Decreased maternal behavior and anxiety in ephrin-A5<sup>−/−</sup> mice

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During development of the nervous system, molecular signals mediating cell–cell interactions play critical roles in the guidance of axonal growth and establishment of synaptic functions. The Eph family of tyrosine kinase receptors and their ephrin ligands has been shown to mediate neuronal interactions in the development of topographic axon projection maps in several brain regions, and the loss of Eph activities result in defects in select axonal pathways. However, effects of deficiencies of the Eph signals on animal behavior have not been well documented. In this study, we showed that inactivation of a ligand of the Eph receptors, ephrin-A5, resulted in defects in maternal behavior and alterations in anxiety. Female ephrin-A5<sup>−/−</sup> mice show significant defects in nest building and pup retrieval. In addition, lower levels of anxiety were observed in both male and female null mice. These changes were not due to deficiencies in estradiol, progesterone or corticosterone levels. Our observations suggest that ephrin-A5 plays a key role in the development and/or function of neural pathways mediating mouse maternal care and anxiety.

Keywords: Anxiety, Eph receptor, ephrin-A5, estrogen, maternal care, nest building, progesterone, pup retrieval

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Proper maternal behavior is essential for survival of the offspring (Numan & Insel 2003). It is important for growth and normal development of the young and can influence their physiological, behavioral and cognitive functions in adult life (Franklin et al. 2012). Early life stress in the form of maternal neglect, separation or limited bedding/nesting, produces behavioral and emotional changes as well as long lasting alterations in cognitive functions (reviewed in Meaney 2001; Molet et al. 2014; Veenema 2009). Adult rats that were separated from their mothers earlier in life had exaggerated inter-male aggression, increased depression-like behavior and altered levels of arginine vasopressin (AVP) and serotonin (5-HT) in the hypothalamus (Veenema et al. 2006). In addition, differences in the hypothalamic-pituitary-adrenal (HPA) response to stress were found in the offspring of low vs. high licking and grooming (LG) rats (Liu et al. 1997). Adult rats that were born and raised by high-LG mothers had decreased plasma corticosterone (CORT) levels in response to restraint stress compared to those raised by low-LG mothers (Liu et al. 1997). Maternal care can also affect the development of maternal behavior in the female offspring (Melo et al. 2006; Shoji & Kato 2009). Shoji & Kato (2009) compared the maternal behavior of the offspring of two inbred mice, CBA/Ca and BALB/c, which differ in their levels of maternal care; CBA/Ca females exhibit nursing and pup licking more frequently and retrieve their pups faster than BALB/c females. They found that low levels of maternal care earlier in life (by BALB/c dams) decreased maternal behavior in the offspring.

In rodents, a wide range of behaviors directed toward the care of the young have been reported. These include nest building, pup retrieval, aggression towards a male intruder, nursing, pup LG and crouching over the pups to provide thermoregulation (Weber & Olsson 2008). Interestingly, these behaviors can be seen in non-parental virgin female rodents. When initially exposed to pups virgin female rats will avoid them. Yet, after continuous daily exposure of about 7 days the female will start caring for the young. This pup-induced behavior is referred to as sensitized maternal behavior (Numan 2014). In contrast to rats, however, mice do not require the ‘sensitization period’ and show spontaneous maternal behavior when presented with pups (Numan 2014). The level of maternal care is higher in postpartum female mice which are faster to retrieve the pups, spend more time crouching over them (Gandelman et al. 1970; Stolzenberg & Rissman 2011) and are able to build a better, more complex nest than virgin female mice (Bond et al. 2002). In addition, maternal motivation is lower in virgin compared to postpartum females (Stolzenberg & Rissman 2011).

The use of gene knockout mice has made a significant contribution to our understanding of the genetic regulation of maternal behavior. Mice carrying a null mutation for the prolactin receptor gene (Lucas et al. 1998), the dopamine (DA) β-hydroxylase gene (Thomas & Palmiter 1997), and the forebrain Gq/11 gene (Wettschureck et al. 2004) exhibit defects in maternal behavior compared to wild-type controls. In addition, several brain regions and neural circuits have been implicated in the development of maternal behavior, specifically the medial amygdala (MeA), the ventral tegmental area (VTA) of the midbrain, the medial prefrontal cortex
Materials and methods

Animals

Ephrin-A5−/− mice have been described previously (Frisen et al. 1998). Animals used in this study are on a mixed background (C57BL/6 and 129/SV) and were generated using heterozygous crosses. For all behavioral tests and to check for differences in hormonal levels, knockouts and wild-type controls were obtained from the same littermates. In order to measure body weight from wild-type and knockout pups, their body weight was measured every 2 days until 1 week of age. If the weight of the pups was not within the normal range (within 10% of the mean), they were excluded from the study.

