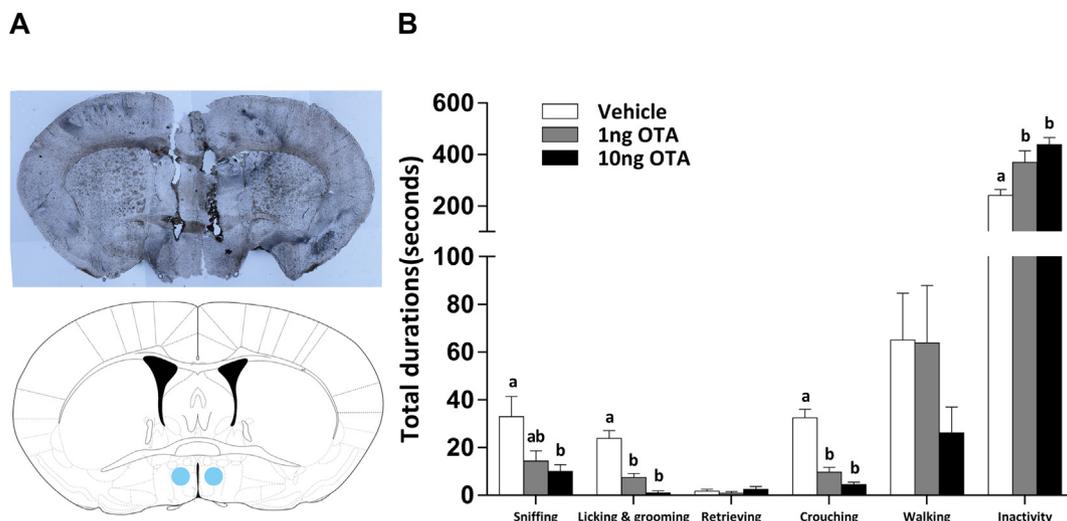


Fig. 6. Effects of the MPOA OTA administration on levels of paternal behavior. (A) Histological representations of the microinjection site. (B) Total duration of paternal behavior after vehicle (n = 7), 1 ng OTA (n = 7) and 10 ng OTA (n = 6) administration. Groups not sharing the same letters are significantly different (P < 0.05). Data are presented as the mean ± SE.

(Akther et al., 2013). Thus, high levels of paternal care in new fathers compared with virgin males may be associated with the high levels of OT obtained in the present study. However, in our study, we did not test the correlation of serum OT and paternal behavior in the virgin males and new fathers because the virgin male mandarin voles behaved aggressively and some even showed high levels of infanticide. Whether paternal behavior can be induced in virgin male mandarin voles after sensitization needs further investigate.

OT exerts its effects by combining OTR and V1AR (Donaldson and Young, 2008; Gimpl and Fahrenholz, 2001). Furthermore, we measured the levels of OTR mRNA and protein expression in the MPOA in the virgin males and new fathers. We found that the new father had higher levels of both OTR mRNA and protein expression in the MPOA than the virgin males. However, no significant difference was found in the expression of V1AR mRNA and protein in the MPOA. This result is similar to the finding in females that expression levels of OTR mRNA increased during parturition. In rats, the changes in OTR mRNA expression had contributed to the induction of oxytocin-dependent behaviors such as maternal behavior (Ostrowski, 1998). In addition, estrogen treatment results in an increase in OTR mRNA expression in the MPOA (Murakami, 2016). The dynamic changes in OTR expression in the MPOA may further mediate maternal behavior onset and establishment (Meddle et al., 2007). Although males do not undergo these physiological processes, we found that the expression of OTR mRNA in the

MPOA also increased after becoming a father, which is consistent with our previous studies showing that new fathers had more OT-IR neurons in the MPOA in mandarin voles (Wang et al., 2015). These results indicated that the increase in OTR in the MPOA in the new fathers relative to the virgin males may be associated with their increases in paternal care, which confirmed the finding of previous studies that oxytocin is central for the initiation of parental behaviors and a role for the MPOA in the transition to paternal behavior (de Jong et al., 2009). The expression level of V1AR in the MPOA did not show a significant difference between new fathers and virgin males, possibly because AVP regulates aggressive behavior in maternal behavior to protect the pup from foreign attack (Bosch, 2013). Whether the AVP system contributes to paternal behavior needs further study. Although the new fathers and virgin males were exposed to an unfamiliar pup for 10 min following 1 h isolation and treated similarly, the father had been exposed to their own pups continuously for 10 days. The 10 days fathering experience may also alter these neuroendocrine parameters as found in previous study (Wang et al., 2018b).

