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The Role of the Estrogen Receptor-α Gene, Esr1, in Maternal-Like Behavior in Juvenile Female and Male Rats

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Abstract

The estrogen receptor-alpha (ER-α) is an important ligand activated transcription factor that works to control gene transcription in many species. Previous studies have shown estrogen to be an important hormone in the regulation of maternal behavior. Like adult female rats, both male and female juvenile rats exhibit increased level of maternal-like behavior when exposed to pups. The aim of this study was to determine whether ER-α is critical for the expression of maternal-like behavior in juvenile male and female rats. ER-α knock-out and wildtype (WT) juvenile male and female rats were generated and tested for maternal behaviors. Latencies to display maternal-like behaviors that included retrieval, grouping and crouching responses, revealed no genotype differences between KO and WT subjects. Male juvenile rats exhibited slightly shorter latencies than WT juvenile female rats indicating a sex difference in the latency to display these responses. Additionally, ER-α KO females exhibited a delay in onset of vaginal opening compared to WT females, indicating a role for ER-α in sexual maturation. The behavioral findings indicate that ER-α is not obligatory for the expression of full maternal-like behavior in male and female juvenile rats. Understanding this neurobiological system will help to elucidate the developmental involvement of the endocrine and brain networks in the regulation of maternal behaviors in mammals.

Keywords

estrogen receptor-alpha; maternal-like behavior; ER-α knockouts; juvenile female and male rats; vaginal opening

Introduction

Maternal behavior is a highly complex response in mammals, influenced not only by genetics and the environment, but also hormonal interactions. One key endocrine component

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Conflict of Interest

Each author has no conflict of interests related to the present manuscript submission.

involved in regulating maternal behavior in rodents as well as sheep is the steroid hormone estradiol (see Bridges, 2015 for a review). Estrogens through their actions on estrogen receptors appear to regulate both the incidence and quality of maternal care. In mice, deletion of estrogen receptor alpha results in a constellation of behavioral deficits, including feminine sexual behavior, aggression, and to a lesser extent the parental behavior of males and females toward pups (Ogawa et al., 1998).

One neural site of estrogenic regulation of maternal behavior is the medial preoptic area (MPOA) as implantation of crystalline estradiol into the MPOA of pregnancy-terminated rats stimulates maternal behavior towards foster young (Numan et al., 1977). In addition, increased estrogen receptor alpha activity in the MPOA is associated with higher levels of pup-licking and grooming in rats (Champagne et al., 2003; Champagne et al., 2006) and mediates increased oxytocin binding to its own receptor, a factor that appears important in rats for the induction of maternal behavior (Champagne et al., 2006; Pedersen, 1997). Together, these data support an important role for estrogen in the regulation of maternal care.

A recent study found that ER-α activity may mediate maternal-like behavior in juvenile female rats (Pena et al., 2014). It is well established that rats of both sexes display a rapid onset of maternal care when first tested daily for responsiveness toward foster young starting at 24 days of age (Bridges, 1974; Brunelli et al., 1990). Pena et al. found that infusions of an adenovirus expressing the estrogen receptor alpha gene, Esr1, into the MPOA during early postnatal development increased the level of maternal care after weaning in juvenile female offspring. Male offspring were not examined in this study. Given that juvenile display high levels of maternal-like responsiveness, that Esr1 may be involved in juvenile maternal behaviors, and that estrogens play an important role in adult maternal care, it is of interest to evaluate whether the lack of the Esr1 gene might interfere with the high level of post-weaning maternal responsiveness in either or both male and female juvenile rats. This possibility was addressed in the present study using the newly established Esr1 rat Esr1 knockout model (Rumi et al., 2014). Our approach was to evaluate the possible effects of deletions of the estrogen receptor alpha gene (Esr1) on the maternal responsiveness of juvenile male and female rats towards foster pups during post-weaning development. The responses of knockout subjects were compared with those of wildtype (WT) male and female littermates. Our hypothesis was that deletion of Esr1 (Esr1 knockouts) would interfere with the expression of maternal responsiveness in both juvenile female and male rats. An understanding of this system will help to further elucidate the involvement of the endocrine system, and specifically estrogen receptor-α, in the regulation of maternal behavior throughout development.