To test for maternal behavior (pup retrieval, maternal aggression, and nest building), pregnant females were housed separately until time of birth and the day of birth was recorded as postnatal 0 (P0). Pup body weights were measured every 4 days until P20 and again at P60. All pups were labeled for individual identification by marking their tails. Genotyping was performed by tail DNA polymerase chain reaction (PCR) as described previously (Frisen et al. 1998). Primers 1: 5′TCCAGCTGTGCGATCTCAGAAAC3′ and primers 2: 5′ATCCAGAGGTTGACTCACCATA3′ were used for amplification of wild-type sequences (397 bp) and primers 1 and 3 (5′AGCCCAAGAAGCGAGGAAGAAAC3′) were used for amplification of null sequences (153 bp).

Nest building

The test was performed as described previously (Deacon 2006) with some modifications. For maternal nest building, ephrin-A5−/−, heterozygous and wild-type virgin female mice (n = 5 per genotype) were housed with a heterozygous male mouse and checked for the presence of a sperm plug; once a plug was observed, the date was recorded as embryonic day 0.5 (E0.5) and the male was removed from the cage. On E18 five pregnant mice were transferred to a new clean home cage and provided with nesting material (nestlets; Ancare Corporation, Bellmore, NY USA). Nests were then observed 1, 6 and 24 h later and assessed on a rating scale of 1–5, according to the nest sample photographs shown by Deacon (2006) by two observers who were blind to the genotype and time of the nest. Observer reliability was determined with Cohen’s kappa method. A score of 1 was given if there was no nest and a score of 5 represented a perfect, fully enclosed nest. In order to assess nest build by nulliparous mice, adult virgin females (n = 6 per genotype; P ≥ 60) were individually housed overnight with food, water and new bedding. The next morning they were provided with the nesting material and the procedure was repeated as described above.

Maternal aggression and pup retrieval test

Ephrin-A5−/− (n = 8), heterozygous (n = 10) and wild-type (n = 8) lactating female mice were exposed to wild-type intruder male in their home cage for 5 min on postpartum day 4 (P4) and 6 (P6). The pups were removed from the cage 2 min before the behavioral test, and the test was recorded for subsequent analysis. The intruder males were sexually naive and group housed. After the maternal aggression test, the pups were randomly distributed throughout the cage and the time to retrieve the first and third pup was recorded. Retrieval counted only when the pup was brought into the nest completely and, a score of 180 seconds was assigned if the dam failed to retrieve her pups in 3 min.

For the cross retrieval of pups with different genotypes, a new set of lactating female mice (ephrin-A5−/−, n = 10, wild-type, n = 9) were used on P2. The pups were removed from their home cage 30 min before the test and kept in a new separate cage. Each female was then introduced to three pups from a different litter such that null dams tested with wild-type pups, and wild-type dams with null pups. The time to retrieve the first and third pup was recorded for a maximum of 5 min.

In both tests the females were housed with a heterozygous male mouse and transferred to a new cage 2–3 days before parturition. The cage was not changed until the end of the experiments (P7 for the retrieval of own pups and P3 for the cross retrieval of pups with different genotypes).

Elevated-plus maze

The maze was constructed of black Plexiglas with four arms in the form of a ‘plus’, 30 cm above the floor. Two opposing arms of the maze (65 cm long) were enclosed in 8 cm high, black Plexiglas walls, while the two remaining arms (30 cm long) were left open. Two experiments were conducted using the elevated-plus maze (EPM). In experiment 1 we tested male ephrin-A5−/− (n = 7) mice that were born and reared by ephrin-A5−/− mothers and wild-type (n = 6) control mice that were born and reared by wild-type mothers. In experiment 2, male and female ephrin-A5−/− (n = 10 per sex), heterozygous (n = 11 per sex) and wild-type (n = 10 per sex) littermates that were born to heterozygous mothers were tested. Mice in the second experiment
were born and reared by the same mother, and thus the effect of rearing was removed. The test began when each mouse was individually placed in the center (6 × 5 cm²) of the maze facing an open arm and the number of times each animal entered an arm (either closed or open) as well as the duration spent in each arm were recorded for 5 min. The number of entrances into the open arm and/or the time spent in the open arms provides indications of anxiolytic-like behaviors, and the total number of entrances (into both open and closed arms) is a measure of locomotor activity. Elevated-plus maze testing was carried out under dim light and an arm entry was recorded only when all four paws crossed into the arm.

