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## Normal Maternal Behavior, But Increased Pup Mortality, in Conditional Oxytocin Receptor Knockout Females

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### Abstract

Oxytocin (Oxt) and the Oxt receptor (Oxtr) are implicated in the onset of maternal behavior in a variety of species. Recently, we developed two Oxtr knockout lines: a total body knockout ( $Oxtr^{-/-}$ ) and a conditional Oxtr knockout ( $Oxtr^{FB/FB}$ ) in which the Oxtr is lacking only in regions of the forebrain, allowing knockout females to potentially nurse and care for their biological offspring. In the current study, we assessed maternal behavior of postpartum  $Oxtr^{FB/FB}$  females toward their own pups and maternal behavior of virgin  $Oxtr^{-/-}$  females toward foster pups and compared knockouts of both lines to wildtype ( $Oxtr^{+/+}$ ) littermates. We found that both  $Oxtr^{-/-}$  and  $Oxtr^{FB/FB}$  females appear to have largely normal maternal behaviors. However, with first litters, approximately 40% of the  $Oxtr^{FB/FB}$  knockout dams experienced high pup mortality, compared to fewer than 10% of the  $Oxtr^{+/+}$  dams. We then went on to test whether or not this phenotype occurred in subsequent litters or when the dams were exposed to an environmental disturbance. We found that regardless of the degree of external disturbance,  $Oxtr^{FB/FB}$  females lost more pups on their first and second litters compared to wildtype females. Possible reasons for higher pup mortality in  $Oxtr^{FB/FB}$  females are discussed.

### Keywords

conditional knockout; stress; maternal behavior; aggression

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Across species, females undergo fundamental changes in behavior during pregnancy and in the postpartum period. After parturition, females display a new repertoire of behaviors collectively referred to as maternal behavior. In rodents, maternal behavior consists primarily of four readily observed behaviors: nest building, nursing and/or crouching over pups, retrieving pups to the nest, and body/genital licking of pups (Lonstein & Fleming, 2001; Rosenblatt, 1975; Rosenblatt, Mayer, & Giordano, 1988). Like many other behaviors, hormones can facilitate maternal behavior and, in particular, oxytocin (Oxt) is convincingly

implicated (Pedersen, Ascer, Monroe, & Prange, 1982; Pedersen & Boccia, 2003; Pedersen, Vadlamudi, Boccia, & Amico, 2006; Ross & Young, 2009).

Oxt is a nonapeptide hormone best known for its role in female reproduction, particularly parturition and lactation. Both its central and peripheral actions are transduced by a single isoform of the Oxt receptor (Oxtr). One well-known role of Oxt is the neural regulation of maternal behavior. Oxt immunoreactivity increases in the female rat brain during pregnancy and in the early postpartum period in regions like the ventral septum (Landgraf, Neumann, Russell, & Pittman, 1992), the paraventricular nucleus (PVN), and the supraoptic nucleus (SON) of the hypothalamus (Caldwell, Greer, Johnson, Prange, & Pedersen, 1987; Landgraf et al., 1992; Mezey & Kiss, 1991), although its regulation at this time appears to be estrogen-dependent (for a recent review of Oxt-estradiol interactions, see Cameron et al., 2008). Oxt expression also increases in the postpartum period within the SON and PVN of several species (see Leng, Meddle, & Douglas, 2008), including rats (Lightman & Young, 1987), voles (Wang, Liu, Young, & Iusel, 2000), rabbits (Caba, Silver, Gonzalez-Mariscal, Jimenez, & Beyer, 1996), and sheep (Broad, Kendrick, Sirinathsinghji, Keverne, 1993).

It has been proposed that the increases in Oxt expression prior to and following parturition facilitate the onset and maintenance of maternal behavior, particularly in rats. Intracerebroventricular (i.c.v.) Oxt administration to virgin female rats increases the display of all aspects of maternal behavior but only in the presence of high endogenous estradiol levels (Pedersen & Prange, 1979). Similarly, ovariectomized female rats primed with estradiol and progesterone have reduced maternal behavior up to 24 hours after i.c.v. anti-Oxt antiserum administration (Pedersen, Caldwell, Johnson, & Prange, 1985). Oxt may be particularly important to the grooming and nursing aspects of maternal behavior as i.c.v. infusion of a selective Oxt antagonist significantly increases self-grooming and the frequency of prone posture over pups, rather than the upright posture that facilitates nursing (Pedersen & Boccia, 2003). However, some studies fail to find a facilitatory effect of Oxt on maternal behavior (Bolwerk & Swanson, 1984; Rubin, Menneti, & Bridges, 1983) or indicate that Oxt can only affect maternal behavior when associated with other systems, such as olfaction (Wamboldt & Insel, 1987).

