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—Original Article—

Puerperal and parental experiences alter rat preferences for pup odors via changes in the oxytocin system

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Abstract. In the rat, induction of maternal behavior depends on the parity of the female. For example, nulliparous (NP) females need longer exposure to pups than multiparous (MP) or lactating (L) females to exhibit similar maternal behavior. In this study, we investigated the role of brain oxytocin in the approaching behavior of these female rats. Olfactory preferences for pup odors were examined for 8 consecutive days. Each preference test was followed by direct overnight exposure to pups. On the 8th day, MP and L, but not NP females showed robust pup-odor preferences. After the behavioral test, half of the females were exposed to pups for 2 h, whereas the other half were not. The females were then sacrificed to analyze brain oxytocin (OXT) and vasopressin (AVP) activities by cFos immunohistochemistry and to quantify their receptor mRNA expression using real-time PCR. In the paraventricular nucleus (PVN), the percentage of cFos-positive OXT neurons was significantly larger in MP and L females than in NP females after pup exposure. No significant differences were found in cFos expression in OXT neurons of the supraoptic nucleus (SON) or in AVP neurons of either the PVN or SON. Expression of OXT receptor mRNA in the medial preoptic area and amygdala of the control groups was also higher in MP females than in NP females. Finally, we demonstrated that infusion of OXT into the lateral ventricle of NP females promoted preferences for pup odors. These results indicate that puerperal and parental experiences enhance the responsiveness of OXT neurons in the PVN to pup stimuli and establish olfactory preferences for these odors in a parity-dependent manner.
Key words: Maternal behavior, Olfactory preference, Oxytocin, Paraventricular nucleus, Supraoptic nucleus, Vasopressin
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Oxytocin (OXT) is known to facilitate maternal behavior in the rat [1], sheep [2] and mouse [3, 4]. However, the first papers that described successful generation of OXT gene knockout mice reported that females deficient in OXT displayed normal parturition and maternal behavior [5, 6]. Recently, detailed research revealed that despite the lack of noticeable change, OXT knockout mice have significantly lower frequencies of pup licking and retrieve a smaller number of pups in inappropriate places of their cages [7]. Similarly, OXT antagonists (OTAs) lower frequencies of pup licking and grooming in rat dams [8, 9]. These findings suggest that OXT in the maternal brain supports the localization of pups retrieval or grooming.

When presented with pups, lactating and multiparous female rats start licking and retrieving immediately or after a short "sensitization" period [10–12]. The sensitization of nulliparous females to pup odors requires a longer period, possibly because pup odors have both repellent and enticing values for them. In support of this contention, the destruction of olfactory epithelia in nulliparous females facilitates the onset of maternal behavior [13]. Moreover, surgical removal of the olfactory bulb (OB) severely impairs maternal behavior in primiparous females but not in multiparous females, in which bilateral bulbectomy causes no deficits in maternal behavior [14]. The longer period of sensitization may be needed in nulliparous females to change the incentive value of pup odors from repellent to enticing [15].

Olfactory input, when combined with increased OXT, establishes maternal and pair bonding in many mammals [16, 17]. In the present study, we elucidated the roles of OXT in approaching behavior during sensitization to pups in nulliparous and multiparous rats. Based on our findings, we discuss a possible role for OXT in the change of motivational values of pup odors from repelling to enticing in dam-pup interactions.

Materials and Methods

Animals

Adult Long-Evans rats were purchased from the Institute for Animal Reproduction (Ibaraki, Japan). All animals were housed one per cage under a controlled temperature (23 ± 2 C) and reversed light/dark illumination (lights off from 1100 h to 2300 h) with free access to food and water. Behavioral tests were conducted under dim red light in the dark phase of the illumination cycle. The experiments

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all adhered to the Guidelines for the Care and Use of Laboratory Animals of Nippon Medical School and were approved by the Committee for Animal Experimentation of Nippon Medical School.

Except for the donor dams and nulliparous females, all female and male rats were used for mating. The female groups prepared for the experiments were (1) the nulliparous (NP) group, which contained sexually naïve rats; (2) the multiparous (MP) group, which contained cyclic rats with 2 parities, with the second pregnancy and parenting finished 2 weeks before the start of the experiments; and the lactating (L) group, which contained rats within 1 or 2 days after delivery and whose pups were removed at the start of the experiments. The ages of the females were matched among the three groups (approximately 6 months old). The stimulus pups used for sensitization were less than 7 days old. The cohorts of littermate pups were equally assigned to all the experimental groups as stimuli for preference tests and sensitization. The experimental females had 3 days of acclimation (30 min per day) to the preference-testing apparatus prior to starting behavior testing.

Behavioral tests

On each test day, the experimental females were given a 5-min period of acclimation to an apparatus followed by an olfactory preference test. The apparatus was an acrylic aquarium (50 (L) × 30 (W) × 40 (H) cm) equipped with two transparent containers (11 × 9 × 24 cm) that had 4 holes in them (l cm in diameter). One of the containers contained only fresh wood chips, and the others contained 3 stimulus pups with wood shavings. The time spent nose poking in each container was recorded during 5 min by an event recorder software.

Following the olfactory preference test, all experimental females were returned to their home cages (25 × 40 × 20 cm) and left alone for 5 min for acclimation followed by a maternal behavior test. For this test, three pups were introduced to each of their cages. Whether or not pup licking, retrieving and defensive burying occurred was recorded every 5 sec for 8 min. Following the maternal observations, the experimental females were allowed to stay with the pups overnight, and the pups were removed each morning (at 1000 h). The tests for preference and maternal behaviors were repeated daily for 8 consecutive days. Different pups were used every day to prevent starvation.

cFos experiment

After the behavioral tests on day 7, all experimental females in each group were randomly divided into 2 groups, the pup-exposure and control groups. As done on previous days, the females of the pup-exposure group were allowed to stay with the pups overnight, and they were tested the next day for olfactory preference and maternal behavior as done on day 8. Following the behavioral tests, the females stayed with the pups in their home cages (pup exposure) for 2 h. In contrast, the females of the control group stayed alone overnight in their home cages. The next day, the females were subjected to the same manipulations as the females in the pup-exposure group but without any pups.