**Light–dark box test**

The test was performed as described previously (Rossi-George et al., 2004), with some modifications. Briefly, the plexiglas box (47 × 24 × 21 cm³, L x W x H) was divided into two compartments, one black-walled fully opaque (14 cm long), and the other (33 cm long) lit from the compartment ceiling by a 20 W bulb. Free passage was allowed between the compartments by a small 4 cm² opening. Male and female ephrin-A5−/− (n = 10 per sex), heterozygous (n = 10 per sex) and wild-type (n = 9 male and 10 female) mice were individually placed in the dark compartment and allowed to freely explore the box for 5 min. All trials were videotaped for subsequent analysis. Latency to emerge from the dark compartments, light–dark transitions, time in the light compartment and a risk assessment, in which the head and fore-paws extended into the lighted area but the remainder of the body stayed in the dark compartment (Bailey & Crawley, 2009) was recorded.

**Corticosterone ELISA**

Blood samples were collected by tail bleeding from male and female ephrin-A5−/− (n = 5 per sex) and wild-type (n = 5 per sex) littermates. Briefly, the tail was dipped in warm water (37°C) for 30 seconds after which the tip of the tail (<0.5 cm) was removed using surgical blade. Blood (20 μl) was collected into microvette tubes (Kent Scientific, Torrington, CT, USA, cat no. MCVT200-7ER) and allowed to clot at room temperature for 1 h. The blood was then centrifuged (10 000 g) for 5 min at 20°C to isolate upper layer serum and the CORT levels were measured by a competitive enzyme immunoassay (Arbor assay, Ann Arbor, MI, USA, cat no. K014). Blood collections were done in <3 min so that sampling is completed before activation of the HPA axis (Vahl et al. 2005). All standards and samples were run in triplicates. In order to compare CORT levels under both basal and stress conditions blood was collected twice from the same mice; once in the morning (first stage of the dark cycle) and again a week later after a 5-min exposure to the elevated plus maze test (stressor).

**Estradiol and progesterone concentration measurements**

Blood samples were collected from lactating females at P4 by tail bleeding (estradiol) as described above or cardiac puncture (progesterone). The blood was spun and the serum isolated. Serum concentration of estradiol were determined using enzyme-linked immunosorbent assay (ELISA) kit (Calbiotech, Spring Valley, CA, USA, cat# ES180S-100) and progesterone concentrations were determined with radioimmunoassay by the core laboratory at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

**Statistical analysis**

Data were analyzed using Statview statistical software. An unpaired Student’s t-test was used for two sample comparisons (EPM, estradiol and progesterone concentration measurements) and multivariate or repeated-measures analysis of variance (ANOVA)

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**Figure 1: Deficits of nesting behavior in primiparous (pregnant) ephrin-A5−/− mice.** Nest building was assessed in ephrin-A5−/−, heterozygous and wild-type pregnant (n = 5 per genotype) and non-pregnant females (n = 6 per genotype). (a) Nest rating was lower in pregnant mutant mice compared to wild-type controls starting 6 h after providing the nesting material (a, right panel). There were no significant differences in nest rating between the non-pregnant females (a, left panel). Data are presented as mean nest score ± SEM. (b) Representative pictures of nests built by pregnant wild-type and ephrin-A5−/− mice 24 h after providing the nesting material. *Indicates significant differences between null and wild-type mice; P < 0.05.
Results