Expression of the Oxtr also increases significantly throughout pregnancy in various hypothalamic regions, particularly the SON, PVN, medial preoptic area (mPOA), and the bed nucleus of the stria terminalis (BNST; Bealer, Lipschitz, Ramoz, & Crowley, 2006; Meddle, Bishop, Gkoumassi, Van Leeuwen, & Douglas, 2007), as well as the amygdala and olfactory bulbs (Meddle et al., 2007), although questions have been raised regarding the specificity of this Oxtr antibody (Yoshida et al., 2009). With Oxt, Oxtr expression is thought to aid in the onset and maintenance of maternal behavior. Evidence supporting this assumption is found in several species. In rats, dams displaying naturally high levels of pup licking, grooming, and arched-back nursing (i.e., High LG-ABN) have higher levels of Oxtr in the BNST, mPOA, and lateral septum than do mothers with low levels of these behaviors (i.e., Low LG-ABN; Champagne, Diorio, Sharma, & Meaney, 2001; Francis, Champagne, & Meaney, 2000). I.c.v. administration of an Oxtr antagonist effectively turns High LG-ABN mothers into Low LG-ABN mothers (Champagne et al., 2001). In female prairie voles, which readily express “spontaneous” maternal behavior, the Oxtr is more highly expressed in the nucleus accumbens compared to species that fail to show “spontaneous” maternal behavior, such as rats, mice, and meadow voles (Olazabal & Young, 2005; Olazabal & Young 2006a). The difference in Oxtr expression within the nucleus accumbens has been found to be physiologically relevant in prairie voles, as administration of an Oxtr antagonist into this area completely abolishes displays of maternal behavior (Olazabal & Young 2006b).

Studies using Oxt and Oxtr knockout (KO) mice ( $Oxt^{-/-}$  and  $Oxtr^{-/-}$ , respectively) have also provided insight into the roles of Oxt and the Oxtr in the neural regulation of maternal behavior. Early studies in two independently derived lines of  $Oxt^{-/-}$  mice found that females have normal parturition, an inability to milk eject, and unaltered maternal behavior (Nishimori et al., 1996; Young et al., 1996). A more recent study also found normal maternal behavior in both postpartum and virgin  $Oxt^{-/-}$  females (Takayanagi et al., 2005). However, Pedersen et al. (2006) revealed moderate maternal behavior deficits in virgin  $Oxt^{-/-}$  females, including a decrease in pup licking and impaired pup retrievals by virgin  $Oxt^{-/-}$  females compared with wildtype ( $Oxt^{+/+}$ ) females. A limitation exists with this study in that the  $Oxt^{+/+}$  and  $Oxt^{-/-}$  females used were not littermates and not obtained from genetically identical mothers. Possible differences in genetic background and intrauterine environment could contribute to the reported differences in maternal behavior. For example,  $Oxt^{-/-}$  males derived from homozygous parents have higher levels of aggression than  $Oxt^{-/-}$  males derived from heterozygous parents (Takayanagi et al., 2005; Winslow et al., 2000).

Initial studies in postpartum  $Oxtr^{-/-}$  females have also reported deficits in maternal behavior compared with wildtype females, as measured by longer latencies to retrieve pups and reduced crouching over the retrieved pups (Takayanagi et al., 2005). Recently, we developed two lines from conditional Oxtr KO mice, a total knockout ( $Oxtr^{-/-}$ ) and an Oxtr knockout ( $Oxtr^{FB/FB}$ ) in which the Oxtr is absent or reduced in most regions of the forebrain (Lee, Caldwell, Macbeth, Toln, & Young, 2008), sparing peripheral receptors.  $Oxtr^{FB/FB}$  females can eject milk (as verified by the presence of milk spots in their offspring's abdomens), thus allowing their offspring to survive and maternal behavior to be assessed. In the current study, we first observed maternal behavior in post-partum  $Oxtr^{FB/FB}$  and wildtype ( $Oxtr^{+/+}$ ) littermates, as well as maternal responsiveness of virgin  $Oxtr^{-/-}$  females to foster pups. We then examined the likelihood of  $Oxtr^{+/+}$  and  $Oxtr^{FB/FB}$  dams to care for their pups following the application of an external stressor.

## Methods

### Animals and Housing

All subjects were littermates obtained from approximately eight breeders per line; equivalent numbers of wildtype and knockout subjects were taken from each breeder (as suggested in Crusio, Goldowitz, Holmes, & Wolfer, 2009). Females were group housed (two to five animals per cage) upon weaning at 21–28 days old in single-sex cages until at least 8 weeks old. Unless noted otherwise below, all females were virgins prior to testing. All animals were maintained under a 12:12 light–dark cycle (lights on at 0300h) with food and water available ad libitum. All procedures were approved by the National Institute of Mental Health Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines for the care and use of animals.

The development and genotyping of the  $Oxtr^{-/-}$  and  $Oxtr^{FB/FB}$  females was as described previously (Lee et al., 2008; Macbeth, Lee, Edds, & Young, 2009). Briefly, we crossed the L7ag13 transgenic line (C57BL/6J genetic background) that expresses Cre recombinase under the control of the Camk2a promoter (Dragatsis & Zeitlin, 2000; Zakharenko et al., 2003) with  $Oxtr^{+/flox}$  or  $Oxtr^{flox/flox}$  mice.  $Oxtr^{flox/flox}$  male mice were crossed with female  $Oxtr^{+/flox}$  mice that contained one transgenic allele expressing Cre recombinase ( $Oxtr^{+/flox,cre}$  or  $Oxtr^{+/FB}$ ). The offspring thus had the following genotypes: (a)  $Oxtr^{+/flox}$ , (b)  $Oxtr^{flox/flox}$ , (c)  $Oxtr^{Cre, +/flox}$ , and (d)  $Oxtr^{Cre, Flox/flox}$ . The first two are considered wildtypes, the third a heterozygous forebrain inactivation, and the fourth a forebrain-specific Oxtr knockout ( $Oxtr^{FB/FB}$ ). Whole-body Oxtr knockout mice were generated by breeding male  $Oxtr^{Cre, +/flox}$  with female  $Oxtr^{flox/flox}$  mice, leading to heterozygous progeny

(*Oxtr*<sup>+/-</sup>). These mice were crossed to obtain homozygous total *Oxtr* knockout (*Oxtr*<sup>-/-</sup>) females. As both the transgenic lines expressing Flp or Cre recombinase were on a C57BL/6J background, the resulting *Oxtr*<sup>-/-</sup> and *Oxtr*<sup>FB/FB</sup> mice studied here were approximately 88% and 81% C57BL/6J, respectively (the remainder being 129/S). All *Oxtr*<sup>FB/FB</sup> mice are heterozygous for the Cre recombinase transgene.