After this, half of the animals were anesthetized with an overdose of sodium pentobarbital (> 80 mg/kg, *ip*) and perfused transcardially with 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by ice-cold 4% paraformaldehyde fixative buffered by 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, each brain was removed and postfixed in the same solution overnight and then placed in 30% sucrose solution buffered by PB. The brains were serially sliced into 25-μm coronal sections by cryostat. Every third section was stained with cresyl violet for determination of the hypothalamic nuclei, and the other two sections were processed for double immunohistochemistry of cFos with OXT or arginine vasopressin (AVP).

Immunohistochemistry

Free-floating coronal sections were incubated twice in 3% H_2O_2 in 20% methanol PBS for 15 min to remove endogenous peroxidase activities. The sections were then treated with 3% normal goat serum (NGS) in 0.25% Triton X-100/PBS (PBST) for 1 h and incubated with polyclonal anti-cFos antibody (rabbit polyclonal IgG, PC38; dilution ratio, 1:20000; Calbiochem, Gibbstown, NJ, USA) in PBST containing 3% NGS for 4 days at 4 C, followed by biotinylated anti-rabbit IgG antibody (goat polyclonal IgG, 1:200, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Then the sections were processed using an ABC Elite Kit (Vector Laboratories). Visualization of antibodies was performed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.25 mg/ml), 0.03% H_2O_2 and 0.25% $NiCl_2$ in PBS.

For double labeling, sections were retreated with 3% bovine serum albumin (BSA) in PBST for 1 h and incubated with polyclonal anti-OXT antibody (rabbit polyclonal IgG, PA1-18007; 1:60000; Affinity BioReagents, Golden, CO, USA) or anti-AVP antibody (rabbit polyclonal IgG, PA1-18008; 1:60000; Affinity BioReagents) in PBST containing 3% BSA for 2 days at 4 C. The sections were then incubated with biotinylated goat anti-rabbit IgG antibody (1:200) for 1 h at room temperature, and processed using an ABC Elite Kit. DAB (0.25 mg/ml) in 0.03% H_2O_2 in PBS was used as a chromogen but without $NiCl_2$. Finally, sections were mounted on coated slides and covered with coverslips for microscopic observation.

The paraventricular nucleus (PVN) and supraoptic nucleus (SON) were identified by cresyl violet staining with reference to the rat atlas [18], and the corresponding series of sections were analyzed. Areas (400 × 400 μm each) in the PVN and SON were counted bilaterally. Cells with nuclei densely stained with anti-cFos antibody were counted as cFos-immunoreactive (ir) neurons. Cells with densely stained somata with the anti-OXT or anti-AVP antibodies were counted as OXT-ir or AVP-ir neurons, respectively. The mean percentages of cFos-ir cells in the OXT-ir or AVP-ir neurons in the respective areas were determined.

Total RNA isolation and reverse transcription

Following the behavioral test, the other half of the females were decapitated under ether anesthesia, and their brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid. Each brain was blocked and sliced in serial coronal sections at a thickness of 300 μm by a vibratome to examine the medial preoptic area (POA) and the amygdala (Amy), which wholly included the posterodorsal part of the medial Amy. These regions were quickly dissected from the sections using micro knives. The OB was also dissected from serial sagittal sections at a thickness of 300 μm by a vibratome. The OB included the dorsal third of the main OB but excluded the

accessory OB. The tissues were stored in liquid nitrogen until use.

Total RNAs were extracted using RNAiso Plus (Takara Bio, Shiga, Japan) following the manufacturer-supplied instructions. Total RNA was treated with Turbo DNase (RNase-free DNase I, Ambion, Austin, TX, USA) to remove genomic DNA and then repurified. The reaction was carried out at 42 C for 60 min and stopped by heating at 75 C for 15 min. The cDNA was treated with RNase H (Takara Bio) and stored at –20 C until use.

Reverse transcription-PCR (RT-PCR)

The detailed procedures of RT-PCR and DNA sequencing are described elsewhere [19]. PCR was performed in 25 µl of PCR mixture containing 10 ng of cDNA. Shuttle PCR was performed over 22 cycles (for β-actin, Actb) or 40 cycles (for OXT receptors (OXTr) and AVP receptors (AVPr1a, AVPr1b, and AVPr2)). Initial denaturing was conducted at 95 C for 10 min, with repetitions of the annealing and elongation steps at 60 C for 35 sec and denaturing at 95 C for 15 sec, followed by a final elongation at 60 C for 5 min. The sequences of the oligonucleotide primers used in the PCR are shown in Table 1.

PCR products (5 µl) were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining under UV irradiation. The amplicons were cloned into pGEM-T Easy Vectors (Promega) and DNA sequenced to confirm their identities. The cloned plasmid vectors were used as standard curve templates for absolute quantification.

Quantitative PCR

Quantitative PCR (qPCR) was carried out using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a SYBR GreenER qPCR SuperMix (Life Technologies, Carlsbad, CA, USA). The qPCR was performed over 40 cycles: repeated cycles of 95 C for 15 sec and 60 C for 35 sec with an initial step of 50 C for 2 min and 95 C for 10 min. The primers for the housekeeping genes (*Gapdh*, *Actb*, *CypA* and *Tbp*) were designed with reference to Langnaese *et al.* [20]. The genes used for normalization were selected by stability analysis of the housekeeping genes (*Gapdh*, *Actb*, *CypA* and *Tbp*) using the NormFinder software [21].

Administration of exogenous OXT

Brain surgeries were carried out under ketamine HCl (25 mg/kg, *im*) and sodium pentobarbital (25 mg/kg, *ip*) anesthesia. Brain infusion cannulae (Alzet Brain Infusion Kit 2 3–5 mm, DURECT Corporation, Cupertino, CA, USA) were implanted into the lateral ventricles (anteroposterior –2.0 mm, mediolateral +1.5 mm, dorsoventral –0.8 mm from bregma) in nulliparous females, and the extracranial end of each cannula was then connected to an Alzet Mini-Osmotic Pump (model 2002, delivery rate 0.5 µl/h for 14 days) filled with either OXT (100 ng/h) or saline as a vehicle control. After 2 days of recovery, olfactory preference testing and maternal behavior testing were carried out for 8 consecutive days. On the last day (day 8), all animals were sacrificed by cardiac perfusion of fixative as described above following an overdose of sodium pentobarbital. To confirm the locations of the cannulae, the brains were sliced into 40-µm sections and stained with cresyl violet.

Statistical analyses

Differences between the experimental groups in their incidences of and latencies in exhibiting maternal behavior and defensive burying during the 8-day pup exposure were examined by Kaplan-Meier survival analysis with the log-rank test and chi-square test. Other statistical differences were examined using one-way analysis of variance (ANOVA) followed by Tukey's test, or two-way ANOVA followed by the *t*-test and/or Tukey's test, if the interaction was significant.

