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Oxytocin mediates the acquisition of filial, odor-guided huddling for maternally-associated odor in preweanling rats

Sayuri Kojima and Jeffrey R. Alberts

Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN 47405.

Sayuri Kojima: skojima@indiana.edu; Jeffrey R. Alberts: alberts@indiana.edu

Abstract

The present study was designed to examine possible roles of oxytocin (OT) in the acquisition of a filial huddling preference in preweanling rats. We used a procedure in which a scented, foster mother can induce an odor-guided huddling preference in preweanling pups, following a single, 2-h-long co-habitation (Kojima & Alberts, 2009, 2011). This single, discrete period for preference learning enables us to observe the mother-pup interactions that establish the pups' preferences and to intervene with experimental manipulations. Four, 14-day-old littermates interacted with a scented foster mother that provided maternal care during a 2-h session. Two of the pups were pretreated with an intracerebroventricular injection of OT or an oxytocin antagonist (OTA), and the others received a vehicle injection. Filial preference for a maternally-paired odor was measured in a huddling test the next day. OT is necessary for acquisition of the filial preference: Odor learning was blocked in the pups treated with OTA, but not in their vehicle-treated littermates who experienced the same mother at the same time. Injection with exogenous OT did not augment the pups' preference. Manipulating pups' central OT also altered the contact interactions of the mother and pups. When some pups received OT, mother-litter aggregations formed as frequently and with similar combinations of bodies, but contact aggregations were significantly more cohesive than when some pups in the litter received OTA. We discuss dual, behavioral and neuroendocrine roles of OT in social learning by preweanling rats.

Keywords

oxytocin; affiliative behavior; huddling; attachment; maternal care; associative learning; skin-to-skin contact; touch; rat pup

Introduction

Maternal care, though species-typical in form and function, nevertheless varies in quantity and quality when it is expressed to offspring. The offspring's development can reflect such variation, as seen in their exploratory behavior (Francis et al., 1999), emotionality (Denenberg et al., 1969; Paschke et al., 1971; Rosenberg et al., 1970), sexual behavior (Moore et al., 1996; Fillion and Blass, 1986) and maternal behavior (Boccia and Pedersen,

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Corresponding author: Sayuri Kojima. Department of Psychological and Brain Sciences, Indiana University. 1101 E. 10th Street, Bloomington, IN 47405. Office phone number: (812) 855-3309, Fax number: (812) 855-2100.

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2001; Shah et al., 2002). Some of these maternal effects can even be transmitted across generations (Fleming et al., 2002; Francis et al., 1999). It has been speculated that maternal stimulation acts on offspring social behaviors through the actions of oxytocin (OT) (Melo et al., 2006), a nonapeptide neurohormone known to affect social behaviors and emotions (Insel and Young, 2001), including social recognition (Engelmann et al., 1998; Ferguson et al., 2001; Ferguson et al., 2000; Popik and van Ree, 1998), pair bonding (Carter, 1998; Young and Wang, 2004), and maternal behavior (Fahrbach et al., 1984; Keverne and Kendrick, 1992; Pedersen, 1997; Pedersen and Boccia, 2003) in rodents and ungulates, as well as love (Bartels and Zeki, 2004), trust (Kosfeld et al., 2005) and fear (Kirsch et al., 2005) in humans. The expression of OT receptors in adult rodents is associated with maternal care received (Francis et al., 2000; Francis et al., 2002), and disrupted maternal behavior alters levels of OT receptors in pups (Noonan et al., 1994).

To date, most studies of OT effects on social behavior have been with adult animals, leaving relatively unexplored the OT mediation of infant behaviors, including early attachment. Likewise, little is known about OT's roles in the developing brain, but the likelihood of its involvement is suggested by knowledge that manipulating OT early in life induces variations in adult social behaviors, including parental behavior, partner preferences, and inter-male aggression (Carter, 2003). The few data available concerning OT in infants implicate it in early olfactory learning of social stimuli (Nelson and Panksepp, 1996) and in aggregative behavior in rat pups (O'Dya et al., 2002, see Alberts, 2007). Reportedly, OT also affects ultrasonic vocalization in rodent pups, albeit with contrasting results. Exogenous OT reduces ultrasonic vocalization in rat pups (Insel and Winslow, 1991), whereas OT-knockout mouse pups have been found to emit fewer vocalizations (Winslow et al., 2000). With increasing interest in the possible roles of OT in neurodevelopmental disorders such as autism (e.g., Carter, 2007; Hollander et al., 2003; Insel et al., 1999; Lim et al., 2005; Whitaker-Azmitia, 2005), there is a clear need for developmental research in this area.

The present study was designed to examine possible roles of OT in early, filial odor-guided huddling preference, a species-typical affiliative behavior displayed by Norway rats (*Rattus norvegicus*) (Alberts and Brunjes, 1978). It is well established that a rat pup's filial huddling preference is acquired by postnatal day (PD) 15 through experiences and interactions with a mother (cf. Alberts, 2007). If exposed daily for the first 14 postnatal days to an arbitrary odor borne on the ventrum of a mother that regularly provides maternal care, whether its own mother or a foster mother, pups acquire and express a huddling preference for a target bearing the maternally-paired odor (Brunjes and Alberts, 1979; Alberts and May, 1984). This huddling preference can be acquired even with a single, short period of interaction with a mother (e.g., 2 h), indicating that maternal care can serve as a potent, unconditioned stimulus (UCS) for the pups' acquisition of a preference for a maternal odor as an olfactory conditioned stimulus (CS) for huddling (Kojima and Alberts, 2009, 2011).