Methods

Generation of ESR1 KO Rats

Two heterozygous male and female pairs of Holtzman Sprague Dawley Esr-1 KO (RRRC:0701 [Esr1-d482]) rats were purchased from University of Missouri Metagenomics Center (MUMC) & Rat Resource and Research Center (RRRC) (University of Missouri) at about 50 days of age. The Esr1 KO homozygous rats were initially produced by Rumi et al., (2014). At 8 weeks of age the male and female heterozygous rats were bred with WT male

or female rats to produce a ratio of approximately ½ heterozygous ER-α KO to ½ WT male and female rats. Heterozygous ER-α KO male and female rats were then mated to produce an expected ratio of ¼ ER-α WT to ½ heterozygous ER-α KO to ¼ homozygous ER-α KO male and female rats. Only ER-α WT and homozygous ER-α KO male and female rats were used in this experiment.

Eleven litters were generated for this experiment, with an average litter size of 12–14 pups. In the event of an abnormally large litter, pups were culled to 10–12 pups on postpartum day two. Newborn litters were sexed, based upon urogenital distance, and then genotyped at 18 days of age by ear punching each rat and sending the samples for genotype processing (WT (+/+), heterozygous Esr1 (+/−) or homozygous Esr1 (−/−) by TransnetYX® (Cordova, TN).

Animals

Using the breeding protocol described above, 47 experimental rats were generated and assigned to one of four experimental groups based upon their genotypes: 12 WT males, 12 homozygous ER-α KO males, 11 WT females, and 12 homozygous ER-α KO female juvenile rats. All subjects were individually housed in translucent cages (45 × 25 × 20 cm) at 21 days of age. At day 23 of age, the day prior to the start of behavioral testing, one inch tall, quadrant dividers were placed into the cages of the experimental animals to prevent test young from crawling to the test subjects' nests. Animals were maintained in light (14:10 light: dark cycle; lights on at 0500 h) and temperature (21°C–24°C) controlled rooms throughout the study. Food and water were provided ad libitum, and Teklad Sani-chips (Envigo, South Easton, MA) were used for cage bedding material with two paper wheels provided for nesting material.

A group of 25–30 donor lactating mothers (Sprague-Dawley CD strain) were maintained throughout this experiment to generate a set of 3–8-day old test pups used in the behavioral testing. Test pups were used, if they were well fed and were not used on repeated test days. Since lactating rats readily accept pups from other mothers, it was not necessary to control for which donor mothers provided the source of test pups. Donor lactating rats were maintained in a separate colony room. All rats in this study were maintained in accordance with the guidelines of the Division of Teaching and Research Resources at Tufts University, Cummings School of Veterinary Medicine following the procedures for animal care prepared by the National Research Council Committee of the Care and Use of Laboratory Animal Resources. The research protocol was approved by Tufts IACUC (#G2016–80).

Maternal Behavior Testing

All ER-α KO and WT rats were tested daily for maternal-like behavior starting at day 24 of age. To commence testing, three healthy rat pups (3–8 days of age) obtained from donor dams were placed into each of the three cage quadrants (excluding the nest quadrant) of the test subject's test cage. Testing began daily between 0930 h and 1000 h. Each subject was observed continuously for 15 minutes during which time the latencies to display the following behaviors were recorded: making first contact with one of the three pups, retrieval of one pup, retrieval of all three pups to the nest, grouping all pups in the nest, and crouching over all three pups. After the initial 15-minute test period, four additional spot checks were

made at 30−, 45−, 60− and 120−minute time points. The relative locations of the three rat pups to the experimental animal were recorded at each time interval. Test pups were left with the experimental animals until the next test session on the following day, and a pre-test spot check was performed in the morning prior to the pups' removal from the cage to note the location of the pups and test subject. The positions of the rat pups from the previous day's testing were observed (pre-test check for overnight grouping) to determine whether pups were grouped in the subject's nest. Pups were then removed from the test cages and returned to donor dams. An hour later, three recently fed pups obtained from donor mothers were placed into each test cage to initiate another test session.