Altered nesting behavior in primiparous (pregnant) ephrin-A5<sup>−/−</sup> mice

Primiparous ephrin-A5<sup>−/−</sup> mice showed impairment of nesting behavior compared to heterozygous and wild-type littermates (Fig. 1a, right panel). There were no significant differences between heterozygous and wild-type controls at all three time points tested ($P > 0.05$). However, null mice achieved significantly lower nesting scores at 6 h ($U = 0.5$, $P = 0.012$) and 24 h ($U = 2$, $P = 0.028$) compared to wild-type controls and lower scores at 24 h compared to heterozygous mice ($U = 3$, $P = 0.047$), showing a lower quality nest. One hour after providing the nesting material wild-type mice were already starting to make a nest, with the nesting square partially torn compared to the null mice that left the nesting square mostly intact. By 6 h wild-type mice built a nearly perfect nest with more than 90% of the nesting square torn, whereas null mice left most of the material intact. Although by 24 h the null mice shredded most of the nesting square, a well formed nest was usually not found in the cage (Fig. 1b). There were no significant differences in nest score between heterozygous and wild-type mice in all three time points tested. However, heterozygous mice had a significantly higher nest score then null mice at 24 h ($U = 3$, $P = 0.047$), suggesting that deletion of one copy of the gene is not sufficient to decrease nesting performance in these mice. The nest scores were determined by two blind raters, and the Interrater reliabilities as determined by Cohen’s Kappa method were $k = 1$, 0.785 and 0.916 for time points 1, 6 and 24, respectively.

Since non-pregnant, nulliparous mice are also able to build nests (Bond et al. 2002; Sherwin 1997) and nesting requires normal sensorimotor behaviors (Gaskill et al. 2012), we analyzed nests built by nulliparous, virgin female mice (Fig. 1a, left panel). There were no significant differences in nest score between the genotypes at all three time points suggesting that ephrin-A5 deletion does not affect the motor ability of the mice to build a non-maternal nest. The Interrater reliabilities of the two blind raters for these results as determined by Cohen’s Kappa method were $k = 0.913$, 1 and 1 for time points 1, 6 and 24, respectively. However, 24 h after introducing the nesting material, the nests of primiparous wild-type females rated significantly higher than those of nulliparous females ($U = 3$, $P = 0.028$). This difference was not observed in ephrin-A5<sup>−/−</sup> females where both primiparous and nulliparous mice built equal quality nests suggesting that the maternal contribution to nest quality is absent in the knockout mice.

No changes in maternal aggression between ephrin-A5<sup>−/−</sup> and wild-type mice

We examined maternal aggression in lactating female mice on postpartum days 4 and 6 because it has been shown that aggression is highest during the early lactation period (Svare et al. 1981). Pups were removed from the cage 2 min before the introduction of a wild-type male intruder into the home cage and the behavior of the dam was recorded for 5 min. A repeated measure ANOVA was used to analyze the latency to first attack as well as the number of attacks made by the dam. There were no statistically significant differences between the genotypes on either measurement (Fig. 2a, b).

Reduced pup retrieval in lactating ephrin-A5<sup>−/−</sup> mice

To further evaluate the role of ephrin-A5 on maternal behavior, we examined postpartum female mice in the...
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Figure 3: Pup retrieval is impaired in lactating ephrin-A5\(^{-/-}\) mice. The latencies to retrieve the 1st and 3rd pup on postnatal day 4 (a) and 6 (b) were tested in ephrin-A5\(^{-/-}\) (n=9 per test day), heterozygous (n=10) and wild-type (n=8) lactating female mice. There were significant differences between the genotypes on both test days where the mutant females took longer to retrieve their pups than both the wild-type and the heterozygous mice. Data are presented as mean latency to retrieve the x pup ± SEM. (c) The percentage of females that retrieve their pups were significantly lower in ephrin-A5\(^{-/-}\) mice compared to wild-type and heterozygous control females. (d) No differences were observed across the days tested. Data are presented as mean latency to retrieve the first pup ± SEM. *Indicates significantly different from wild-type mice; \( P < 0.05 \). **Indicates significantly different from heterozygous mice; \( P < 0.05 \).

Figure 4: Retrieval of wild-type pups is impaired in lactating ephrin-A5\(^{-/-}\) mice. The latencies to retrieve the first and third pup were significantly higher in ephrin-A5\(^{-/-}\) dams (n=10) compared to wild-type (n=7) controls. Data are presented as mean latency to retrieve the x pup ± SEM. *Indicates significantly different from wild-type mice; \( P < 0.05 \).