### Experiment 1: Maternal Behavior in *Oxtr*<sup>FB/FB</sup> and *Oxtr*<sup>-/-</sup> Females

***Oxtr*<sup>FB/FB</sup> females**—*Oxtr*<sup>+/+</sup> (*n* = 9) and *Oxtr*<sup>FB/FB</sup> (*n* = 10) females were used to assess maternal behavior. The average age of the dams at the time of their first litter was 12.9 ± 0.8 weeks for *Oxtr*<sup>+/+</sup> and 13.7 ± 1.0 weeks for *Oxtr*<sup>FB/FB</sup> females. Subject females were singly housed in a new, clean cage and mated to a C57BL/6J male (Jackson Laboratories). Mating was confirmed by the detection of a sperm plug, and males were removed approximately one week after mating. Females remained singly housed throughout parturition and during testing. Gestational length and litter sizes did not significantly differ between genotypes for either experiment. Upon completion of this experiment, all mothers and pups were euthanized.

Day of parturition (first day pups observed in the cage by 0900 hours) was designated postnatal day (PND) 0. Dams were tested for maternal behavior in their home cages beginning on PND 1 (first day where the pups had the presence of a milk spot) and continued through PND 3. On PND 1-PND 3, maternal behavior was videotaped for three 10-min observation sessions during “lights on” (light phase) at 900, 1100, and 1300 hours and one 20-min observation session during “lights off” (dark phase) at 1500 hours. Cages were moved to the testing room at least 30 minutes prior to testing and returned to the animal room immediately after each observation session. The observation session during the dark phase was videotaped under dim red light illumination with an infrared camera. From 900 to 1520 hours, the cages were not disturbed but remained stationary during videotaping. Following the last observation session at 1500 hours, pup retrieval by the dams was quantified by removal of pups from the dam for five minutes, during which time the female was videotaped. Pups were then scattered opposite of the dam in the home cage and the dam’s behavior was videotaped for an additional 10 minutes. Maternal aggression was tested on PND 4–6 at 1300 hours by removing the pups and immediately introducing an intruder Balb/c male to the home cage. The pups were removed to reduce their risk of injury; removal of pups has not been found to affect displays of maternal aggression (Svare, Betteridge, Katz, & Samuels, 1981).

Maternal behavior and aggression were later viewed and scored by an observer blind to genotype using Observer 5.0 (Noldus, Leesburg, VA). For the data collected on PND 1–3, dams were scored for 1) pup interactions (sniffing/licking and nursing pups); 2) nonsocial behaviors (resting alone, feeding, and moving around the cage); 3) nest building; and 4) self-grooming. Pup retrieval latencies were measured by determining the amount of time it took to the dam to retrieve the first pup (this was used because the number of pups varied among the dams). On PND 4–6, dams were scored for maternal aggression. Any dams failing to attack the intruder male in the first 5 minutes of testing were given a latency score of 300 seconds. If an attack occurred, the female’s behavior was scored for an additional 2 minutes. Behaviors measured included tail rattles, fleeing, attack behavior (lunge-bite), nonattack aggression (pushing), defensive behavior (upright and sideways defensive posturing), and nonsocial behavior (eating, sleeping, and climbing sides of the cage).

Maternal behaviors, with the exception of pup retrievals, were analyzed within each day using a two-way ANOVA with genotype and light phase as the main factors. For this analysis, the amount of time the dams engaged in all behaviors (maternal and nonmaternal) over the three light-phase observational sessions were first summed, and the percentage of

time the females engaged in each behavior was determined. The percentage of time the females engaged in each behavior was then compared between the light and dark phases and between genotypes. Percentages were used, rather than durations, because of the difference in the amount of time the data were collected in the two different light conditions; that is, 30 minutes during “lights on” and 20 minutes during “lights off.” Pup retrieval latency was analyzed within each day using a one-way ANOVA. A  $p$  value of  $\leq 0.05$  was considered statistically significant. No statistical analyses were performed for measures of maternal aggression as too few animals attacked.

For this experiment, the number of pups surviving was also recorded. At the completion of the measures of maternal behavior and maternal aggression, females used in this experiment were mated a second time to determine whether the number of pups surviving differed across the two genotypes on a second litter.