Experimental animals were tested once daily for maternal-like behavior. Full maternal-like behavior (FMB) was defined as the retrieval, grouping, and crouching over of all three rat pups within the two-hour testing period on two consecutive days. A score was assigned to each animal equivalent to their first day of displaying full maternal-like behavior. For example, if an experimental animal reached FMB on test days 2 and 3, it would be assigned a FMB latency score of 1. Individual behaviors (retrieval, grouping, crouching) were recorded daily as components of maternal responsiveness can occur independent of full responsiveness (FMB). Testing continued until an animal reached the criterion of two consecutive days of full maternal-like responsiveness or until the 10-day test period elapsed. Experimental rats were again weighed at 33 days of age at the end of the 10-day testing period. Starting at 33 days of age, female ER-α KO and WT rats were checked for vaginal opening daily until full vaginal opening was observed.

Statistics

Behavioral and body weight data were analyzed using IBM® SPSS® Statistics 24.0 software. Normally distributed data were analyzed using an ANOVA (2 × 2 design with planned comparisons). Vaginal opening data were analyzed using a one-way analysis of variance with genotype as the variable. When data that were not normally distributed (i.e. retrieval of the first pup), the results were analyzed using non-parametric statistical analyses (Kruskal-Wallis and Mann Whitney tests). GraphPad® Prism® 7 software was used to generate figures. Statistical significance was set as a probability of $P < 0.05$.

Results

Maternal-Like Behavior

No significant difference was found between genotype and/or sex in the rates of display of what was defined as maternal-like behavior, i.e. retrieval, grouping and crouching responses, although there was a trend toward a significance between sex and genotype. Male WT rats exhibited FMB faster (mean = day 1.7) than WT female rats (mean = day 4.1) (see Figure 1). When the latency to display just the initial day of full responsiveness is compared among treatment groups, a significant sex difference emerged between the WT males and females ($P = 0.05$). WT males retrieved, grouped and crouched over the three test pups in their nests faster $\overline{X} \pm SEM = 0.8 \pm 0.2$ days) than did WT females $\overline{X} \pm SEM = 3.4 \pm 0.9$ days). It is noteworthy that both KO female and male juvenile rats displayed full maternal-like responsiveness with latencies of less than three days.

Pup Retrieval

The data for first pup retrieval was analyzed using nonparametric statistics due to the lack of variability in the responses of Esr1 KO females. Analysis revealed a significant overall statistical difference across groups for first retrieval latency score ($H = 4.157$, $df = 3,43$; $P = 0.006$). Post hoc testing revealed a significant difference in retrieval of the first pup between male and female Esr1 KO homozygous juvenile rats ($U = 48.0$, $P < 0.05$). All Esr1 KO homozygous female subjects retrieved the initial pup on the first test day, faster than Esr1 KO homozygous male subjects whose average response latency was 1.6 days. Overall, the presence or absence of Esr1 did not affect the rate of retrieval of the first pup in either sex.

No significant differences were found among the groups in terms of latencies to retrieve the second and third test pups. All juvenile rats retrieved the second and third test pups in a similar amount of time - pup two $\overline{X} \pm SEM = 1.9 \pm 1.2$ days) and pup three $\overline{X} \pm SEM = 2.2 \pm 1.4$ days).These data indicate that there is no effect of sex or genotype in the retrieval of the second or third pups.

Grouping and Crouching Responses

Individual grouping and crouching responses for each group paralleled those for retrieval responses and were included as a component of full responsiveness. No significant differences were found in the grouping and crouching responses among test groups. Grouping and crouching responses occurred concurrent with the test animals' scores for retrieving all three test pups. Hence, these responses are not presented separately.

Pre-Test Score – Overnight Grouping

No significant differences were found among any groups in the juvenile rat's pre-test scores ($\overline{X} \pm SEM = 0.8 \pm 0.2$ days; see Table 1). Thus, there is no effect of sex or genotype on the juvenile rats' pre-test scores.