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mice regardless of the pups’ genotype, we concluded that the defects are in the dams and not the pups.

**Reduced pup survival in ephrin-A5−/− mice**

We observed decreased survival rate in pups that were born and reared by ephrin-A5−/− females compared to those born and reared by wild-type controls. Within the first postpartum week, pups reared by null female had a 55% survival rate compared to an 80% survival rate for pups reared by wild-type females (Fig. 5a). It is important to note that survival rate was highly correlated to the shape of the nest and to the level of pup gathering by the mother on the day of parturition; when a well-formed nest was not observed in the cage, and the mother was not gathering the pups after birth, the pups would not survive well (Fig. 5b).

**Body weight in ephrin-A5−/− mice**

Since we previously observed that ephrin-A5−/− animals had lower body weight (Sheleg et al. 2013), we sought to determine whether this reduction depends on the dam’s behavior. Body weights of heterozygous pups born and reared by ephrin-A5−/− (n=8), heterozygous (n=6) and wild-type (n=8) dams were measured every 4 days until P20 and again at P60 (Fig. 6a). The rationale here is that if heterozygous pups that were reared by null dams weigh less than those reared by wild-type or heterozygous control dams, then the dam behavior probably contribute to these differences. There were no genotypic differences between the groups (F2,61 = 1.444, P = 0.244; Fig. 6b). However, there was a significant effect of sex (F1,61 = 43.674, P < 0.001), postnatal day (F5,305 = 11096.859, P < 0.001), and postnatal day x sex interaction (F10,305 = 0.724, P = 0.70). Post hoc testing revealed that on P60 male mice weigh significantly more than female mice. The fact that heterozygous pups from all 3 dams (ephrin-A5−/−, heterozygous and wild-type) had similar body weights (Fig. 6b), suggests that the growth differences between the null and wild-type mice are not due to differences in maternal behavior. Finally, we wanted to confirm our previous results (Sheleg et al. 2013) and see if the differences between null and wild-type mice are consistent with the current breeding scheme: ephrin-A5−/− and wild-type females bred with heterozygous males (see Materials and methods and Fig. 6a). As expected, there was a significant effect of genotype (F1,27 = 22.719, P < 0.0001) and postnatal day (F5,135 = 4666.440, P < 0.0001) as well as postnatal day x genotype (F5,135 = 18.431, P < 0.0001) and postnatal day x sex (F5,135 = 35.739, P < 0.0001) interaction. Post hoc tests revealed that null mice weigh significantly less than wild-type mice on all the days tested (Fig. 6c). In addition, a sex difference was observed on P60 with female mice weighing less than male mice. These data support our previous results, and show that null mice weigh less than wild-type mice and that the decreased level of maternal care is not likely to be responsible for this difference.

**Serum estradiol and progesterone levels are comparable between the genotypes**

Since changes in steroid hormones around parturition have been shown to play a role on maternal behavior in
mammals (Knobil & Neill 1994), we sought to determine whether serum estradiol and progesterone levels are affected by ephrin-A5 deletion. We found no differences in estradiol (Fig. 7a; \( t = -0.490, P = 0.631 \)) and progesterone (Fig. 7b; \( t = -1.121, P = 0.5240 \)) between the genotypes.