Results

Olfactory preferences and maternal behavior

Daily changes in the percentages of female rats that exhibited maternal behavior during the pup exposures were determined (Fig. 1A). All L females exhibited pup-licking or retrieving behavior throughout the 8 exposure days. No MP females showed these maternal behaviors on the first day. However, they began to gradually exhibit these behaviors, and almost all of them engaged in maternal behaviors after the 4th day. In contrast, no NP females displayed maternal behavior during the first 6 days. Then, out of the eight NP females, five on the 7th day and all on the 8th day exhibited maternal behavior. Kaplan-Meier survival analysis indicated that all differences among groups were significant ($\chi^2 = 33.08$, df = 2, $P < 0.001$). All of the NP females also showed defensive burying toward pups on the 1st and 2nd day of exposure, but ceased this behavior after the 3rd day of exposure, whereas no MP or L females exhibited defensive burying during the 8 days of pup exposure (Fig. 1B). Although Kaplan-Meier survival analysis failed to show significance among groups, the incidences of the first 2 days were significantly different between NP and MP females by chi-square test ($\chi^2 = 24.0$, $P < 0.01$).

To analyze odor preference for pups, the percent time spent nose poking toward a container with a pup out of the total time nose poking toward both containers was calculated (Fig. 1C). MP and L females exhibited high rates of nose poking toward the pup side of the container throughout the 8 days of exposure. In contrast, NP females initially showed high rates of nose poking toward pups, but this behavior gradually decreased. After the 5th day, their percent time spent nose poking in a pup container almost equaled that of nose poking an empty container, even though the NP females continued to display maternal behaviors on days 7 and 8. A two-way ANOVA with Tukey's post hoc test indicated that the percent time spent nose poking in a pup container was significantly lower in the NP females than the MP and L females after the 3rd day of testing (Fig. 1C; test days, $F_{(7, 238)} = 1.21$, *ns*; groups, $F_{(2, 238)} = 96.84$, $P < 0.001$, interaction; $F_{(14, 238)} = 4.84$, $P < 0.001$; group comparisons on test days 3–8, $F_{(2, 33)} = 6.30$, 14.53, 15.19, 25.17, 6.30 and 115.15, $P < 0.01$).

cFos expression in OXT and AVP neurons

To evaluate the activation of OXT and AVP neurons after pup stimulation, the expression of cFos was identified by double-staining immunohistochemistry (Fig. 2). The percentages of cFos-positive OXT neurons in the PVN were significantly increased by pup stimulation in the L and MP groups (Fig. 3A; main effects of parity, $F_{(2, 18)} = 6.83$, $P < 0.01$; pup exposure, $F_{(1, 18)} = 14.27$, $P < 0.01$; interaction, $F_{(2, 18)} = 4.36$, $P < 0.05$). Tukey's post hoc test indicated

Table 1. Primer sequences and amplicon lengths

Target gene	Direction	Sequence (5' to 3')	Product size [bp]
Gapdh	Forward	CAACTCCCTCAAGATTGTCAGCAA	118
	Reverse	GGCATGGACTGTGGTCATGA	
Actb	Forward	AAGTCCCTCACCCTCCCAAAAG	97
	Reverse	AAGCAATGCTGTCACCTTCCC	
CypA	Forward	TATCTGCACTGCCAAGACTGAGTG	126
	Reverse	CTTCTTGCTGGTCTTGCCATTCC	
Tbp	Forward	TGGGATTGTACCACAGCTCCA	131
	Reverse	CTCATGATGACTGCAGCAAACC	
OXTr	Forward	GGCTGCCGAGGGGAATGAC	222
	Reverse	ATGGCAATGATGAAGGCAGAAGC	
AVPr1a	Forward	GATCCGCACTGTGAAGATGACCT	149
	Reverse	GCAACGCCGTGATTGTGATGG	
AVPr1b	Forward	GGGATGAGAATGCCCCCAATGA	278
	Reverse	ACGGAGGCTGAGGTTAAGGCTGAGT	
AVPr2	Forward	CTTCCGGGAGATACACGCCAGTC	260
	Reverse	TAGCCAGCAGCATGAGCAACACAA	

The primers for the housekeeping genes (*Gapdh*, *Actb*, *CypA* and *Tbp*) were designed with reference to Langnaese *et al.* [20].