Body Weights

Analysis revealed an overall significant difference between body weights of WT male and female juvenile rats on Day 33 of testing ($F = 3.044$, $df = 3, 42$, $P = 0.039$). There was significant effect of sex ($P = 0.005$) with males weighing more than females and WT male juvenile rats weighing more on day 33 $\overline{X} \pm SEM = 139.9 \pm 3.5$ grams) than did WT female juvenile rats (.$\overline{X} \pm SEM = 126.7 \pm 3.0$ grams)Body weights of Esr1 KO males and females at 33 days of age did not differ (see Table 1).

Vaginal Opening

A significant difference was found between WT and homozygous Esr1 KO female juvenile rats in the time of vaginal opening ($t = 3.57$, $df 1,20$; $P = 0.002$). Female WT juvenile rats displayed full vaginal opening, an indicator of sexual maturation, earlier $\overline{X} \pm SEM = 33.6 \pm 0.4$ days). than homozygous Esr1 KO female juvenile rats $\overline{X} \pm SEM = 38.6 \pm 1.3$ days).

Discussion

The results of the present study demonstrate that a rapid onset of maternal-like behaviors is displayed in both juvenile female and male rats in the absence of the estrogen receptor-alpha. Subjects lacking the Esr1 responded with equally short latencies as did WT controls when testing was initiated at 24 days of age. The average latencies to exhibit individual components of maternal-like care and full responsiveness averaged less than 2 days, responses consistent with those of earlier published studies in juvenile male and female rats (Bridges et al., 1974; Brunelli et al., 1990). These findings indicate that the expression of maternal-like care in the post-weaned, juvenile rat is not dependent upon the presence of the Esr1 gene and likely is not physiologically affected by the actions of estrogens on this receptor at this stage of development.

An earlier report by Pena et al., (2014) found that female juvenile rats given early postnatal MPOA infusions of an adenovirus expressing Esr1 increased the level of maternal-like care. It was somewhat unexpected based on earlier studies that the control females' responses averaged about 4 days; latencies that were lowered to 2-day latencies. It is noted that in prior studies control latencies for juvenile females and males typically average about 1–2 days (Bridges et al., 1974; Brunelli et al., 1990) It is possible that under conditions where response latencies in juvenile females appear to be are delayed (Pena et al., 2014) enhancement of Esr1 gene expression may instate a more rapid responsiveness. However, data from the present report indicate that Esr1 activity does not appear to be necessary for full rapid responsiveness. Rather, an involvement of this receptor complex under specific conditions (Pena et al., 2014) may help modulate and restore rapid responsivity in juvenile female rats.

The involvement of the ER-α receptor in maternal behavior in the adult female rodents is more definitive. Champagne and colleagues (Champagne et al., 2003, 2006) demonstrated that the quality of maternal care transmitted across generations in rats is mediated by ER-α. Likewise, silencing ER-α RNA in the MPOA severely attenuates maternal care in mice (Ribeiro et al., 2012). These findings together with the earlier work of Numan et al (1977) demonstrating that the MPOA is a site of estrogen regulation of maternal behavior are supportive of the idea that ER-α plays an important role in given aspects of maternal behavior. It is of interest, however, that the induction of maternal behavior brought about by constant pup exposure in nulliparous rats is similar in Esr1 knockout and WT adult female rats (Gallagher et al., 2019). Thus, both in juvenile development, as shown here, and as adults, nulliparous females rats do not display major impairments in their expression of maternal care, i.e. induction rates. This would indicate that ER-α is not involved in the nonhormonal basis of the behavior (Rosenblatt, 1967), but rather mediates the endocrine modulation of maternal care.

It is pointed out that the subjects in this study lacked the Esr1 genotype throughout prenatal and postnatal life thorough the time of behavioral testing as juveniles. It is possible that these animals experienced some form of neural compensation that made their reliance on the estrogen receptor alpha during juvenile development less obligatory. It would also be interesting to examine a conditional KO during juvenile development to compare maternal

responsiveness in both juvenile female and male WT and conditional KO rats. Furthermore, a conditional region-specific Esr1 KO, such as one directed at the MPOA in mice using siRNA to silence ER-α (Riberio et al., 2012), an area vital for the expression of maternal behavior (Numan et al., 1977) would help to solidify the role of Esr1 in juvenile rats on maternal-like responsiveness.