In the present study, we tested whether the offspring OT system is actively involved in the acquisition of filial huddling preference in 14-day-old pups. To accomplish this, we employed our single-session odor conditioning procedure (Kojima and Alberts, 2009, 2011) and manipulated central OT via intracerebroventricular (ICV) injection. We also recorded the behavior of mother and pups during the 2-h odor conditioning session for subsequent analyses of how the OT manipulation affected their social interactions in the litter. Previously, we reported that mother's hovering over pups is positively associated with the strength of a pup's huddling preference for a maternally-paired odor (Kojima and Alberts, 2009). Indeed, the thermotactile component of contact received by a 14-day-old pup, via skin-to-skin contact with the mother, is critical for the acquisition of the pups' preference (Kojima and Alberts, 2011). Despite the demonstrable reward value of suckling stimulation and milk transfer in some settings, (e.g., Brake, 1981; Kenny and Blass, 1977; Amsel et al.,

1976), suckling and milk are not necessary for learning a filial huddling preference, nor do they add to this kind of the learned preference (Alberts and May, 1984; Kojima and Alberts, 2009, 2011). Similarly, we found no evidence that maternal licking/grooming or tactile stimulation from stroking contribute to the acquisition of filial huddling preference in 14-day-old pups (Kojima and Alberts, 2009, 2011), although stroking a pup's body with a brush can facilitate olfactory learning in rats aged 9-days and younger (e.g., Moriceau and Sullivan, 2005; Sullivan and Wilson, 1994; Woo and Leon, 1987) due to assumed commonalities between the brush strokes and maternal licking/grooming. Thus, it was important to ask whether and how OT manipulations modify the social interactions among mother and pups, as we expected to find effects consistent with our previous findings.

Here, we report that OT is necessary for the acquisition of pup's huddling preference for a maternally-paired odor. Moreover, manipulating central OT in two pups within a litter of four, altered mother-pup social interactions, specifically changing contact cohesion in the whole litter. Overall, the present study shows that a pup's filial huddling preference for an odor associated with mother is established through OT involvement in both neuroendocrine function and behavioral mechanisms.

Method

Animals

A total of 112, 14-day old rat pups from 28 litters served as subjects. In addition, 28 lactating foster mothers, matched by postpartum age to the subject pups' mother, were used for odor conditioning. All animals were derived from Sprague-Dawley stock originally purchased from Taconic Farms (Germantown, NY) and bred in the Animal Behavior Laboratory colony at Indiana University. Litters were born and reared in standard maternity cages (48 × 20 × 26 cm) with food and water available ad libitum. The vivarium was maintained on 12:12 h light/dark cycle (lights on at 0700 h) at 22.0 °C ± 2 °C. Litters were reduced to 8 pups (4 males and 4 females) on PD 1 (day of birth = PD 0). A needle-punch (30-gauge) implement (Ketchum Manufacturing Inc., Ottawa, Canada) was used to tattoo the ventral aspect of the paw(s) by PD 7 or ears after PD 7 for individual identification. Animal care and experimentation were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Bloomington Institutional Animal Care and Use Committee at Indiana University.

Treatment

Prior to a 2-h pairing with a scented foster mother, pups received an ICV treatment (see below) with OT, an oxytocin antagonist (OTA), or vehicle only. Separate litters were used for OT and OTA treatments, and within-litter vehicle controls were run with each treatment. This design was used to assess effects of OT and OTA on pup's acquisition of filial odor preference, while avoiding possible confounding effects that each treatment could exert on interactions with the foster mother. Thus, a pair of male and female pups from a single litter received OT or saline (SAL), and these four littermates comprised a group designated as an OT litter. Likewise, two male-female pairs from another litter were assigned to OTA or SAL, and these four pups formed an OTA litter. Thus, we used 4 pups per litter, and a total of 14 litters were designated as OT or OTA litter groups.

ICV Administration

A pup was anesthetized in a transparent compartment through which flowed a controlled mixture of air and isoflurane (0.3%; Butler Animal Health Supply, Dublin, OH). When the pup was immobile with slow respiration, it was removed and placed in a nose cone that continued to deliver the anesthetic mixture. At this point, it was determined that the pup was

unresponsive to vibrissal stimulation, tail pinch, and touch with a syringe needle. Under this deep level of anesthesia, the pup received an ICV injection into a lateral ventricle with either 250 ng of OT, 25 ng of (d(CH₂)₅¹, Tyr(Me)², Thr⁴, Orn⁸, des-Gly-NH₂⁹)-Vasotocin as OTA (Bachem, San Carlos, CA) in 5 µl saline, or vehicle only. Dosage was based on a previous study with infant rats; we used doses that produce changes in aggregative behavior in 10-day-old rats (Ody et al., 2002, see Alberts, 2007). Because its affinity for receptor binding is much greater than that of natural ligand, lower doses of OTA than an effective OT dose can affect social behavior (Cho et al., 1999). The injection was accomplished by stabilizing the pup's head and injecting through the uncalcified skull into a lateral ventricle with a 27-gauge needle fitted to a 10-µl Hamilton syringe, using anterior, posterior, and lateral landmarks guided needle insertion, similarly to a previous study (Insel and Winslow, 1991). A teflon collar positioned 4 mm from the tip of the 27 gauge needle controlled depth. Injecting into the ventricle was confirmed visually in sacrificed pups by using 10% India ink of the injected solution for post-test verification of injection site.