***Oxtr*<sup>-/-</sup> females**—*Oxtr*<sup>+/+</sup> ( $n = 9$ ) and *Oxtr*<sup>-/-</sup> ( $n = 8$ ) virgin females ( $21 \pm 0.8$  weeks old) were used to assess maternal responsiveness toward foster pups. Foster pups were obtained from eight breeding pairs of C57BL/6J mice (Jackson Labs, Bar Harbor, Maine). Approximately one week prior to testing, subject females were singly housed in new, clean cages. The day pups were found in the C57BL/6J cage was designated PND 0; testing began on PND 1. Subject females were moved into the testing room at least 30 minutes prior to introduction of foster pups. Females were presented with four pups (two male, two female as determined by anogenital distance) from no more than two C57BL/6J breeders for a period of three days (PND 1–3). Only pups with observable milk spots were used as foster pups. Subject females were exposed to pups from the same breeder(s) each day. Testing occurred at approximately 1100 hours. Pups were scattered into the three corners of the cage not containing the nest; the females’ behavior was videotaped for a single 30-min period, after which pups were returned to their biological mothers. If subject females were observed to attack any foster pups, the session was immediately terminated and the injured pup euthanized. Maternal behaviors were scored as described for *Oxtr*<sup>FB/FB</sup> females. Aggression was not measured in these virgin mice.

## **Experiment 2: Effects of Environmental Disturbances on Pup Mortality in *Oxtr*<sup>FB/FB</sup> Females**

*Oxtr*<sup>+/+</sup> ( $n = 30$ ) and *Oxtr*<sup>FB/FB</sup> females ( $n = 30$ ) were mated with C57BL/6J males as described above. The average age of the dams at the time their first litter was born was  $17.6 \pm 0.5$  weeks for *Oxtr*<sup>+/+</sup> and  $19.9 \pm 0.6$  weeks for *Oxtr*<sup>FB/FB</sup> females. Females from each genotype were randomly assigned to one of three disturbance groups: NONE, LOW, and HIGH ( $n = 10$  of each genotype per group). For all three groups, the number of pups present on the day of parturition (PND 0: the day pups were present in the nest by 0900 hours) was counted. Females in the NONE group remained undisturbed in their cage until PND4, when a final pup count was taken. On PND 1–3, cages of females in the LOW and HIGH groups were removed from their shelves, and a pup count was taken at 0900 hours each day. Additionally, at 0900, 1100, and 1300 hours (the same times at which maternal behavior was assessed in Experiment 1), a cage disturbance was administered. For females in the LOW group, this disturbance consisted only of removing the lid of the cage and visually inspecting the inside of the cage. Pups were not moved in any way, and if the dam was blocking the pups for the pup count, she was gently moved off the nest to get an accurate count. For females in the HIGH group, all pups and nest material were scattered to the three previously empty corners of the cage, forcing the mother to rebuild the nest and retrieve all pups three times a day. A final pup count occurred at 0900 hours on PND 4.



In Experiment 1, no pups were found dead after PND 4, indicating the observed pup mortality (see Results) occurs shortly after parturition. Therefore, pups from litter 1 were euthanized by PND 7, and the dams were remated to novel males. Disturbances for the second litter were carried out in an identical manner as for the first litter. For their second litter, the average age of the dams was  $23.8 \pm 0.7$  weeks for *Oxtr*<sup>+/+</sup> females and  $26.3 \pm 0.8$  weeks for *Oxtr*<sup>FB/FB</sup> females. Litter 2 pups were euthanized by PND 7, and the dams mated a third time. As a further manipulation to assess the effects of stress on pup mortality, for the third litter we switched the NONE and HIGH groups (average age of the dams was  $29.3 \pm 0.9$  weeks for *Oxtr*<sup>+/+</sup> and  $32.8 \pm 1.0$  weeks for *Oxtr*<sup>FB/FB</sup> females). Average time between litters was approximately 6 weeks. Females who failed to become pregnant for any litter after two mating attempts were excluded from the study, accounting for the unequal group sizes across litters and groups (Figure 4, legend). Litter sizes did not significantly differ between the genotypes (*Oxtr*<sup>+/+</sup>: litter 1 =  $7.62 \pm 0.46$ ; litter 2 =  $9.03 \pm 0.32$ ; litter 3 =  $8.88 \pm 0.46$ . *Oxtr*<sup>FB/FB</sup>: litter 1 =  $7.61 \pm 0.43$ ; litter 2 =  $8.07 \pm 0.42$ ; litter 3 =  $7.48 \pm 0.50$ ). Upon completion of this experiment, all mothers and pups from litter 3 were euthanized.

Two separate repeated measures three-way ANOVAs were carried out on the dependent variable (number of pups lost), with genotype, disturbance, and litter as the main factors. In the first ANOVA, only litters 1 and 2 were compared (disturbance groups remained identical), and in the second ANOVA litters 1 and 3 were compared to analyze the effect of switching NONE and HIGH groups on litter 3. Significant main effects and/or interactions were analyzed via independent-samples *t* tests. A *p* value of  $\leq 0.05$  was considered significant.