**Altered anxiety-like behavior in ephrin-A5\(^{-/-}\) mice**

It has been suggested that early life care influences anxiety levels in adult life (Meaney 2001). In order to test whether the lower levels of maternal care seen in the null mice affects their offspring anxiety levels, adult ephrin-A5\(^{-/-}\) mice that were born and raised by ephrin-A5\(^{-/-}\) dams and wild-type mice that were born and raised by wild-type females were examined on the EPM (Fig. 8). The EPM test is one of the most commonly used assays to study the psychological and neurochemical basis of anxiety behavior in rodents (Bourin et al. 2007). It takes advantage of the normal preference of mice for a dark and protected space (the closed arm) over an open and exposed area (the open arm). Student’s t-test was used to analyze the absolute number and the percentage of open arm entries, as well as the time and percentage of time spent in the open arm and the total number of
to differences in maternal care, we repeated the above study using male and female ephrin-A5\(^{-/-}\), heterozygous and wild-type littermate mice that were born and reared by the same heterozygous mother (Fig. 9). There were again overall genotypic differences where ephrin-A5\(^{-/-}\) mice had a higher number of open arm entries (\(F_{2,56} = 21.550, P < 0.0001;\) Fig. 9a), and percentage of open arm entries (\(F_{2,56} = 12.657, P < 0.0001;\) Fig. 9b) as well as increased time spent in the open arm (\(F_{2,56} = 9.365, P = 0.0003;\) Fig. 9c) and percentage of time spent in the open arm (\(F_{2,56} = 7.330, P = 0.002;\) Fig. 9d) compared to heterozygous and wild-type littermates. In addition, null mice had an increased number of total entries (\(F_{2,56} = 23.056, P < 0.001;\) Fig. 9e) into both arms. There were no significant effects of sex or genotype \(\times\) sex interactions in the absolute number and the percent of open arm entries as well as in the time and percent of time spent in the open arm. However, a genotype \(\times\) sex interaction was found in the total entries (\(F_{2,56} = 4.446, P = 0.020\)). Post hoc tests revealed that ephrin-A5\(^{-/-}\) male mice had increased total entries compared to ephrin-A5\(^{-/-}\) female mice (\(P = 0.030\)). These data suggest that both male and female null mice are less anxious as revealed by increased entries and time spent in the open arm, which is usually avoided by rodents. In addition, the increase in total entries that was observed in the null mice supports our previous observation of increased locomotor activity in the null mice (Sheleg et al. 2013). Finally, since decreased anxiety was observed regardless of maternal behavior, we conclude that this change in behavior is the result of genetic factors and not exposure to early life stressors.

Next we wanted to examine whether the anxiety-like behaviors of ephrin-A5\(^{-/-}\) mice were specific to the EPM. To test this, we used the light–dark test which takes advantage of the fact that mice tend to avoid light and unprotected areas. Both male and female ephrin-A5\(^{-/-}\) mice showed anxiety-like behavior in the tests (Fig. 10). A multivariate ANOVA test showed a significant genotypic difference where ephrin-A5\(^{-/-}\) mice spent significantly more time in the light chamber of the box (\(F_{2,53} = 11.361, P < 0.0001;\) Fig. 10a), and had a decreased number of head pokes (\(F_{2,53} = 8.367, P = 0.0007;\) Fig. 10b), compared to wild-type and heterozygous mice. In addition, there was a sex difference where male mice had increased head pokes compared to female mice (\(F_{1,53} = 8.367, P = 0.0014\)). There were no statistical differences between the genotypes in the latencies to initially enter the light or the number of transitions between the two compartments (Fig. 10c,d, respectively). However, there was a significant effect of sex (\(F_{1,53} = 30.613, P < 0.0001\)) where female mice had a consistently lower number of transitions between the two compartments across all genotypes. In addition, a genotype \(\times\) sex interaction was found (\(F_{2,53} = 3.809, P = 0.030\)), where post hoc tests revealed that ephrin-A5\(^{-/-}\) null male initially entered the light compartment faster than ephrin-A5\(^{-/-}\) null female (\(P = 0.014\)). These results support our previous data from the EPM test and suggest that ephrin-A5 deletion decreases anxiety in mice.

**CORT levels are comparable between the genotypes**

Activation of the HPA axis has been shown to play an important role in anxiety. In response to stress,
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corticotrophin-releasing hormone (CRH) is released from the hypothalamus, which leads to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary into the blood. ACTH in turn induces the release of glucocorticoid stress hormones from the adrenal (Miller & O’Callaghan 2002). In order to test whether the alteration in anxiety is due to changes in the activation of the HPA axis, we determined CORT concentrations, the major stress steroid, in mice. Serum CORT concentration was not significantly different between the genotypes ($F_{1,16} = 0.963, P = 0.34$) nor was there a significant effect of sex ($F_{1,16} = 0.121, P = 0.73$). However, CORT levels were overall significantly higher under stress conditions compared to baseline ($F_{1,16} = 22.379, P = 0.0002$; Fig. 11).