Odor conditioning

We followed the procedure for odor conditioning used previously (Kojima and Alberts, 2009, 2011), but here added the ICV administration. On PD 14, 1 h prior to the conditioning session, pups were removed from their respective home cages and placed in a standard mouse cage (30 × 13 × 19 cm) with fresh bedding. At the same time, a foster mother was also removed from her home cage and placed for 1 h in the conditioning arena, which was a clean, standard maternity cage (48 × 20 × 26 cm) with fresh bedding and water. Body weight was not measured as a precaution to minimize sensory stimulation, which can potentially affect OT levels (Stock and Uvnas-Moberg, 1988). Nevertheless, this standardized procedure reliably induced pups' body weight losses in previous studies, demonstrating that no milk transfer occurred during the odor conditioning (Kojima & Alberts, 2009, 2011).

During the last 25 min of the separation, pups received their assigned ICV treatment, after which they were marked with a non-toxic pen on the back for identification during behavioral observation, and returned to the cage with their siblings for a recovery period (approximately 15 min) until the end of 1-h separation. During the separation, 400 µl of one of the two test odors (lemon and orange extracts: McCormick & Company, Inc., Sparks, MD) was applied with a pipette to the ventral midline of the foster mother. Conditioned odors were counter-balanced across litters.

After the separation, the pups were placed with the scented foster mother for 2 h, a period sufficient for a foster mother and pups to interact repeatedly, during which time pups would experience the odor CS in combination with receipt of maternal stimulation. After the conditioning session, the pups and foster mother were returned to their respective home cage. All odor conditioning sessions were conducted during the light phase. The conditioning arenas were sanitized in a cage washer after each session.

Huddling preference test

On PD 15, subject pups received a huddling preference test. The apparatus and testing procedure were those used previously (Kojima and Alberts, 2009, 2011). A test arena was a plastic cylinder (15 cm diameter, 12.5cm high) with two rectangular windows 180° apart at the base of the cylinder. Through the window, a swatch of acrylic fur wrapped around a plastic pipe (2 cm diameter, 10cm long) held in place by springs from the outside of the test chamber was accessible to the pups. Prior to the test, 200 µl of a conditioned odor (e.g., lemon) was sprayed on the fur in one side, and 200 µl of a novel odor (e.g., orange) was applied on the fur of the other side. Tests were conducted at room temperature (22.0 °C ± 2

°C) for 4 h after a 25 min-acclimation period, during which pups typically sampled the test odors by sniffing. A video camera was positioned above the test arena to capture behaviors through a wire-mesh lid. After the test, pups were euthanized by CO₂ inhalation and decapitated for verification of injection site. The test arenas were sanitized in a cage washer after each test.

Data collection

During the preference test and odor conditioning, behavior was recorded through a video camera positioned above the test and conditioning arenas, respectively, and connected to a time-lapse recorder set to 12:1 record:playback ratio. We measured huddling as physical contact between the pup's body (tail excepted) and the scented fur lasting at least 30 sec, thus excluding briefer events that might involve physical contact (e.g., sniffing investigation). Video scoring was performed by experienced observers, following training trials with randomly chosen segments of behavioral data with a custom computer program for Mac to monitor behavioral events. Inter- and intra-rater reliabilities were measured at rs 0.90 and 0.95, respectively.

To examine OT effects on the interactions between mother and pups during the odor conditioning, we obtained three types of behavioral data: (1) contact patterns of the mother and litter, (2) contact cohesion of the mother and litter, and (3) individual pup contacts with the mother or with another pup. Thus, there were two measures of group behavior and one measure of individual behavior. Most behavioral observations were accomplished using QuickTime player for Mac. An additional method, described below, was used for contact cohesion.

Contact patterns were captured and encoded once per minute during odor conditioning, yielding 120 observations during the 2-h conditioning session. We adapted a previously devised method for viewing a group of individuals as patterns of aggregation, or *aggregons* (Schank and Alberts, 1997). A group of five individuals (i.e., the mother and four pups in the present study) can form seven contact patterns. The righthand section of Figure 2 displays each of the contact patterns or aggregons, ordered hierarchically by the number of aggregon elements, describing the seven contact patterns with the number of aggregon elements and the number of individuals contained in each aggregon element. Each aggregon in the hierarchic order was indexed with a number, so that the higher aggregon index described the greater contact unity with the fewer aggregon elements. The lefthand section of Figure 2 illustrates four examples from the seven contact patterns. Note that the mother is treated as an equal element, so that the five individuals are always arranged in a combination that can be defined by one of the seven contact patterns, identified by aggregon index. With this categorization method, group structure and group dynamics can be expressed in terms of contact pattern and aggregon frequency.

Contact cohesion was measured from digitalized video frames, captured automatically every minute using MATLAB program (The MathWorks, Inc., Natick, MA), yielding 120 snapshots. In those snapshots, the total aggregon area (cm²) subtended by mother and pups of litter was measured using ImageJ program (National Institutes of Health, Bethesda, MD). Aggregon area was determined by a line encompassing the bodies of animals in contact except tail and paws (i.e., body parts covered by fur). If an animal was not in contact with any other, the area of its body was measured as a separate aggregon element, and the sum of the measured areas determined the aggregon area for each aggregon snapshot. The mother's participation in contact was also recorded with 'yes' or 'no' for every snapshot.