## Results

### Experiment 1

***Oxtr*<sup>FB/FB</sup> females**—Four of 10 *Oxtr*<sup>FB/FB</sup> dams showed total pup mortality by PND 1, with all pups found dead in the cages. In contrast, none of the nine *Oxtr*<sup>+/+</sup> females exhibited pup mortality. Therefore, behavior from only six *Oxtr*<sup>FB/FB</sup> dams was included in the final statistical analysis of maternal behavior. Of the *Oxtr*<sup>FB/FB</sup> dams that displayed maternal care, there were no statistical differences between genotypes in any of the behaviors measured during the light phase of the circadian cycle on PND 1–3 (Figures 1a, b, c). Latency to retrieve the first pup back to the nest also did not differ between *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> dams (within 20s; data not shown). Furthermore, 100% of dams of both genotypes retrieved all pups back to the nest on all three test days. Maternal behavior of *Oxtr*<sup>FB/FB</sup> dams was also assessed during the dark cycle; for all three days, there was decreased percent of time engaged in nest building and nonsocial behavior and increased sniffing/licking of pups in the dark phase compared to the light phase (data not shown). During the maternal aggression task, only three out of nine *Oxtr*<sup>+/+</sup> females and only one out of six *Oxtr*<sup>FB/FB</sup> females displayed any aggression (data not shown), indicating low levels of maternal aggression regardless of genotype.

A Fisher's exact test comparing pup mortality between the genotypes had a two-tailed *p* value of 0.087. These data suggested that offspring from *Oxtr*<sup>FB/FB</sup> dams might have increased mortality. To test this, we mated the same females a second time and only monitored the females for pup death from PND0-4. Unlike with their first litter, we found no genotypic differences in pup mortality (1/7 and 1/10 of *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> dams, respectively).

***Oxtr*<sup>-/-</sup> females**—Data from three *Oxtr*<sup>+/+</sup> females and one *Oxtr*<sup>-/-</sup> female were excluded because they attacked a foster pup on the first day of testing. Therefore, maternal behavior was assessed from six *Oxtr*<sup>+/+</sup> females and seven *Oxtr*<sup>-/-</sup> females. No genotypic differences

in maternal care of foster pups were observed over the three test days (Figures 2a, b, c). Additionally, *Oxtr*<sup>-/-</sup> females did not differ from *Oxtr*<sup>+/+</sup> females in latency to retrieve the first pup on any of the three testing days (data not shown). However, on Day 1, only 40% of *Oxtr*<sup>-/-</sup> females retrieved at least two foster pups, compared to 100% of the *Oxtr*<sup>+/+</sup> females. By Day 3, over 80% of females from both genotypes retrieved all four foster pups (see Figure 3).

## Experiment 2

In this experiment, we assessed whether or not application of an external stress (cage disturbance) influences pup mortality in *Oxtr*<sup>FB/FB</sup> dams. Comparison of the data from litters 1 and 2 via three-way ANOVA (genotype × litter × disturbance) revealed significant main effects of genotype,  $F_{1,101} = 17.61, p < .001$ , and litter,  $F_{1,101} = 11.83, p < .001$ . There were no significant interactions. Analyses of the main effects via *t* test are shown in Figure 4. Regardless of disturbance group, all dams lost more pups on litter 1 (Figure 4, left) compared to litter 2 (Figure 4, middle;  $p < .05$  for *Oxtr*<sup>+/+</sup> dams and  $p < .01$  for *Oxtr*<sup>FB/FB</sup> dams). Additionally, regardless of disturbance group, *Oxtr*<sup>FB/FB</sup> dams lost significantly more pups on both litters 1 and 2 compared to *Oxtr*<sup>+/+</sup> dams ( $p < .001$  for litter 1 and  $p < .05$  for litter 2).

Comparison of the data from litters 1 and 3 via three-way ANOVA (genotype × litter × disturbance) revealed a significant genotype × litter interaction,  $F_{1,92} = 4.60, p < .05$ . Analyses via *t* tests are shown in Figure 4. *Oxtr*<sup>+/+</sup> dams did not significantly differ in number of pups lost from litter 1 to litter 3 ( $p > .10$ ). In contrast, regardless of disturbance group, *Oxtr*<sup>FB/FB</sup> dams lost significantly more pups on litter 1 (Figure 4, left) than litter 3 (Figure 4, right;  $p < .01$ ). There were no genotypic differences in pup loss between *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> dams on litter 3 ( $p > .10$ ).

## Discussion

In the current paper, we used a forebrain knockout of the *Oxtr* gene, as well as a total *Oxtr* knockout to assess the role of the *Oxtr* in female maternal behavior. We had hypothesized that lack of the *Oxtr* in the entire brain, and specifically in the forebrain, would result in impaired maternal behavior as compared to WT littermates. Contrary to our hypothesis, we found that *Oxtr*<sup>FB/FB</sup> females did not significantly differ from *Oxtr*<sup>+/+</sup> females on our measures of maternal behavior, including retrieval latencies, at least in those that did not lose their pups (see below). The normal maternal behavior in the remaining *Oxtr*<sup>FB/FB</sup> females is impressive in the face of the altered pattern of expression with absence in some brain regions and maintained expression to varying degrees in others, such as the medial amygdala, olfactory bulbs, and neocortex (Lee et al., 2008). This altered *Oxtr* expression pattern, developing after weaning in *Oxtr*<sup>FB/FB</sup> females, could allow for the continued maintenance of maternal behavior, including maternal aggression, as *Oxt* activity in the olfactory bulbs and amygdala is implicated in maternal behavior and aggression, respectively (Bosch, Meddle, Beiderbeck, Douglas, & Neumann, 2005; Brennan & Keverne, 1997; Fleming & Rosenblatt, 1974; Lubin, Elliot, Black, & Johns, 2003; Nephew, Bridges, Lovelock, & Byrnes, 2009). However, it should be noted that in a more natural, socially competitive environment, female *Oxtr*<sup>-/-</sup> mice have high levels of infanticide and display high levels of aggression toward intruders (Ragnauth et al., 2005).