Discussion

Here, we demonstrate that genetic deletion of ephrin-A5 significantly decreased maternal behavior. Nesting behavior was reduced in lactating ephrin-A5$^{-/-}$ females at 6 and 24 h after providing the nesting material. Since mice are born without the ability to regulate their body temperature, the construction of the nest is important for their survival (Weber & Olsson 2008). It is therefore not surprising that pups born to ephrin-A5$^{-/-}$ females had a lower survival rate compared to those born to wild-type controls. Although maternal nest building behavior is important for the lifetime reproductive success of the mouse (Bult & Lynch 1997) and primiparous mice tend to build the most complex quality nests (Bond...
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Figure 9: Decreased anxiety-like behavior of ephrin-A5−/− mice in the EPM.

Anxiety-like behavior was measured on the EPM in male and female ephrin-A5−/− (n=10 per sex), heterozygous (n=11 per sex) and wild-type (n=10 per sex) mice. The number and percentage of entries into the open arm (a and b) as well as the time and percentage of time spent in the open arm (c and d) were higher in both male and female null mice compared to heterozygous and wild-type controls. (e) The total number of entries into both open and closed arms were significantly higher in male and female ephrin-A5−/− mice. Data are presented as mean ± SEM. *indicates significantly different from wild-type mice; P < 0.05. **Indicates significantly different from heterozygous mice; P < 0.05.

et al. 2002), non-pregnant, nulliparous mouse are also capable of building a nest (Bond et al. 2002; Sherwin 1997). This nest has been referred to as ‘sleeping nest’ and it is smaller and relatively flat compared to the maternal nest (Gandelman 1973). Here nesting is a spontaneous behavior (Deacon 2012), it provides shelter from predators and is essential for thermoregulation. Non-maternal nesting behavior has been shown to be affected by hippocampal damage and can be used to assess the mouse well-being (Jirkof 2014). In addition, it requires sensorimotor behaviors such as carrying, digging, sorting and pushing (Gaskill et al. 2012). In order to examine whether the impairment in nesting seen in the primiparous ephrin-A5−/− mice was due to sensorimotor defects, we analyzed nesting in nulliparous, virgin females. We found no differences in non-maternal nesting behavior between the genotypes suggesting that the sensorimotor behaviors required for nest building are intact in the null mice. Since the difference in nesting was only observed between primiparous null and wild-type females, the defect is most likely due to responses to maternal factors such as changes in maternal motivation.

In the pup retrieval test more than 50% of the lactating ephrin-A5−/− females failed to retrieve their own pups back to the nest, and those that did retrieve took longer than wild-type and heterozygous littersmates. It has been shown that the latency of pup retrieval decreases in virgin females with repeated exposure to pups (Stolzenberg & Rissman 2011), as well as across the first week of postpartum in primiparous mice (Feierstein et al. 2010). In order to test whether maternal responsiveness to pups in the form of pup retrieval improves in ephrin-A5−/− females, pup retrieval was measured twice (4 and 6 days postpartum). We did not detect differences in retrieval latency across the days tested in any genotype, suggesting that repeated exposure to pups...
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Figure 10: Decreased anxiety-like behavior of ephrin-A5−/− mice in the light–dark box. Anxiety-like behavior was measured in male and female ephrin-A5−/− (n=10 per sex), heterozygous (n=10 per sex) and wild-type (n=9 male and 10 female) mice in the light–dark box. Ephrin-A5−/− mice spend more time in the light compartment (a) and had increased number of head pokes (b) compared to heterozygous and wild-type controls. There were no genotypic differences in the latency to the first exit (c) and the number of transition between the two compartments (d). Data are presented as mean ± SEM. *Indicates significantly different from wild-type mice; P < 0.05. **Indicates significantly different from heterozygous mice; P < 0.05. ***Indicates significant sex differences; P < 0.05.

Figure 11: No differences in CORT levels between ephrin-A5−/− and wild-type mice. CORT levels were measured in male and female ephrin-A5−/− and wild-type mice (n=5 per sex and genotype) under baseline and mild stress conditions. There were no significant differences between the genotypes. However, CORT levels were significantly higher under stress conditions compared to baseline. Data are presented as mean CORT levels (ng/ml) ± SEM. *Indicates significantly different from baseline conditions; P < 0.05.

this study, wild-type mothers were able to retrieve null pups, suggesting that ephrin-A5 deletion did not affect the pups' cues toward the dams at least with respect to retrieval behavior. Moreover, wild-type pups did not elicit this behavior in the null mothers indicating that the deficit is indeed due to a maternal defect in the null mother.