Individual contacts (pup-mother and pup-pup contacts) were observed for each minute in the digitalized videos during the 2-h odor conditioning session. Data were collected so that the

mother's and pups' locations in each contact pattern were known and could be analyzed in terms of the identified individual or individuals with which each pup was in contact. When pups were hidden under the mother's body, the existence of contact among pups was sought by checking pup's positions before and after mother's body obscured them. If these efforts could not confirm the pup positions under mother's body, contact was counted for those pups.

Statistical Analysis

Due to unsuccessful ICV injections and technical failures, 12 pups or 6 litters were excluded in the analyses of huddling preference test (huddling preference and contact time with the test odor) and group contacts (contact patterns and contact cohesion), respectively. Of litters included in the analyses of group contacts, inability to identify individuals led to exclusion of 2 litters in the analysis of individual contacts (pup-mother and pup-pup contacts).

Huddling preference was determined with the Wilcoxon signed rank test, accommodating the two related variables (time with the conditioned odor vs. time with the novel odor for each subject) as previously conducted (Kojima and Alberts, 2009, 2011). The Mann-Whitney test was used to examine the effects of treatment on huddling preference and huddling time with a scented target.

Effects of OT manipulation on contact patterns over the 2-h odor conditioning session, the distribution of contact pattern over the seven aggregon indices, contact cohesion over the 2-h odor conditioning, and contact cohesion over the seven aggregon indices were each assessed with linear mixed model analysis (Littell et al., 2000), which offers advantages and its flexibility with the repeated-measures over repeated-measures ANOVA and multivariate models (Bagiella et al., 2000). Linear mixed model analyses were conducted with treatment (OT/OTA) and time or aggregon index as fixed factors and with covariation between aggregon areas, aggregon indices, or frequencies of aggregon index in a same litter at different time or aggregon index, and at a given time or a given aggregon index if a significant group difference was identified, using Proc Mixed in SAS for Windows (SAS Institute Inc. Cary, NC) (Littell et al., 2006). Mean values of aggregon index and aggregon area calculated at 10 min intervals during the 2-h odor conditioning session were used when time was entered as a fixed factor in the model for contact pattern and for contact cohesion, respectively. Effects of treatment on individual contacts were accessed in one-way ANOVA with Tukey's post hoc tests when significant effects on ANOVAs were found. Association of aggregon area with mother's participation in contact averaged per litter was examined using Pearson correlation (r). All analyses were performed on SPSS (SPSS Inc. Chicago, IL) for Mac, except linear mixed model analyses described above. Differences and correlations were considered significant when $p < 0.05$.

Results

Huddling preference for a maternally-paired odor

Figure 3 illustrates odor-guided huddling preferences of pups in OT and OTA litters. Both OT-treated pups and their control siblings huddled more with a maternally-paired odor than with a novel odor (median score: +115 min, $z = -3.46$, $p = 0.001$ and +98 min, $z = -2.77$, $p = 0.006$, respectively). OT-treated pups huddled preferentially with the maternally-paired odor, as did their littermate controls ($z = -0.04$, $p = 0.968$).

In contrast, OTA-treated pups failed to express a huddling preference (+18 min, $z = -1.05$, $p = 0.292$), whereas their control siblings preferred the maternally-paired odor (+141 min, $z = -3.32$, $p = 0.001$). Indeed, the huddling preferences of OTA-treated pups were significantly

lower than those of their control siblings ($z = -2.92$, $p = 0.003$). The two saline-control groups in OT and OTA litters had comparable preference in huddling for an odor paired with maternal care ($z = -1.22$, $p = 0.222$).

Total time spent huddling and time spent huddling with a test odor

Figure 4A illustrates total time spent huddling with a target bearing a test odor during the 4-h huddling preference test. Median total time spent huddling across treatment groups was 126 min (53% of the 4-h test session huddling), replicating the robust levels of contact behavior seen in previous studies without pharmacological manipulation (Kojima and Alberts, 2009, 2011). Time spent huddling was comparable for OT-treated pups and their control siblings (median: 124 min and 143 min, respectively. $z = -0.62$, $p = 0.535$). In contrast, OTA-treated pups spent huddling less than their control siblings (102 min and 161 min, respectively. $z = -2.60$, $p = 0.009$).

Figure 4B displays time spent huddling with a target bearing a maternally-paired odor. OT-treated pups and their control siblings spent similar amounts of time huddling with the maternally-paired odor (median: 119 min and 104 min, respectively. $z = 0.00$, $p = 1.000$). In contrast, OTA-treated pups huddled with the maternally-paired odor less than did their control siblings (39 min and 144 min, respectively. $z = -2.68$, $p = 0.007$).

Figure 4C shows time spent huddling with a target bearing a novel odor. OT-treated pups and their control siblings spent equivalent time with a novel odor (median: 4 min and 4 min, respectively. $z = -0.01$, $p = 0.992$). In contrast, time spent huddling with a novel odor by OTA-treated pups was greater than that by their control siblings (22 min and 1 min, respectively. $z = -2.46$, $p = 0.014$).