Previous studies have indicated subtle deficits in maternal behavior, specifically longer retrieval latencies, in both total *Oxtr* and *Oxt* KO lines (Takayanagi et al., 2005; Pedersen et al., 2006, respectively). We also found deficits in pup retrieval but only on the first presentation of pups. Even about half of the virgin wild-types left some pups scattered about the cage initially. This may indicate that our *Oxtr*<sup>-/-</sup> females may have a greater initial

aversion to pups that is overcome by repeated exposure. It is interesting to note that by day three, both virgin genotypes' patterns of behaviors begin to resemble that of postpartum wildtype mice, with trends toward increased pup interactions and decreased non-social behaviors. While we did not assess maternal behavior for longer than minutes across a day, similar short durations have been used in previous studies (Keller, Pawluski, Brock, Douhard, & Bakker, 2010; Olazabal & Young 2006a; Olazabal & Young 2006b). It may be useful to have a more prolonged measure of time spent in contact with pups, although this becomes difficult when using foster pups. Based upon the methods used in this study, we find it remarkable just how robust basic maternal behaviors appear to be despite the absence of the *Oxtr*.

Given that Oxt cannot be involved in the expression of maternal behavior in *Oxtr*<sup>-/-</sup> and *Oxtr*<sup>FB/FB</sup> (at least where it is absent in the latter) females, other neural mechanisms must compensate for the loss of Oxt action. One possibility is the vasopressin (Avp) system. Avp is closely related to Oxt and regulates many of the same behaviors as Oxt. In terms of parental care, Avp has primarily been studied for its importance in rodent paternal behavior (see Caldwell, Lee, Macbeth, & Young, 2008, for review). However, recent studies indicate that Avp does contribute to maternal behavior, predominantly through actions at the Avp 1a receptor (Bosch & Neumann, 2010; Bosch, Pfortsch, Beiderbeck, Landgraf, & Neumann, 2010; Nephew & Bridges, 2008a; Nephew & Bridges, 2008b). Obviously, maternal behavior is critical for species propagation so it would be logical for the species to have evolved as much redundancy as possible for essential functions.

In Experiment 1, we found that in *Oxtr*<sup>FB/FB</sup> dams whose pups survive, there are no impairments in maternal behavior. However, we did find a unique pup mortality phenotype in which offspring from 40% of *Oxtr*<sup>FB/FB</sup> dams died by PND 4. This phenotype was present only in *Oxtr*<sup>FB/FB</sup> dams and only with their first litter. As these animals had been disturbed daily from PND 1–3 to test for maternal behavior, we hypothesized that *Oxtr*<sup>FB/FB</sup> dams were unusually susceptible to an environmental disturbance, which resulted in increased pup mortality.

To test this hypothesis, in Experiment 2, the home cage environment of *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> females was disturbed from PND 1–3. We found no significant overall effect of type of disturbance type (NONE, LOW, or HIGH) on pup mortality. Both nondisturbed (NONE) and disturbed (LOW and HIGH) *Oxtr*<sup>FB/FB</sup> dams lost similar numbers of pups from litters 1 and 2, indicating that the stress applied in this experiment did not influence the pup mortality phenotype. However, *Oxtr*<sup>FB/FB</sup> dams lost significantly more pups than *Oxtr*<sup>+/+</sup> dams from each of the first two litters, confirming the pup mortality phenotype in *Oxtr*<sup>FB/FB</sup> dams observed in Experiment 1. By their third litter, *Oxtr*<sup>FB/FB</sup> dams no longer lost a significantly greater number of pups than *Oxtr*<sup>+/+</sup> dams and switching the NONE and HIGH disturbance groups did not alter pup death in either genotype. The discrepancy between Experiment 2 (in which *Oxtr*<sup>FB/FB</sup> dams still lost more pups than *Oxtr*<sup>+/+</sup> dams from litters 2), and Experiment 1 (in which *Oxtr*<sup>FB/FB</sup> dams did not lose more pups than *Oxtr*<sup>+/+</sup> dams from litter 2) may indicate that disturbing the cages in Experiment 2 has a subtle effect on pup loss.

The results of this study indicate that the pup mortality phenotype in *Oxtr*<sup>FB/FB</sup> dams is not significantly facilitated by disturbances to their environment and the underlying cause of the pup death remains unknown. In this study, none of the offspring were homozygous, as all females were mated to C57BL/6 males, so *Oxtr*<sup>+/+</sup> offspring were WT, and *Oxtr*<sup>FB/FB</sup> offspring were heterozygotes. We have not previously observed increased pup deaths of heterozygotes from wildtype or heterozygous dams in development of the line. However, there may be a fundamental difference in the offspring of *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> females.



For example, ultrasonic vocalizations in both *Oxt*<sup>-/-</sup> and *Oxtr*<sup>-/-</sup> male infant pups are decreased compared with wildtype littermates (Winslow et al., 2000; Takayanagi et al., 2005, respectively). It is possible that the heterozygous offspring of *Oxtr*<sup>FB/FB</sup> dams have impaired vocalizations. It does not appear that the dams are attacking their pups, as the pups are whole when found dead in the cage. Both *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> mothers clean their pups and engage in placentophagia as would be expected. The pups do not appear to die from starvation, as milk spots are noted in their stomachs. However, given that Oxt plays a causal role in prolactin release and subsequent milk ejection (Kennett et al., 2009; Bertram et al., 2010; Tabak et al., 2010), the possibility remains that *Oxtr*<sup>FB/FB</sup> mothers' milk supply, while intact, may be diminished compared to *Oxtr*<sup>+/+</sup> mothers. If this is the case, lactation would seem to improve in subsequent litters.