What is the evidence that the endocrine system might in part regulate juvenile maternal-like behavior? Numerous studies have examined the role of specific hormones and neuropeptides in the expression of juvenile maternal-like behavior. Efforts to establish a role for testosterone and gonadal function in male juvenile rats were without success (Bridges et al., 1974). Likewise, efforts to assess whether progesterone might mediate the rapid onset of juvenile maternal care failed to identify a role for progesterone in this process (Nephew et al., 2008). In another study, young rats were treated with the opiate antagonist naltrexone during the transition period from rapid to slower onset rates (days 24 to 30 of age) to see whether a shift in opiate activity might account for the increase in latencies in rats first tested at 30 days of age (Zaias et al., 1996). The responsiveness of the young rats was unaffected by treatment with an opiate antagonist. The only report that demonstrated possible endocrine involvement in juvenile maternal-like care was reported when juvenile male rats were treated with the dopamine agonist, bromocriptine, to suppress prolactin secretion (Kinsley & Bridges, 1988). Injections of prolactin in similarly treated bromocriptine-injected juvenile males partially restored faster onsets of maternal care. Therefore, the preponderance of the experimental evidence indicates that the expression of short-latency maternal-like behavior in juvenile male and female rats occurs relatively independent of major endocrine mediation, including an involvement of Esr1.

That the strain of rat used to develop the knockout model was generated from different sources (Harlan Farms, Sprague Dawley) likely did not impact the basal responsiveness of the juvenile test subjects. The maternal-like latencies of control female and male test subjects in the present study were almost identical to those previously reported using Sprague-Dawley rats obtained from Charles River Laboratories (Kinsley & Bridges, 1988; Brunelli et al., 1990) as well as Wistar juvenile rats (Bridges et al., 1974). Thus, it appears that the knockout model itself using a different animal provider did not impact the underlying rapid expression of maternal-like responsiveness in these juvenile rats. The ethological significance of the high level of maternal-like responsiveness in juvenile female and male rats is not known. One can speculate that that given postpartum ovulation and mating by the mothers that the juvenile subjects may participate in some form of alloparenting directed towards pups from a second litter born during this juvenile period of an initial litter or minimally not behave aversively towards the new litter. However, such ethological studies under more naturalistic conditions remain to be conducted. Hence, it is not possible to state whether the maternal-like responses of female and male juvenile rats towards younger pups can be considered as a form of alloparenting.

It is noted that whereas the physiological and reproductive effects of Esr1 KOs have previously been reported (Rumi et al., 2014), we were able to extend their findings by showing that the onset of vaginal openings in the Esr1 KO females was delayed by as much as 6 to 7 days. Whereas almost all WT females displayed full vaginal openings when first

examined at 32 days of age, the average age of full vaginal opening in the Esr1 KO females was approximately 39 days of age. This observation provides further documentation of the effects of ESR1 gene deletion.

In summary, Esr1 knockout male and female juvenile rats displayed normal rates of behavioral responsiveness to rat pups. Whereas ER-α activity is not required for this rapid onset of maternal-like care at this developmental stage, it is possible that under some conditions ER-α may modulate expression of maternal care.

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Highlights

- Estrogen receptor alpha knockout juvenile rats display normal maternal behavior.
- Male and female *Esrl* KO rats respond rapidly with similar onset latencies.
- Vaginal opening onset is delayed in female *Esrl* knockouts.
- Presence of the *Esrl* gene is not needed for juvenile rapid onset maternal-like care.