Contact pattern during odor conditioning and its variation

Averaged aggregon index per 10 min over the 2-h odor conditioning and its frequencies over the seven aggregon indices are described in Table 1 and Table 2, respectively. No difference in contact patterns during the odor conditioning session was found between OT and OTA litters (6.41 ± 0.08 and 6.37 ± 0.06 , respectively; $F(1, 20) = 0.12$, $p = 0.736$). Likewise, there was no difference in the distribution of contact pattern over the aggregon indices between OT and OTA litters ($F(1, 20) = 1.21$, $p = 0.284$) with a significant interaction between treatment and aggregon index ($F(6, 20) = 3.23$, $p = 0.022$).

Contact cohesion during the odor conditioning

Figure 5 and Table 1 illustrate averaged aggregon areas per 10 min during the 2-h odor conditioning. In general, OT litters had smaller aggregon areas than did OTA litters ($126.5 \pm 2.8 \text{ cm}^2$ and $141.3 \pm 3.7 \text{ cm}^2$, respectively; $F(1, 20) = 10.69$, $p = 0.004$) with a significant interaction between treatment and time ($F(11, 220) = 3.00$, $p = 0.001$). Specifically, after the first 10 min, aggregon areas in OT litters were smaller than those in OTA for 60 min: at 20 min ($F(1, 220) = 10.94$, $p = 0.001$), 30 min ($F(1, 220) = 20.99$, $p < 0.0001$), 40 min ($F(1, 220) = 8.50$, $p = 0.004$), 50 min ($F(1, 220) = 7.27$, $p = 0.008$), 60 min ($F(1, 220) = 10.46$, $p = 0.001$), and 70 min ($F(1, 220) = 8.95$, $p = 0.003$). After the 70 min point, OT litters and OTA litters again had similar aggregon areas until the end of the conditioning session.

Contact cohesion by aggregon index

Figure 6 and Table 2 show averaged aggregon area per aggregon index. Overall, OT litters created smaller aggregon areas than did OTA litters ($126.5 \pm 2.8 \text{ cm}^2$ and $141.3 \pm 3.7 \text{ cm}^2$, respectively; $F(1, 20) = 10.28$, $p = 0.004$) with a significant interactions between treatment and index ($F(6, 100) = 3.23$, $p = 0.006$). More specifically, the aggregon areas of OT litters

were smaller than those of OTA litters at Index 3 ($F(1,100) = 11.42, p = 0.001$), Index 4 ($F(1,100) = 6.03, p = 0.016$), Index 5 ($F(1,100) = 5.55, p = 0.020$), Index 6 ($F(1,100) = 14.56, p < 0.001$), and Index 7 ($F(1,100) = 4.44, p = 0.038$).

Individual contacts with mother and with pup

We assessed the effects of treatment on all combinations of individual contact (pup-mother and pup-pup, Figure 7). Figure 7A illustrates frequency of pup contact with the mother during odor conditioning. Pups in OT litters had more contact with the mother than did pups in OTA litters (352.9 ± 18.7 and 289.3 ± 18.7 , respectively, $F(1, 20) = 5.804, p = 0.027$). Accordingly, differences were found among OT-treated, OT-control, OTA-treated, and OTA-control groups ($F(3, 60) = 6.00, p = 0.001$). Specifically, OT-treated pups had more contact with the mother (90.4 ± 3.3) than did OTA-treated ($73.0 \pm 3.7, p = 0.004$) and OTA-control pups ($73.7 \pm 3.3, p = 0.008$). In contrast, OT-control pups had contact with the mother (86.2 ± 4.4) similarly to OTA-treated ($p = 0.102$) and to OTA-control pups ($p = 0.153$). There was no difference in maternal contact between OT-treated and OT-control pups ($p = 0.893$) and between OTA-treated and OTA-control pups ($p = .999$).

In contrast to pup-mother contact, no differences were identified in pup-pup contacts among the four pup groups (OT-treated pups: 257.9 ± 8.5 , OT-control pups: 253.0 ± 12.2 , OTA-treated pups: 253.7 ± 4.1 , and OTA-control pups: $245.4 \pm 5.8, F(3, 60) = 0.59, p = 0.632$), as depicted in Figure 7B.

Association between maternal participation in contact and aggregon area

Because OT manipulations affected both contact between the mother and pups (Figure 7A) and aggregon area (Figures 5 and 6), we examined the association between maternal participation in contact and aggregon area. Figure 8 illustrates the correlation between the amount of maternal participation in contact and the family aggregon area, using the average ratio of maternal participation in contact and averaged aggregon area during the 2-h odor conditioning per litter. There was a negative correlation between the maternal participation and aggregon area ($r = -0.44, p = 0.039$): The more a mother participated in contact, the smaller the litter's aggregon area was. Thus, in aggregons in which the mother contacts pups, a pup will tend to experience not only the mother's body but also more of its littermates' bodies, as seen in the reduced aggregon area with increasing presence of the mother in the aggregon.

Discussion

The present study extends the well-known roles of OT in the social behavior of adult animals to an affiliative behavior of preweanling rats. We found that blocking central OT in pups prevented pups from acquiring the huddling preference for a maternally-paired odor and that the central OT manipulations altered social interactions among mother and pups. These findings provide new insights into several aspects of early olfactory learning, the establishment of social affiliation in infant animals, and parent-offspring relations.

Oxytocin is necessary for the acquisition of a filial huddling preference

Pups treated intraventricularly with an OTA failed to acquire a huddling preference following 2-h interaction with a scented foster mother. In contrast, saline-treated siblings that experienced the same foster mother at the same time as the OTA-treated pups acquired a huddling preference for the mother-associated odor. When exogenous OT was administered, the filial preference was acquired, but was no greater than that displayed by saline-treated controls (Figure 3).