The pup mortality phenotype is difficult to quantify. The pups did not die in the same manner across both experiments. In Experiment 1, all dead pups were found in the cage by PND 1. In Experiment 2, most pups died by PND 1, yet some were found stillborn, and others did not die until PND 2–3. Lastly, the pup mortality phenotype is not universal across all *Oxtr*<sup>FB/FB</sup> dams. In Experiment 1, only 40% of *Oxtr*<sup>FB/FB</sup> dams were observed to have the phenotype. A slightly higher percentage (50–60%) was observed to have the phenotype on the first litter in Experiment 2, but still not 100%. *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup> females from both experiments were tested in the same animal facility, were obtained from like breeding pairs, and all came from litters containing both male and female offspring, indicating that testing environment, parenting, and/or early life experience likely did not play a role in pup mortality differences across the two experiments. It is possible that some unobserved part of the delivery process is impaired with their first litter, inducing pup mortality, which is avoided in subsequent litters. For example, stress, induced by fear of peripartum events and usually dampened by the actions of Oxt in the amygdala (Hansen & Ferreira, 1986), could be elevated in the *Oxtr*<sup>FB/FB</sup> females, leading to greater corticosterone exposure and untoward consequences (Rangon et al., 2007; Zahwa, Yorty, & Bonneau, 2008). Perhaps prior experience produces a less stressful second pregnancy, parturition, and postpartum, leading to reduced pup mortality.

Oxt is heavily involved in the regulation of affiliative behaviors in rodents, with Oxt administration increasing social interactions, partner preference, and parental behavior (reviewed in Lee, Macbeth, Pagani, & Young, 2009). As discussed above, Oxt is particularly involved in the onset and maintenance of maternal behavior. Pups are initially anxiety provoking when encountered by females (Fleming, Cheung, Myhal, & Kessler, 1989; Fleming & Luebke, 1981); as Oxt aids in decreasing anxiety in the postpartum period, it may aid in forming proper pup attachment. Oxt administration inhibits infanticide normally observed in virgin and pregnant wild mice (McCarthy, 1990; McCarthy, Bare, & vom Saal, 1986). In women, higher levels of Oxt during the third trimester correlate with self-reported feelings of “bonding” with the fetus (Levine, Zagoory-Sharon, Feldman, & Weller, 2007), and in the postpartum period plasma Oxt levels are correlated with maternal bonding behaviors, such as infant-directed gaze, vocalizations, and affectionate touch (Feldman, Weller, Zagoory-Sharon, & Levine, 2007). Recent work also indicates that a less efficient variant of the *Oxtr* gene in human mothers is correlated with lower levels of maternal responsiveness toward their toddlers (Bakermans-Kranenburg and van Ijzendoorn, 2008). The unique *Oxtr* expression pattern present in our *Oxtr*<sup>FB/FB</sup> females may result in impaired pup attachment, resulting in the pup mortality phenotype. It does result in an altered ability for social recognition (Macbeth et al., 2009). Yet the attachment failure is not complete, as not all *Oxtr*<sup>FB/FB</sup> dams exhibit the phenotype, and they are able to overcome the deficit, as by litter 3 we observed no genotype differences in pup mortality. Future studies are planned to assess whether nonmaternal behavior differences exist between *Oxtr*<sup>+/+</sup> and *Oxtr*<sup>FB/FB</sup>

females in the postpartum period (such as altered depressive-like behaviors and responses to stress and/or rewarding stimuli) that could indirectly influence the pup mortality phenotype.

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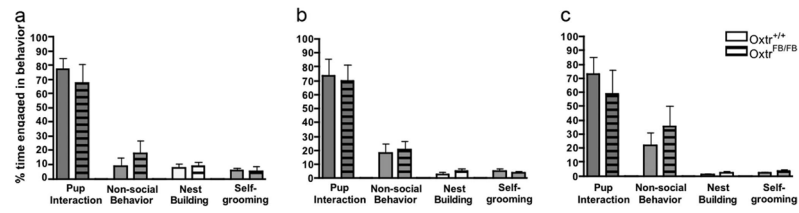
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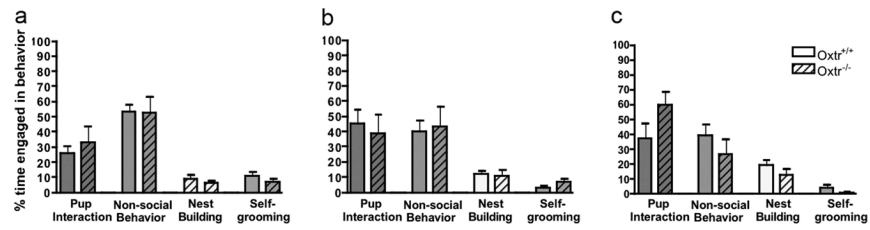
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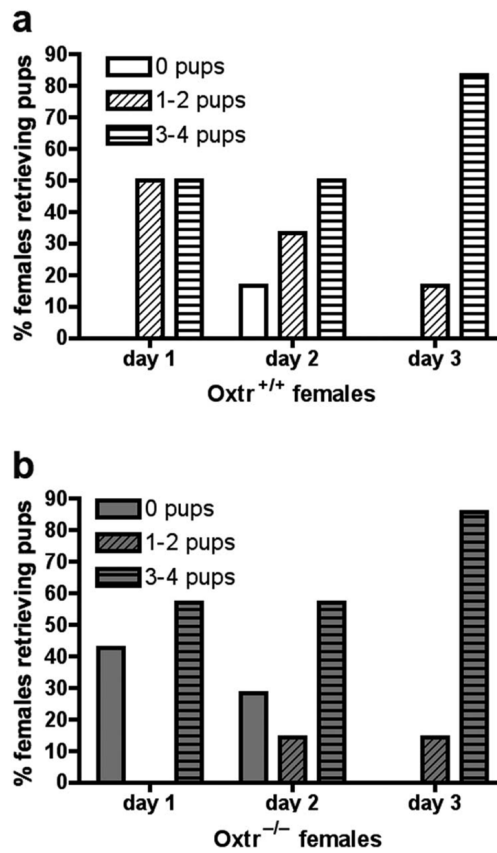