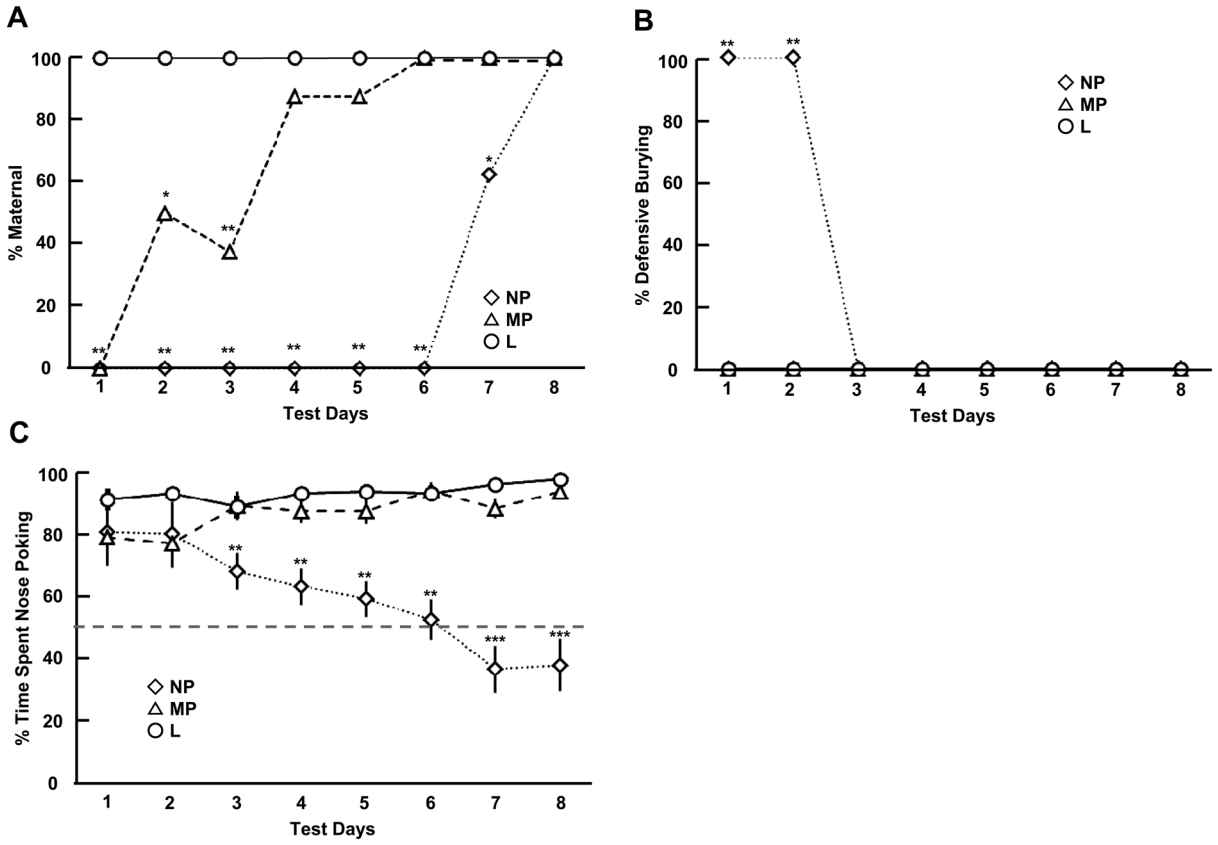

Fig. 1. (A, B) Percentage of the female rats that showed pup licking and/or retrieval (A) or defensive burying (B) in the 8-min maternal behavior tests conducted over 8 consecutive days (n = 8 in each group). The differences were statistically significant as assessed by Kaplan-Meier survival analysis ($P < 0.001$). In both panels A and B, significant differences on each test day were also indicated by the chi-square test (*$P < 0.05$; ** $P < 0.01$). (C) Percentage of time spent nose poking toward a container with a pup out of the total nose poking time (both containers with pups and empty containers). The horizontal dotted line represents equivalent access, that is, no preference (n = 12 in each group). Data are expressed as the mean ± SEM. Asterisks indicate statistically significant differences among the nulliparous, multiparous and lactating groups: ** $P < 0.01$; *** $P < 0.001$ (Tukey's test). Diamonds, NP (nulliparous); triangles, MP (multiparous); circles, L (lactating).

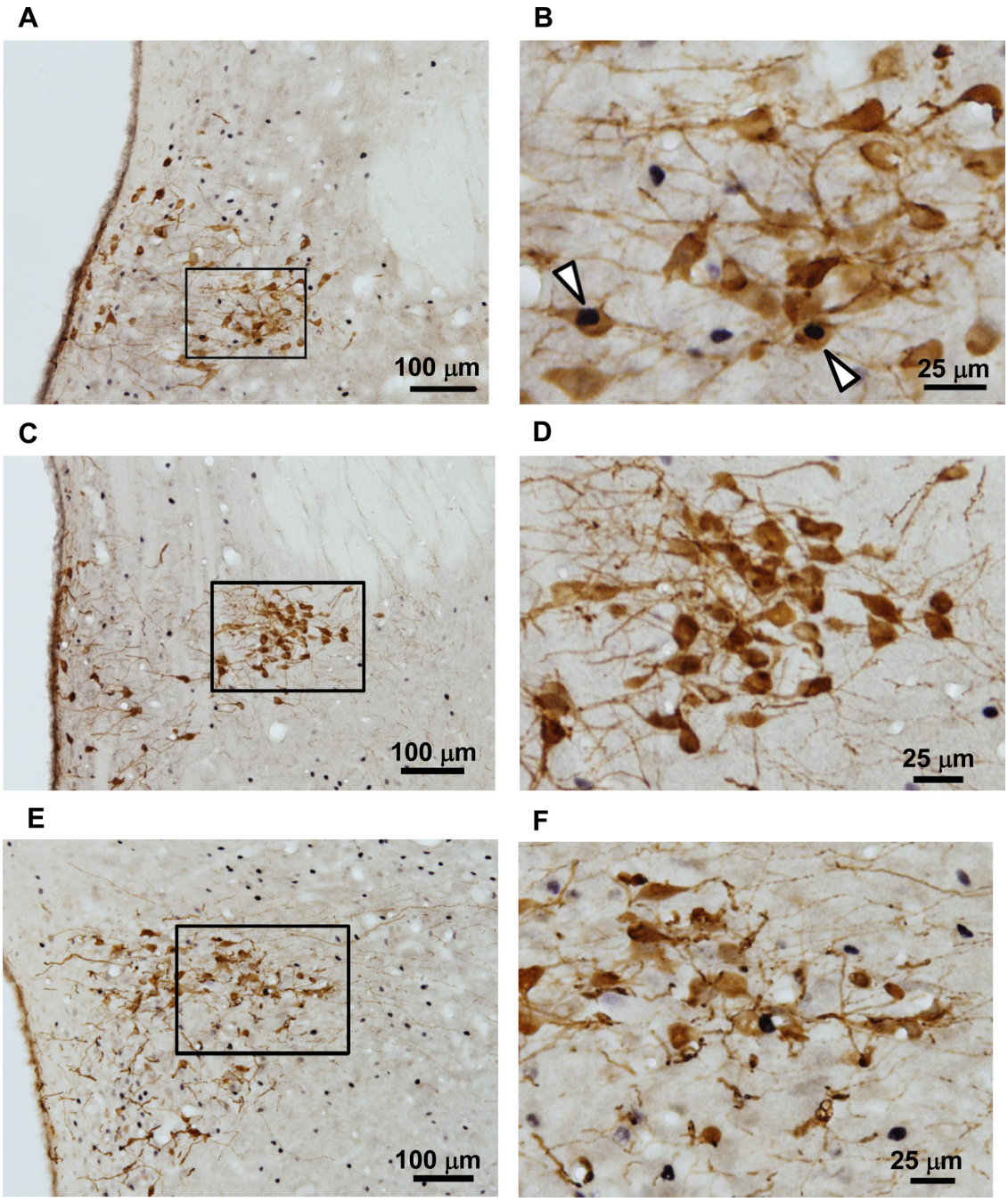

Fig. 2. Representative photomicrographs of cFos/OXT and cFos/AVP double-immunostained sections in the PVN. The right panels are higher magnification images of the regions outlined by rectangles in the left panels. (A, B) Lower (A) and higher (B) magnification images of the cFos (black by DAB with nickel intensification)- and OXT (brown by DAB)-immunostained section in L females after pup exposure. The open arrowheads indicate cFos postive OXT neurons. (C, D) Lower (C) and higher (D) magnification images of the cFos (black)- and OXT (brown)-immunostained section in NP females after pup exposure. (E, F) Lower (E) and higher (F) magnification images of the cFos (black)- and AVP (brown)-immunostained section in NP females without pups.

that these effects were attributable to differences of the NP group from the MP and L groups during pup exposure ($P < 0.01$). In contrast, activation of OXT neurons in the SON was not observed (Fig. 3B). Pup exposure did not alter cFos immunoreactivities of AVP neurons in the PVN or SON (Fig. 3C and D). The numbers of OXT and AVP neurons in the PVN and SON were not significantly changed by their experiences (Table 2).