We are able to characterize the effects of the experimental manipulations in several ways, because the preference measure was based on choice behavior in a well-controlled setting. Examination of the total time spent huddling (time with mother-associated stimulus + time with novel stimulus) revealed that OTA-treated pups huddled less during the test than did their control siblings (Figures 4A). In fact, we noted here and in other experiments (Kojima and Alberts, unpublished data) that within groups expressing a learned preference, the magnitude of a pup's huddling preference is positively associated with its total huddling time.

Additional manifestations of the absent filial preference in the OTA-treated pups were an overall diminution in absolute time spent in contact with the maternally-associated stimulus, as well as a significant increase in contact time with the novel stimulus, relative to vehicle-treated littermate controls (Figures 4B and 4C). Nevertheless, the decrease and increase of contact time with the maternally-associated odor and the novel odor, respectively, did not lead to huddling preference for the novel odor in OTA-treated pups (Figure 3). Thus, the lack of huddling preference seen in the OTA group was due to a diminished preference for the maternally-paired odor, rather than due to an attractiveness of the novel odor. Because the OTA-treated pups and their control siblings spent comparable time with the foster mother (Figure 7A), we cannot attribute their lack of filial preference to reduced maternal contact. Rather, OTA may attenuate maternal effects on this pup's preference learning. Together, the present results suggest that OT is necessary for the acquisition of a filial huddling preference for a maternal odor, neuroendocrinologically attenuating pup's olfactory learning of social stimuli.

Exogenous OT does not increase filial huddling preference

Pups that received exogenous OT prior to the 2-h session with the scented foster mother subsequently displayed a filial preference for the mother-associated odor, but there was no evidence for an augmented preference. Figure 3 illustrates the similarity in huddling preferences in OT-treated pups and their vehicle-treated littermate controls, which expressed filial preference similarly to the other, vehicle-treated controls that shared the foster mother with OTA pups.

It is possible that the absence of enhanced preference with exogenous OT is due to the normal engagement of the pups' available OT receptors. Skin-to-skin contact with mother appears to stimulate central OT in pups (Kojima et al., 2011). If most of the receptors are already bound and activated by the increased endogenous OT, additional exogenous OT cannot add stimulation through the receptors. This could account for a "ceiling effect" in the present experimental setting, and additional doses of OT and OTA could add support to such interpretation.

Another possibility is that the role of OT in the acquisition of pups' filial preference is to modulate other neurochemical systems. Norepinephrine and corticosterone, for example, have been shown to be involved in olfactory learning in rat pups (Moriceau and Sullivan, 2006; Moriceau et al., 2006; Shionoya et al., 2007; Sullivan and Wilson, 1994). OT-norepinephrine interactions appear important for social recognition in adult rodents (Dluzen et al., 2000). OT can also modulate activities in the hypothalamo-pituitary-adrenal (HPA) axis affecting secretion of adrenocorticotrophic hormone (ACTH) and corticosterone (Neumann, 2002; Neumann et al., 2000; Windle et al., 1997). Moreover, contact with an anesthetized mother can suppress norepinephrine in hypothalamic paraventricular nucleus and circulating corticosterone in pups, affecting their olfactory learning (Shionoya et al., 2007). Thus, neurochemicals other than OT may participate in affiliative learning in pups, and OT may play a role as a modulator for such neurochemicals which might have a rate-limiting role in OT's effects.

Central oxytocin in pups mediates individual behavior and group behavior

The results of the present study, combined with other findings (Kojima and Alberts, 2009, 2011; Ody et al, 2002, see Alberts 2007), provide new data and novel perspectives on how central OT may modulate both individual and group behavior of rat pups. We now know that various measures of contact behavior among pups and between the mother and offspring correlate with the formation of the pups' affiliative preferences (Kojima and Alberts, 2009, 2011). Behavioral interactions involving sustained contact between the mother's body and the pups' body (i.e., skin-to-skin contact) correlate with the magnitude of the subsequent filial preferences (Kojima and Alberts, 2011). Here we reported that manipulation of central OT in the pups did not affect pup-pup contact, but did alter contact behavior between mother and pups in the 2-h induction session, during which the filial preference is acquired by the offspring via maternal skin-to-skin contact. In our previous studies (Kojima and Alberts, 2009, 2011), maternal contact was identified as the significant factor for acquiring a filial preference, and here we found that OT litters displayed more frequent pup-mother contacts during the conditioning session than did OTA litters (Figure 7A).

OT-related regulation of contact behavior was found with some, but not all measures of group behavior that we used to characterize the social interactions in the litter. Including the mother as one of the behaving agents that combines with the individual pups to form the various contact patterns (Figure 2) was a useful innovation in the present study. Coding and quantifying the dynamic interactions of groups of such agents provided a valuable overview of the geometry of the mother-pup interactions. Interestingly, neither the frequency distribution of contact patterns nor the level of aggregation described by aggregon index across time differed between OT and OTA litters. The lack of a group difference in terms of contact pattern indicates that the total number of ways that the pups and mother oriented and positioned their bodies in relation to each other was the same when pups' central OT receptors were pharmacologically blocked with OTA or when receptor binding was presumably augmented by exogenous OT.