**Figure 1.** Maternal behavior of postpartum OxtR<sup>+/+</sup> and OxtR<sup>FB/FB</sup> females toward biological offspring. No statistically significant genotypic differences in maternal behavior were observed on (a) Day 1, (b) Day 2, or (c) Day 3.

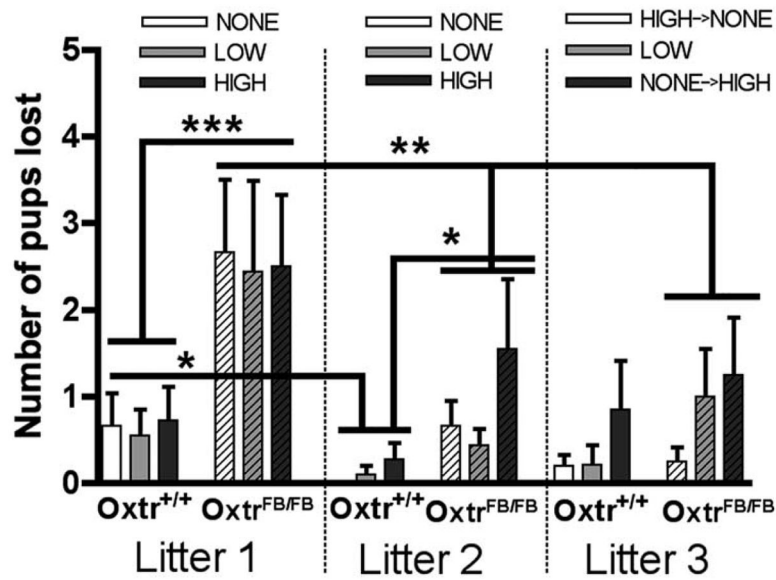


**Figure 2.** Maternal behavior of virgin OxtR<sup>+/+</sup> and OxtR<sup>-/-</sup> females toward foster pups. No statistically significant genotypic differences in maternal behavior were observed on (a) Day 1, (b) Day 2, or (c) Day 3.



**Figure 3.**

Pup retrievals by virgin *Oxttr*<sup>+/+</sup> and *Oxttr*<sup>-/-</sup> females. 100% of *Oxttr*<sup>+/+</sup> females (a) retrieved at least one pup to the nest on Day 1 of testing. In comparison, over 40% of *Oxttr*<sup>-/-</sup> females (b) failed to retrieve any pups to the nest on Day 1. However, by Day 3 both genotypes had identical levels of pup retrieval, with 100% retrieving at least one pup and over 80% retrieving 3–4 pups.



**Figure 4.**

Comparison of pup mortality in *Oxttr*<sup>FB/FB</sup> females across three litters. Both *Oxttr*<sup>+/+</sup> and *Oxttr*<sup>FB/FB</sup> females lost significantly more pups on their first litter compared with their second litter, regardless of disturbance group. Only *Oxttr*<sup>FB/FB</sup> females lost significantly more pups on their first litter as compared with their third litter. On both litter 1 (*left*) and litter 2 (*middle*), *Oxttr*<sup>FB/FB</sup> females lost significantly more pups compared with *Oxttr*<sup>+/+</sup> females, regardless of disturbance group. On litter 3, there were no significant differences in pup loss between the two genotypes, and switching the NONE and HIGH groups did not significantly affect pup mortality. Litter 1: *Oxttr*<sup>+/+</sup>: NONE = 9; LOW = 9; HIGH = 10; *Oxttr*<sup>FB/FB</sup>: NONE = 9; LOW = 9; HIGH = 10. Litter 2: *Oxttr*<sup>+/+</sup>: NONE = 8, LOW = 10; HIGH = 10; *Oxttr*<sup>FB/FB</sup>: NONE = 9; LOW = 9; HIGH = 9. Litter 3: *Oxttr*<sup>+/+</sup>: NONE = 10; LOW = 9; HIGH = 7; *Oxttr*<sup>FB/FB</sup>: NONE = 8; LOW = 5; HIGH = 8. \**p* < .05; \*\**p* < .01; \*\*\**p* < .001.