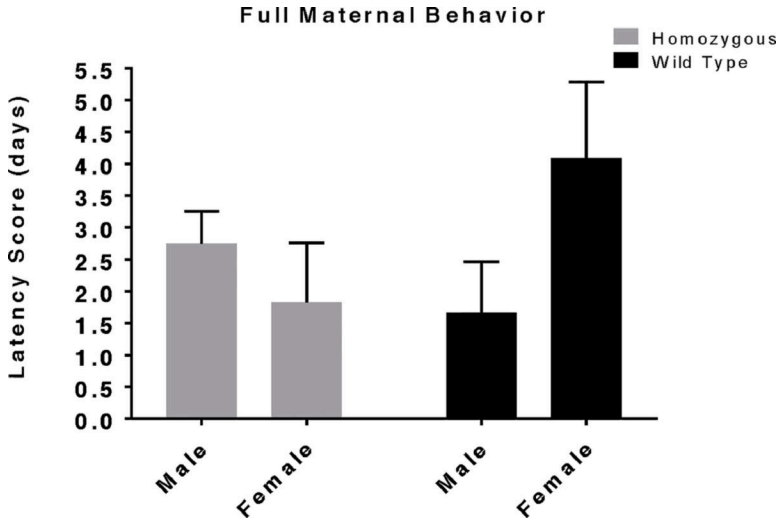

Figure 1:

Latencies in days (mean ± SEM) for WT and ER-α KO male and female juvenile rats to display retrieval and grouping of all three test pups and assume a crouching position over the test young. This composite set of responses is defined as full maternal-like behavior. All groups were composed of 12 animals, except WT female juvenile rats, which consisted of 11 animals. Male WT juvenile rats exhibited a trend towards a shorter latency to show full maternal-like behavior than WT female juvenile rats.

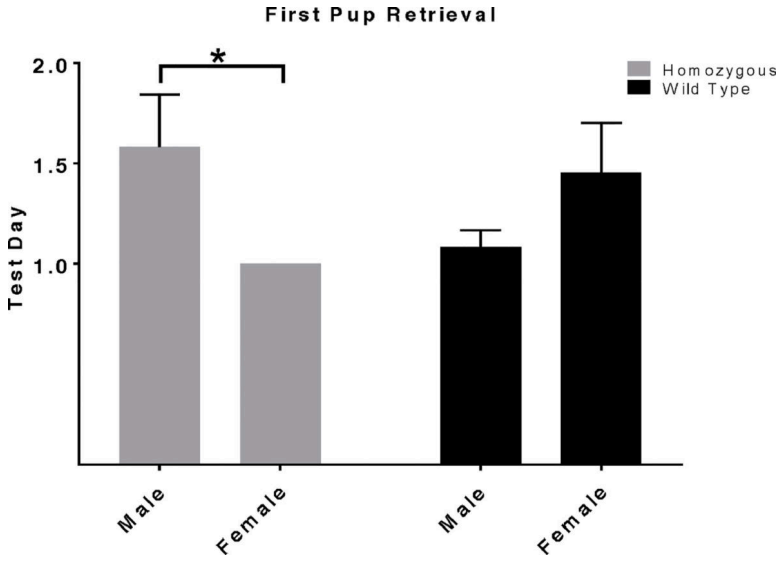

Figure 2:

Latency to retrieve a pup (means ± SEM) for WT and ER-α KO male and female juvenile rats. All groups were composed of 12 animals, except WT female juvenile rats, which consisted of 11 animals. Note that all KO females retrieved a pup during the first test session. Hence, there is no error bar for this group. Esr1 homozygous KO juvenile female rats retrieved the initial pup faster than did male Esr1 homozygous KO juvenile rats. *P < 0.05.

Table 1.

Pre-test responsiveness, body weights, and vaginal opening data for WT and Esr1 KO subjects.

	Endpoints		
Treatment Group	Positive Pre-Test Score†	Body Weight Day 33 (grams)	Vaginal Opening Age (in days)
WT Female (11)	0.8 ± 0.4	126.7 ± 3.0	33.6 ± 0.4
Esr1 KO Female (12)	0.6 ± 0.4	132.3 ± 2.7	38.6 ± 1.3*
WT Male (12)	1.0 ± 1.0	139.8 ± 3.5*	-
Esr1 KO Male (12)	0.9 ± 0.4	138.2 ± 4.3	-

†A latency score of 0 means that a positive pre-test score was noted on test day 2, the first pre-test observation. Sample sizes, Ns, are shown in parentheses. Values are expressed as means ± SEM.

*P < 0.01 versus WT females.