Importantly, planimetric data on the *cohesion* of such social contacts nonetheless revealed that central OT mediated these interactions, and that these effects conformed to the consequences seen in the pups' learned preferences. Figure 5, for example, shows that following ICV treatment, the areas subtended by the OT litters were smaller than those of the OTA litters at each 10-min interval from the 10-min point to the 70-min point of the 2-h odor conditioning session. Even more impressive was the finding that the geometry of the more contactful aggregons (Index 3 – 7, illustrated in Figure 6) were each smaller and more compact when two pups of the four in a litter were OT treated than when they were OTA treated. Figure 9, below, illustrates this finding with a sample of two families arranged as the most unified aggregon (Index 7: mother and all 4 pups in contact forming single aggregation). In this representative comparison, the OTA family shown in the lefthand panel has an aggregon area that subtends about twice that of the OT litter shown in the righthand panel.

At this point in the analysis, it is not possible to discern whether these effects on family geometry involve increased cohesion with OT, decreased cohesion with OTA, or both. We emphasize here the basic phenomenon, as well as the complexity of interactions, since the remaining two pups in each aggregon were saline-treated and the mother was untreated, but they were nonetheless actively involved. We strongly suspect, however, that OT enhancement of pup contact was a significant factor in these results. Figure 7A shows that pups in OT litters had more contact with the mother than did pups in OTA litters. Specifically, OT-treated pups had a higher level of the contact than did OTA-treated pups and OTA-controls, whereas the OT-control pups did not. OT enhancement of physical contact has also been reported previously; OT enhanced group cohesion and OTA

diminished cohesion in comparison to saline-treated controls in litters of rat pups, in which all 8 pups received the same treatment but with no mother present (Ody et al., 2002, see Alberts, 2007), and side-by-side contact was enhanced by central infusion of OT in adult prairie voles (Cho et al., 1999; Williams et al., 1994).

Bi-directional roles for oxytocin and behavior in the acquisition of the pups' filial preference

Although we treated the mother as one of five equal agents in the group analysis, we recognize that the mother has a distinct position in regulating group contacts. The significant correlation shown in Figure 8 indicates that the amount of maternal participation in family contact is associated with greater cohesion in the litter. When a mother reduces the aggregon area, it is most often by covering pup bodies (see Figure 9), and this is typical of "hovering", a maternal behavior often used to define maternal responsiveness (e.g., Lonstein et al., 1999; Moffat et al., 1993; Rees et al., 2004; Seip and Morrell, 2008; Stern and Johnson, 1990). Mothers who provide greater amounts of such contact (i.e., skin-to-skin contact) are providing stimulation that modulates central OT concentrations in the pups (Kojima et al., 2011), which, in turn, is associated with higher levels of filial preference for a mother-associated odor (Kojima and Alberts, 2011). Thus, when we observe mothers with OT litters producing smaller aggregon areas per aggregon index, we are seeing the pups' central OT affecting maternal behaviors that create conditions that enhance acquisition of a maternal odor preference – via the pups' central OT system. Thus, the present findings suggest that OT mediates the acquisition of filial, huddling preference not only neuroendocrinologically, but also *behaviorally*.

Importantly, such dual action appears *bi-directional*. Mother-pup contact modulates OT concentrations in the pups (Kojima et al., 2011), potentially affecting pup's olfactory learning as well as altering the pup's behavior, so that the pup makes more contact with the mother. Such active contact by pups can stimulate maternal responses that are expressed in forms (e.g., greater contact cohesion through maternal hovering) that potentiate skin-to-skin contact with pups (Sterns and Johnson, 1989, 1990). Such maternal contact facilitates the pup's preference learning (Kojima and Alberts, 2011) via the OT system, which, in turn, further augments the mother-pup interactions and the conditions for learning an odor-based social affiliation.

Highlights

- Oxytocin mediates social behaviors in adults, but is largely unexplored in infants.
- We examined oxytocin roles in pup's filial huddling preference for maternal odor.
- Manipulation of central oxytocin revealed its necessity for this pup's learning.
- This manipulation also affected contact cohesion among mother and pups.
- Oxytocin mediates pup's filial preference neuroendocrinologically and behaviorally.

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Figure 1.
Timeline of pups' experiences.

Figure 2. Possible contact patterns (aggregons) established by one mother and four pups. Four of those contact patterns in the conditioning arena are also described: (a) none are in contact (1, 1, 1, 1, 1: Index 1), (b) two pairs of two individuals form two aggregon elements and one pup is in no contact forming one aggregon element (2, 2, 1: Index 3), (c) a mother and one pup form an aggregon element whereas three pups form another aggregon element (3, 2: Index 5), and (d) all individuals are in contact forming one aggregon element (5: Index 7).

Figure 3.

Box plot of huddling preference for a maternally-paired odor in pups treated with oxytocin (OT; $n = 25$), oxytocin antagonist (OTA; $n = 26$) or saline-vehicle (SAL) sharing the same mother either with OT-treated pups ($n = 24$) or with OTA-treated pups ($n = 25$). Data points show median differences in contact time between a maternally-paired odor and a novel odor, with quartiles depicted by the brackets and with 1.5 times interquartile range (IQR) depicted by the whiskers. Scores significantly above or below 0 (no difference) reflect preference for a maternally-paired odor or for a novel odor, respectively. Lack of difference in contact time between test odors indicates no odor bias. The asterisk (*) indicates that a huddling preference was expressed. Dagger (†) indicates a significant difference in huddling preference between experimental pups and control siblings.

Figure 4.