The hormonal changes that accompany pregnancy and parturition have also been implicated in the regulation of maternal behavior, specifically changes in progesterone and estradiol levels (Sheehan & Numan 2002; Terkel & Rosenblatt 1968). For example, a state of high progesterone and low estrogen has been suggested to control maternal nest-building in primiparous mice (Lisk et al. 1969). However, we did not detect changes in hormonal levels between null and wild-type control. Differences with respect to maternal aggression were not observed between the genotypes, but since it has been shown that different neuronal circuits influence separate maternal behaviors (Gammie 2005), it is possible that ephrin-A5 deletion affects circuits that control pup retrieval and nest building but not those that regulate maternal aggression. Consistent with this notion, lesion studies have shown that defects in maternal aggression do not affect pup retrieval in rats (Factor et al. 1993; Hansen 1989).

Previously we reported that ephrin-A5−/− mice have decreased body weight compared to wild-type control (Sheleg et al. 2013). Here, we confirmed these results and showed that the differences in body weight were not due to the differences in maternal behavior between the genotypes since heterozygous pups that were born and raised by...
ephrin-A5−/− females had similar body weight to those that were born and raised by heterozygous and wild-type control females.

Ephrin-A5−/− mice had lower levels of anxiety-like behavior as revealed by increased entrance and time spent in the open arm of the EPM, as well as increased time in the light compartment of the light–dark box and decreased number of head pokes. Although it has been suggested that the quality and quantity of early life care influences anxiety (Meaney 2001), we did not find a correlation between the two. Decreased anxiety in the EPM test was observed in the null mice regardless of the levels or quality of maternal care. Additionally, in response to mild stress (exposure to the EPM) both ephrin-A5−/− and wild-type controls showed similar activation of the HPA axis; blood CORT concentration was increased under stressful condition in both genotype with no significant differences between the genotypes. Our observations reported here suggest that genetic factors play key roles in anxiety levels of mice.

The interaction between the MPOA and the mesolimbic system plays a pivotal role in regulating maternal behavior in rats (Numan & Stolzenberg 2009). Numan (2014) proposed a model in which MPOA projections to the VTA induce the release of DA into the nucleus accumbens (NA). This release stops the inhibitory effect of the NA on the ventral pallidum (VP) which then becomes responsive to the stimulation from the pups. Since the mesolimbic DA system is involved in motivated behavior, it is suggested that the MPOA-VTA connection governs the appetitive aspect of maternal responses to pup stimuli which includes retrieval behavior (Numan 2014; Stolzenberg & Numan 2011). Previously, we have shown that ephrin-A5 and one of its receptors, EphA5, are expressed in the mesolimbic DA system and affects dopaminergic neurite growth (Cooper et al. 2009; Deschamps et al. 2009; Kimura et al. 2011). Both in vivo and in vitro data suggested that ephrin-A5 has an adhesive effect on EphA5-expressing dopaminergic neurons from the midbrain, and its absence leads to a decrease in neuronal targeting to the striatum. Thus, it is possible that the decreased maternal behavior seen in the null mice is due to alteration in the mesolimbic DA system. However, since ephrin-A5 is expressed broadly in the brain (Deschamps et al. 2009; Gao et al. 1998), and has been shown to regulate neurogenesis (Depaepe et al. 2005; Gerstmann et al. 2015; Noh & Park 2016; Shu et al. 2016) and formation of several other neural circuits (Carvalho et al. 2006; Guellmar et al. 2009; Steinbeck et al. 2014; Tadesse et al. 2013; Yates et al. 2014), the precise mechanism underlying maternal care deficiency remains to be determined by future investigations. Thus, the mechanistic connection of ephrin-A5 function to maternal behavior is at present unclear, and this gene may not regulate maternal care exclusively. In addition, other ephrins may also compensate for the loss of ephrin-A5 functions. Consistent with a potential compensatory mechanism by functionally similar ephrin genes, heterozygous ephrin-A5 mice which retained one copy of ephrin-A5 gene showed a transient defect in maternal nest making at 6 h, but not at 24 h (Fig. 1a) and a delay in pup retrieval at P4 but not P6 (Fig. 3). Nevertheless, the maternal care defects we have observed in the homozygous ephrin-A5−/− mice are robust and very reproducible, which support the notion that ephrin-A5 plays a role in modulating maternal behavior functions.

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