Table 2. Numbers of OXT- and AVP-ir neurons

		Control			Pup exposure		
		NP (n = 4)	MP (n = 4)	L (n = 4)	NP (n = 4)	MP (n = 4)	L (n = 4)
OXT	PVN	86.75 ± 9.23	114.50 ± 5.58	81.50 ± 13.91	69.50 ± 22.94	77.75 ± 14.67	71.75 ± 7.59
	SON	58.25 ± 14.13	44.75 ± 4.71	44.50 ± 5.20	40.00 ± 4.78	50.75 ± 9.59	49.25 ± 2.29
AVP	PVN	50.25 ± 13.42	38.50 ± 7.79	81.00 ± 13.87	64.50 ± 13.11	66.50 ± 5.78	78.25 ± 15.56
	SON	48.75 ± 7.19	42.75 ± 17.78	38.75 ± 10.93	50.25 ± 6.57	49.50 ± 7.75	48.25 ± 3.45

The numbers of OXT- and AVP-ir neurons were bilaterally counted in the areas (400 μm × 400 μm each) of the PVN and SON regions. The data represent the mean ± SEM. The numbers of OXT- and AVP-ir neurons in the PVN and SON were not significantly changed by experiences or pup stimuli.

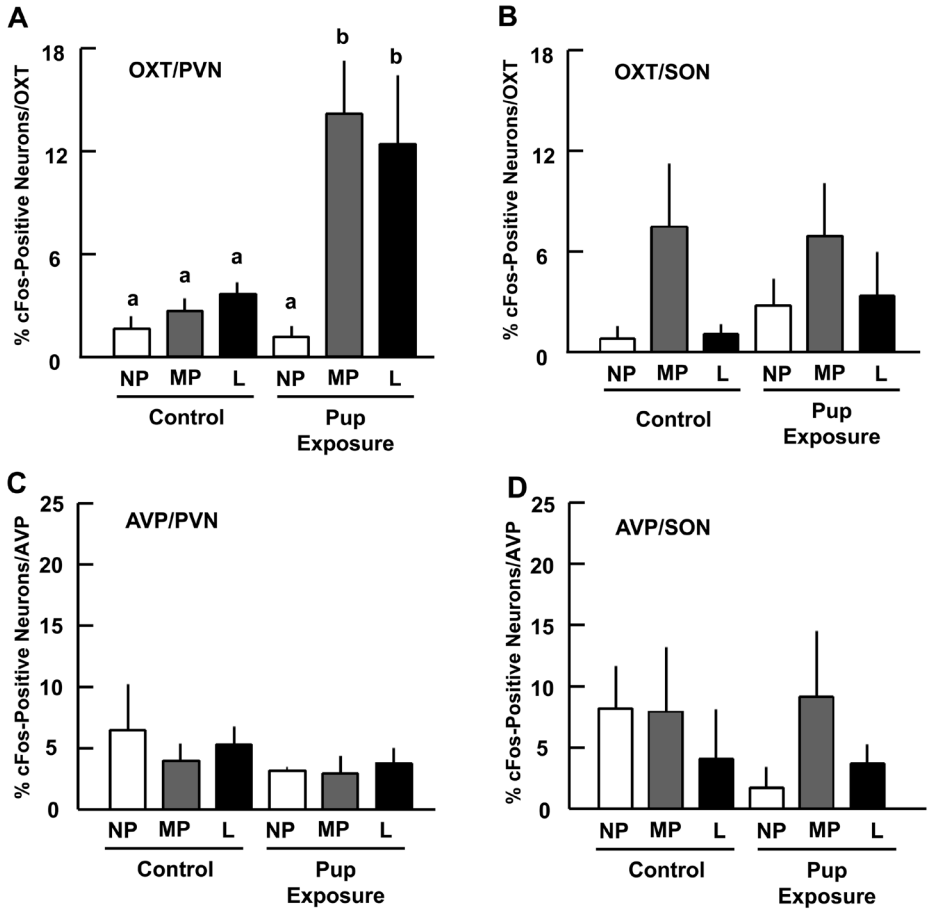

Fig. 3. Quantification of cFos-positive OXT and AVP neurons in female rats with or without pup exposure. (A, B) Percentage of cFos-positive OXT neurons in the PVN (A) and SON (B). (C, D) Percentage of cFos-positive AVP neurons in the PVN (C) and SON (D). Data are expressed as the mean ± SEM (n = 4). Columns indicated with different letters are statistically different from each other ($P < 0.01$, Tukey's test and *t*-test). White columns, NP (nulliparous); gray columns, MP (multiparous); black columns, L (lactating).

OXT and AVP receptor expression

Brain OXT and AVP systems play key roles in the regulation of social behaviors. Since both OXT and AVP can bind to their receptors with different affinities [22–24], we comprehensively quantified expression levels of the OXT and AVP receptor (OXTr, AVPr1a, AVPr1b, and AVPr2) mRNAs in the OB, POA and Amy, as these areas have been implicated in the processing of olfactory signals for maternal behavior [25]. As a preliminary experiment, we performed conventional RT-PCR to analyze the expression in these regions. Since specific amplicons from AVPr2 mRNAs were not detectable except in the reference tissue (the kidney), we excluded AVPr2 from the following quantitative analysis.