Box plot of total huddling time with a scented target (A), huddling time with a target bearing a maternally-paired odor (B), huddling time with a target bearing a novel odor (C) during the 4-h huddling preference test in pups treated with oxytocin (OT: $n = 24$), oxytocin antagonist (OTA: $n = 26$), or saline-vehicle (SAL) sharing the same mother either with OT-treated pups ($n = 24$) or with OTA-treated pups ($n = 25$). Data points show medians with quartiles depicted by the brackets and with 1.5 times interquartile range (IQR) depicted by the whiskers. Dagger (†) indicates a significant difference between experimental and control groups.

Figure 5.

Aggregon area over the 2-h odor conditioning in oxytocin (OT: open circle, $n = 12$) and oxytocin antagonist litters (OTA: closed circle, $n = 10$). Data points show average area (cm² $\pm$ SEM) every 10 min. Dagger (†) and asterisk (*) indicates that a significant difference between OT and OTA litters over the conditioning session and at a given time point, respectively.

Figure 6.

Aggregon area by aggregon index in oxytocin (OT: open bar, $n = 12$) and oxytocin antagonist litters (OTA: shaded bar, $n = 10$). Data points show average area (cm² $\pm$ SEM). Dagger (†) and asterisk (*) indicates that a significant difference between OT and OTA litters over the seven aggregon indices and at a given index, respectively.

Figure 7.

Pup-mother contact (A) and pup-pup contact (B) during the odor conditioning in pups treated with oxytocin (OT: $n = 20$), oxytocin antagonist (OTA: $n = 20$), or saline-vehicle (SAL). The SAL pups shared the same mother either with OT-treated pups ($n = 20$) or with OTA-treated pups ($n = 20$). The bar graph describes frequency means ($\pm$ SEM) of pup's contact with mother or with another pup counted every minute during the conditioning. The dagger (†) indicates a significant difference between OT and OTA litters. The asterisk (*) indicates significant differences from OTA-treated pups and OTA-control pups.

Figure 8.

Association between maternal participation in contact and aggregon area, using averaged ratios of maternal participation and of averaged aggregon area for each litter over the 2-h session of the odor conditioning. Line expresses a significant correlation at $p < 0.05$ ($r = -0.44$, $p = 0.039$).

Index7

Contact area = 165.4 cm²

Contact area = 88.9 cm²

Figure 9.

Examples of aggregon areas at Index 7 (each of all animals in litter have at least one contact, forming single aggregation). Although both litters have the same contact pattern, the right litter has smaller aggregon area than did the left litter, showing more cohesive contact.

Table 1
Mean ($\pm$ SEM) aggregon index and aggregon area during the 2-h odor conditioning

		Time											
		10	20	30	40	50	60	70	80	90	100	110	120
Aggregon Index	OT	4.29 ± 0.25	6.28 ± 0.15	6.60 ± 0.09	6.45 ± 0.17	6.46 ± 0.16	6.20 ± 0.17	6.78 ± 0.09	6.75 ± 0.15	6.79 ± 0.12	6.82 ± 0.09	6.83 ± 0.10	6.63 ± 0.15
	OTA	4.25 ± 0.33	6.49 ± 0.14	6.55 ± 0.09	6.49 ± 0.11	6.53 ± 0.11	6.58 ± 0.12	6.76 ± 0.10	6.61 ± 0.15	6.60 ± 0.15	6.59 ± 0.16	6.60 ± 0.18	6.41 ± 0.24
Aggregon Area (cm ²)	OT	137.2 ± 2.4	126.7 ± 2.0	121.9 ± 2.4	123.2 ± 3.8	126.3 ± 5.9	122.3 ± 3.0	121.8 ± 5.1	131.2 ± 5.9	130.8 ± 4.9	124.8 ± 4.7	126.5 ± 4.5	125.7 ± 7.3
	OTA	146.8 ± 4.8	137.4 ± 2.4	148.1 ± 5.6	139.9 ± 5.1	147.1 ± 6.6	146.2 ± 6.9	145.9 ± 5.6	145.5 ± 5.9	129.6 ± 5.1	136.3 ± 7.2	136.7 ± 7.7	137.4 ± 2.4

Table 2Mean ($\pm$ SEM) frequency and aggregon area per aggregon index

		Aggregon Index						
		1	2	3	4	5	6	7
Frequency	OT	0.6 $\pm$ 0.2	2.4 $\pm$ 0.4	1.5 $\pm$ 0.3	5.1 $\pm$ 1.0	6.1 $\pm$ 2.1	22.0 $\pm$ 4.3	82.3 $\pm$ 6.1
	OTA	1.1 $\pm$ 0.2	2.0 $\pm$ 0.5	1.9 $\pm$ 0.6	4.2 $\pm$ 1.0	3.1 $\pm$ 0.8	32.8 $\pm$ 4.7	75.0 $\pm$ 5.3
Aggregon Area (cm ²)	OT	149.1 $\pm$ 7.2	143.7 $\pm$ 4.1	134.0 $\pm$ 5.0	137.9 $\pm$ 3.3	126.8 $\pm$ 6.0	134.7 $\pm$ 3.5	120.5 $\pm$ 2.7
	OTA	153.6 $\pm$ 4.3	149.3 $\pm$ 7.5	160.7 $\pm$ 6.0	152.5 $\pm$ 5.6	154.3 $\pm$ 5.5	157.5 $\pm$ 5.8	129.9 $\pm$ 3.1