4.3. Effect of OTA on paternal behavior

Given the increase of OTR in the MPOA of new fathers, to further identify the involvement of OT in the MPOA in the regulation of paternal behavior, we investigated whether microinjections of OTA into

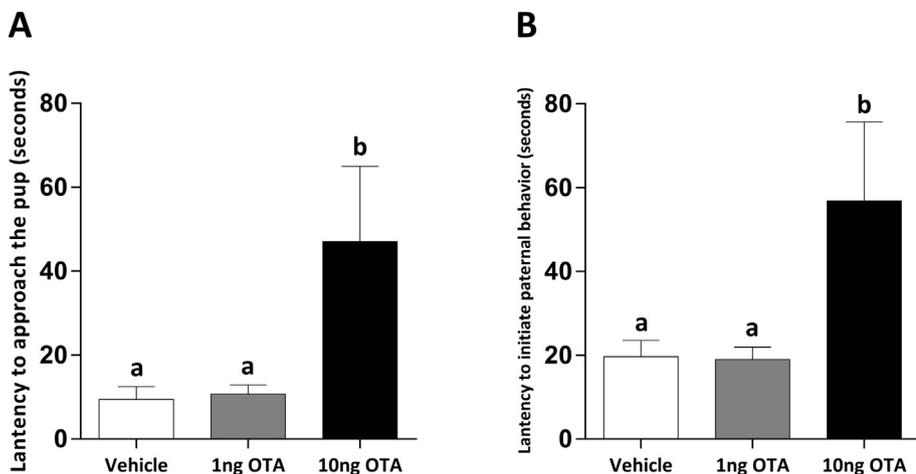


Fig. 7. (A) Latency of fathers to approach their own pups following vehicle (n = 7), 1 ng OTA (n = 7) or 10 ng OTA (n = 6) administration in the MPOA. (B) Latency of fathers to initiate paternal behavior affected by vehicle (n = 7), 1 ng OTA (n = 7) or 10 ng OTA (n = 6) administration in the MPOA. Groups not sharing the same letters are significantly different (P < 0.05). Data are presented as the mean ± SE.

the MPOA can reduce the levels of paternal care in new fathers. We tested the effects of two different doses of OTA on paternal behavior toward the fathers' own 10-day-old pups. The data indicated that 1 ng and 10 ng OTA microinjections into the MPOA reduced paternal behavior, such as licking and grooming, and crouching behaviors directed to their own pups. This result is in agreement with previous studies that cerebral ventricle (Bales et al., 2004) or MPOA OTA microinjection (Okabe et al., 2017) reduced maternal behavior in rodents. We found that both doses of OTA impaired paternal behavior, but no difference in effects between the two doses was found. In addition, we found that treatment with both doses of OTA did not influence the total duration of walking but significantly increased the total duration of inactivity. The reason is possibly that the OTA microinjection decreased paternal behavior by influencing the total duration of inactivity. Remarkably, we found that different doses of OTA had different effects on the latency to approach the pup and initiate paternal behavior. Compared to vehicle and 1 ng OTA, 10 ng OTA treatments significantly increased the latency to either approach the pup or initiate paternal behavior. This result is consistent with the study that OTA regulates social behavior in a dose-dependent manner (Yu et al., 2016).

A great number of studies have indicated that the MPOA and ventral bed nucleus of the stria terminalis (BNST) neurons project to the ventral tegmental area (VTA) (Numan and Numan, 1996; Numan and Numan, 1997; Simerly and Swanson, 1988), and these brain areas have strong interactions with the limbic dopamine system and regulate maternal responsiveness to pups (Numan and Stolzenberg, 2009). In rats, unilateral lesions of the MPOA combined with contralateral VTA impaired maternal behavior (Numan and Smith, 1984), and similar results were found when OTA bilateral infusions into the VTA influenced maternal behavior in rats (Pedersen et al., 1994). These reports suggest that OTR in the MPOA may interact with the dopamine system to regulate the motivation of males to take care of pups.

In conclusion, our study identified the involvement of the OT system in the MPOA in the regulation of paternal care.

Competing interest statement

The authors declare no conflict of interest.

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