The expression levels of the OXTr, AVPr1a and AVPr1b mRNAs were assessed by absolute quantification (Fig. 4). The quantification

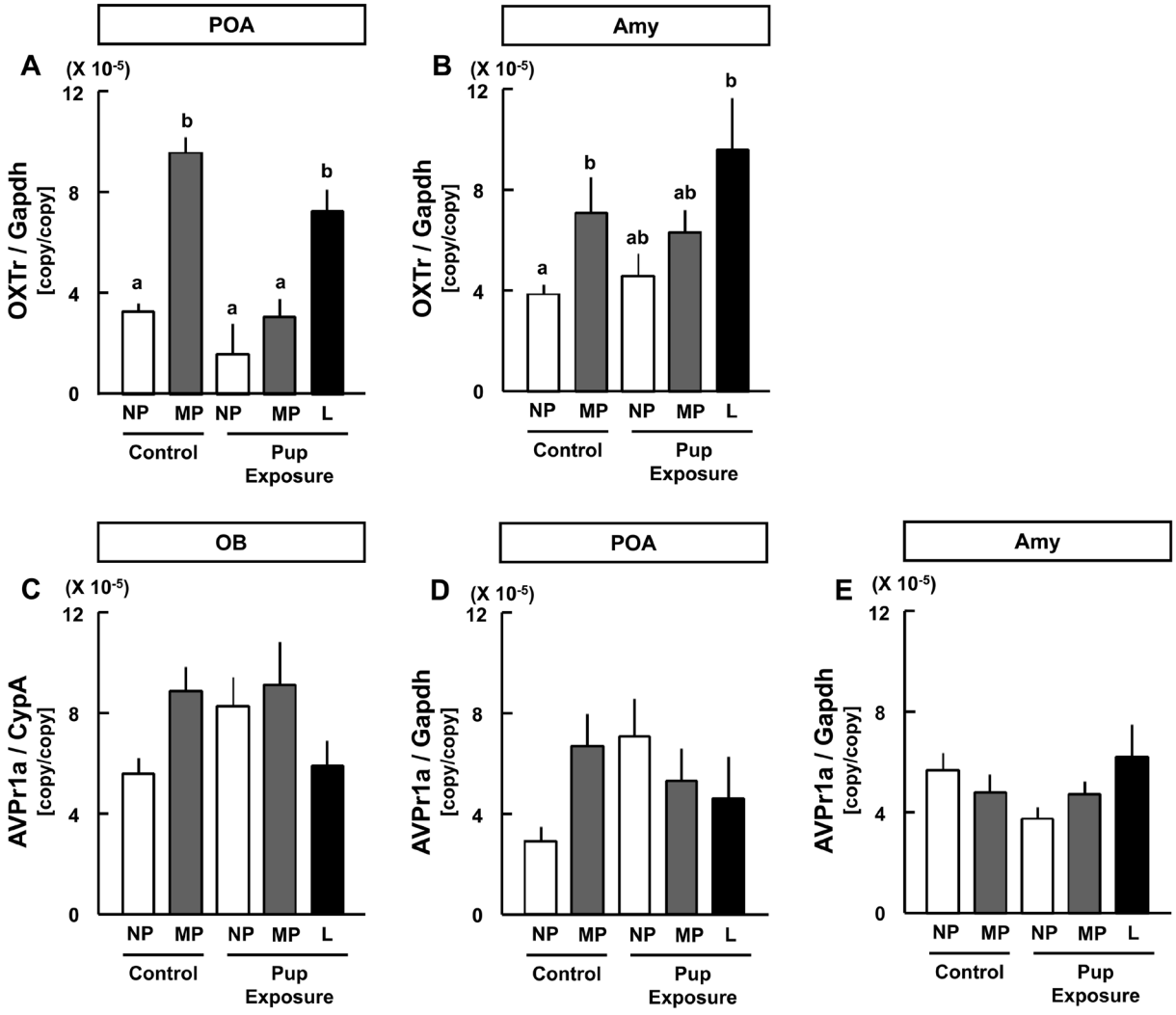

Fig. 4. Quantitative RT-PCR analysis of mRNAs encoding OXTr (A, B) and AVPr1a (C, D, E) with and without pup exposure in the OB, POA and Amy. The expression levels of OXTr mRNA were compared in the POA (A) and Amy (B), but not in the OB because it was below the limit of detection. The expression levels of AVPr1a mRNA were also measured in the OB (C), POA (D) and Amy (E). Mean ± SEM values were calculated based on values normalized with the housekeeping genes (n = 8 in each group). Columns indicated with different letters are statistically different from each other ($P < 0.05$, Tukey's test and *t*-test). White columns, NP (nulliparous); gray columns, MP (multiparous); black columns, L (lactating).

ranges determined from serial plasmid dilutions were from 10^2 to 10^8 copies in our qPCR procedure. Since the expression levels of OXTr in the OB and of AVPr1b in the OB, POA and Amy were below the detection limit, the values were excluded from the quantitative analysis. The housekeeping genes used for normalization were selected from four genes (Gapdh, Actb, CypA and Tbp) by estimation of stability values using NormFinder (Table 3). CypA was used as an internal control for the OB, and Gapdh was used as an internal control for the POA and Amy.

Subsequently, two-way ANOVA [parities (NP and MP) × stimulations (with and without pups)] was carried out in each brain region except in the L group. In the POA of the control group, the expression level of OXTr in MP rats was higher than that in NP rats, but there was no significant difference between the NP and MP rats after pup exposure (Fig. 4A; group, $F_{(1, 28)} = 19.07$, $P < 0.001$; pup stimulation, $F_{(1, 28)} = 16.99$, $P < 0.001$; interaction, $F_{(1, 28)} = 9.29$, $P < 0.01$; NP *vs*. MP rats of the control, $t = 4.76$, $df = 14$, $P < 0.001$). In the POA of the pup exposure group, the OXTr expression level of L rats was higher than those of NP and MP rats (Fig. 4A, $F_{(2, 21)} = 5.41$, $P < 0.05$ with Tukey's test, $P < 0.05$).

In the Amy of the control group, the OXTr expression level of the MP rats was higher than that of the NP rats (Fig. 5B, $t = 2.20$, $df = 14$, $P < 0.05$). However, there were no significant differences between control and pup exposure in the NP and MP rats (Fig. 4B; group, $F_{(1, 28)} = 5.46$, $P < 0.03$; pup exposure, $F_{(1, 28)} = 0.63$, $P > 0.05$; interaction, $F_{(1, 28)} = 0.13$, $P > 0.05$). In the Amy, the OXTr levels of the MP rats in the control group and the L rats in the pup-exposure group were higher than that of NP in control group (Fig. 4B, t =

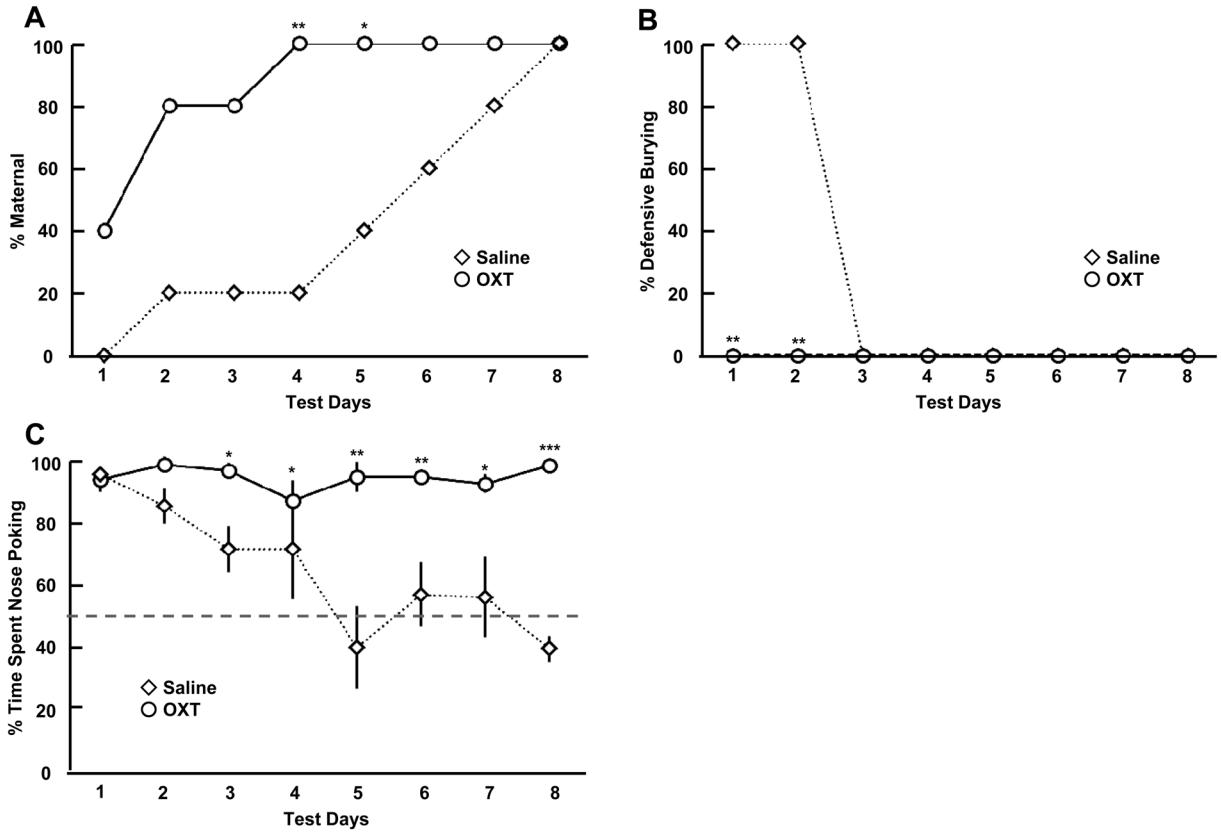

Fig. 5. Effects of OXT administration on maternal behaviors and olfactory preferences in NP female rats. (A, B) Percentage of the female rats that showed pup licking and/or retrieval (A) or defensive burying (B) in the 8-min maternal behavior tests conducted over 8 consecutive days (n = 5 in each group). The differences were statistically significant as assessed by Kaplan-Meier survival analysis (P < 0.05). In both panels A and B, significant differences on each test day were also indicated by the chi-square test (*P < 0.05; ** P < 0.01). (C) Percentage of time spent nose poking toward a container with pups out of the total nose poking time (both containers with and without pups). The horizontal dotted line represents equivalent access to pup and empty containers. Data are expressed as the mean ± SEM. Asterisks indicate statistically significant differences between saline- and OXT-injected groups: * P < 0.05; ** P < 0.01; *** P < 0.001 (*t*-test). Diamonds, saline injected; circles, OXT injected.

Table 3. Stability values of the housekeeping genes revealed by NormFinder

Gene name	OB	POA	Amy
Gapdh	0.134	0.182	0.210
Actb	0.151	0.288	0.254
CypA	0.119	0.220	0.248
Tbp	0.207	0.361	0.253
Best gene	*CypA*	*Gapdh*	*Gapdh*

2.20, *df* = 14, P < 0.05).

Although we measured the expression levels of AVPr1a in the OB, POA and Amy, no significant difference was detected in those data (Fig. 4C–E).

Effects of OXT administration on maternal behaviors and olfactory preferences

To determine whether OXT is involved in pup odor preferences, exogenous OXT was chronically injected into the lateral ventricle of NP females and behavioral changes were observed (Fig. 5). OXT injection significantly decreased the time required to exhibit maternal behaviors compared with vehicle injection (Fig. 5A). This treatment difference was significant as assessed by Kaplan-Meier survival analysis (χ^2 = 6.47, df = 1, P < 0.05). Defensive burying was also suppressed in OXT-injected females (Fig. 5B, χ^2 = 9.00, df = 1, P < 0.01). Nose poking in the pup-odor preference test is shown in Fig. 5C, using the same calculations as the data presented in Fig. 1C. OXT-injected females continued to exhibit strong preferences for pup odors throughout the 8 consecutive exposure days. ANOVA indicated a significant difference after the 3rd day (days 3–8, t = 3.27, 3.05, 3.86, 3.61, 2.77 and 13.68, df = 8, P < 0.05). Microscopic observation of Nissl-stained sections confirmed the localization of all cannula tips inside the lateral ventricle (data not shown).

Discussion

The present study demonstrated that NP female rats showed transient preferences for pup odors, whereas MP and L female rats showed persistent preferences. Pup stimuli have been reported to

contain not only positive but also negative components, which provoke aversion to pups [26, 27] or infanticide [27], especially in male and NP female rats. Anosmia caused by zinc sulfate lesions in the olfactory epithelium suppresses infanticide, indicating that the negative response to pups may require an intact olfactory epithelium [27, 28]. In addition, deafferentation of the vomeronasal nerve and/or olfactory bulb promoted maternal behavior in NP female rats [27].

In our olfactory-preference-test apparatus, the pup stimuli were presented through holes in pup containers so that experimental females could not directly contact the pups. Thus, the presented stimuli were airborne chemicals (and possibly ultrasonic vocalizations). Since the vomeronasal organ is known to process involatile chemicals, the experimental females could receive pup stimuli via the olfactory epithelium. Despite the negative components of the pup stimuli, the NP females showed frequent behavior directed at the pup container for the first 2 days. This may be because of the novelty of the stimulus, as the NP females were naïve to pups except during their own infantile periods. In the first two days, NP females may not be able to judge whether pup odor is harmless or harmful, and they may lose interest in pup odor after three days. Although anosmia caused by zinc sulfate lesions shortens the induction period for maternal behavior [28], the current results imply that the stimuli required for this induction are not airborne odors. A shortened induction period may occur as a result of indirect changes in the social interactions of anosmic females.

The MP and L females in our experiment exhibited maternal behavior immediately or after a short sensitization period and further displayed persistent preferences for pup odors. Although long sensitization to the pups induced maternal behavior and suppressed a defensive response to pups in the NP females, the NP females eventually lost their pup odor preferences. These results indicate that the development of pup odor preferences is mediated via processes independent of the induction of maternal behavior and suppression of defensive response and further suggest that activation of the processes requires pup stimuli and certain parity-associated events such as marked changes in circulating hormones during pregnancy and lactation.

Our immunohistochemical analysis revealed that pup stimuli activated OXT neurons in the PVN in a parity-dependent manner. The effects of pup exposure on OXT neurons in the PVN were well concordant with the effects of pup exposure on behavior in the olfactory preference test. Furthermore, quantitative PCR analysis demonstrated significant alteration of OXTr mRNA among three groups. There were no significant differences in the number of cFos-positive AVP neurons. Expression of AVPr1b or AVPr2 was scarcely detectable in the brain subregions tested. Although the AVPr1a gene was moderately expressed in the OB, POA and Amy, the expression levels of AVPr1a mRNA did not differ significantly among the three groups. Therefore, these results indicate that the OXT system may be involved in the acquisition of preferences for pup odors in the parous females. Intracranial treatment of OXT confirmed this. Administration of OXT evoked pup-oriented behavior as well as maternal behaviors, suggesting that activation of a distinct neural circuit is required for specific induction of olfactory preferences and that OXT neurons in the PVN are associated with the circuit. In a physiological condition, drastic changes in the OXT system during pregnancy, delivery, and maternal care may mediate acquisition of olfactory preferences.

Lesions in the PVN disrupt the acquisition of maternal behaviors [29], decrease pup retrieval [29, 30] and increase maternal aggression [31]. Since the PVN contains both OXT and AVP neurons, these effects may be compounded by dysfunction in OXT and AVP neurons. It has been reported that treatment of AVP facilitates maternal care [32], whereas administration of an AVPr1a antagonist decreases maternal care [33, 34]. Moreover, increased expression of AVPr1a in the POA by viral vectors augments maternal behavior, whereas decreased expression AVPr1a in the POA by antisense oligodeoxynucleotides suppresses maternal behavior [32]. These previous findings demonstrate the involvement of AVP in the neural regulation of maternal behavior, although our current study failed to reveal the different responsiveness of AVP neurons to pup stimuli by parity of the females. Accordingly, OXT and AVP neurons in the PVN may by differentially regulated by exposure to pup stimuli and play distinct functional roles in controlling maternal behavior.

It is well known that circulating OXT induces uterus contractions during periparturitional periods. Indeed, a cFos study showed increased activity of OXT and OXTr-positive neurons in the PVN during parturition [35], suggesting that the activity of brain OXT systems is also elevated. Studies in mice genetically void of the OXT gene reported that OXT is not essential for parturition and maternal behavior [5, 36], although deletion of the OXT gene affects various components of maternal behavior [7, 37].

Intracranial administration of OXT can induce maternal behavior in virgin females, although priming with estrogen is also required [38–41]. Conversely, intracranial OTAs treatment decreases pup licking and nursing postures over pups, increases lying prone on pups [42], and delays pup retrieval [8]. The effects of brain OXT have been shown to be region specific in observations of maternal aggression in the resident-intruder test. Specifically, OXT infused into the central nucleus of the Amy reduces the frequencies of biting and frontal attacks, but OXT infused into the bed nucleus of the stria terminalis decreases only biting. Moreover, OXT infused into either area exerts no influence on pup retrieval [43]. In contrast, infusion of an OTA into the POA or the ventral tegmental area (VTA) reduces the frequency of pup retrieval and nursing postures [44, 45]. Since high-licking mothers have been reported to have denser projections from the PVN to the VTA and a higher dopamine level in the nucleus accumbens than low-licking mothers, OXT in the VTA may modulate the dopamine system to enhance maternal motivation.

A considerable body of evidence indicates that OXT is involved in various olfactory behaviors. Infusion of an OTA blocks preferences of rat pups for odors associated with their dams [46]. Local application of OTA in the OB suppresses all components of maternal behavior, whereas OXT injections in the OB induce a full range of maternal behaviors in ovariectomized virgin rats primed with estrogen [47]. Furthermore, electrophysiological studies of the accessory OB demonstrate that OXT increases spontaneous glutamatergic excitatory postsynaptic currents in granular cells [48] and diminishes $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents in mitral cells [49]. Since loss of memory for the odors of conspecifics has been reported in mice deficient in the OXT gene [50], OXT may be involved in both maternal behavior and general social behavior.

Our quantification of the OXTr and AVPr mRNAs in the OB, POA and Amy demonstrated that their expression is drastically changed in parity- and stimuli-dependent manners. These differences may also contribute to differences in behaviors. At present, however, we do not know the functional relevance of these changes, and further elucidation is required. Although OXTr expression varies across the different brain regions [51, 52], increase of OXTr expression levels after parturition is not essential for the onset of maternal behavior [53]. On the other hand, our results indicate that OXTr expression can be changed within a few hours by pup exposure, suggesting the participation of OXTr in the quick sensitization to pups in MP females.

In conclusion, the present study indicates that puerperal and parental experiences enhance the responsiveness of OXT neurons in the PVN to pup stimuli and establish olfactory preferences for these odors in a parity-dependent manner. In addition, the results of the behavior tests suggest that the mechanisms underlying acquisition of the olfactory preference are different from those inducing maternal behaviors. We believe that these findings provide new insights into the OXT-mediated processes by which maternity is induced in females.

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