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Paternal Influence on Female Behavior: The Role of *Peg3* in Exploration, Olfaction, and Neuroendocrine Regulation of Maternal Behavior of Female Mice

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Genomic imprinting represents a mechanism through which parent-of-origin effects on offspring development may be mediated. However, investigation of the influence of imprinted genes on behavior has been limited. Here the authors investigate the role of the maternally imprinted/paternally expressed gene, *Peg3*, in several aspects of behavior using both 129Sv- and B6-*Peg3* mutant female mice. Virgin *Peg3* females on both genetic backgrounds were less exploratory and had higher rates of defecation with strain-dependent effects on activity levels and olfactory discrimination. Reproductive success, pup retrieval, and postnatal maternal care of pups were reduced in these females whereas indices of maternal aggression were higher among B6 *Peg3*-KO females. Differences in maternal care were apparent in females caring for biological or cross-fostered offspring and deficits in pup retrieval apparent beyond the immediate postpartum period. Oxytocin receptor binding in the MPOA and LS was reduced in *Peg3*-KO females. Thus, the authors demonstrate that disruptions to *Peg3* influences aspects of female behavior that are critical for mediating maternal effects on offspring development, such as postpartum licking/grooming, and that effects of *Peg3* are dependent on the maternal genetic background.

Keywords: *Peg3*, maternal, oxytocin, strain differences, behavior

The discovery that maternal and paternal genomes do not contribute equally to offspring phenotype reveals a unique mechanism through which parent-of-origin effects may be mediated. The bases of this discovery were the growth and developmental abnormalities observed among chimeric mouse embryos in which there was either a high maternal (Pg) or paternal (Ag) genome content (Allen et al., 1995; Barton, Surani, & Norris, 1984; Surani & Barton, 1983). Pg embryos were found to be growth restricted relative to normal embryos whereas Ag embryos were much larger than normal. Conversely, brain size was reduced in Ag embryos and enhanced in Pg embryos. Further exploration of the mechanisms that drive these effects implicated germline epigenetic modification of either the maternal or paternal genome leading to uni-parental gene expression (Keverne, Fundele, Narasimha, Barton, & Surani, 1996; Lees-Murdock & Walsh, 2008). Since the discovery of this phenomenon, now referred to as genomic imprinting, approximately 100 genes have been identified that are maternally expressed/paternally silenced (imprinted) or paternally expressed/maternally imprinted. To understand the function of these genes and the evolutionary role of genomic imprinting, mouse models have been developed in which the effects of targeted deletion of these genes can be explored.

Though the principal focus of functional studies of imprinted genes has been physiology and growth (Frontera, Dickins, Plagge, & Kelsey, 2008), the effects of these genes on brain development and the expression pattern of these genes within the brain suggest that genomic imprinting would have a role in parent-of-origin effects on behavior (Davies, Isles, Humby, & Wilkinson, 2007; Wilkinson, Davies, & Isles, 2007) that is not limited to the unique contributions of sex chromosomes. Paternally expressed/maternally imprinted genes are highly expressed within the hypothalamus whereas maternally expressed/paternally imprinted genes are highly expressed within the cerebral cortex and striatum (Allen et al., 1995; Keverne et al., 1996). Corresponding to the function of these brain regions, targeted deletion of paternally expressed genes is associated with suckling deficits, longer latencies to pup retrieval, impairments in male sexual behavior, and aberrant circadian rhythms (Curley, Barton, Surani, & Keverne, 2004; Kozlov et al., 2007; Lefebvre et al., 1998; Li et al., 1999; Swaney, Curley, Champagne, & Keverne, 2007) whereas disruption of maternally expressed genes is associated with increased cognitive deficits (Davies et al., 2007; Plagge et al., 2005). Based on the function of these genes, several hypotheses have emerged regarding the evo-

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lution of genomic imprinting, including conflict hypothesis and mother-infant coadaptation (Curley et al., 2004; Hager & Johnstone, 2003; Haig, 1993).

In the current study, we explore the role of the paternally expressed gene *Peg3* in the behavior of adult female mice. *Peg3* is strongly expressed in the placenta and the developing embryo, particularly in the hypothalamus (Li et al., 1999). The *Peg3* gene is located on mouse proximal chromosome 7 and encodes a large zinc finger protein (Kuroiwa et al., 1996; Relaix et al., 1996) that has been implicated in p53 mediated apoptosis (Deng & Wu, 2000; Relaix et al., 2000). Because of the transmission pattern of paternally expressed/maternally imprinted genes, the biological offspring of females with the *Peg3* mutation do not possess the mutation if the male used to generate the litter is genetically wild type. As such, we can differentiate the effects of the mutation within the mother and offspring. Mutant offspring exhibit impairments in sucking and rate of growth with associated developmental delays (Curley et al., 2004). As adults, male *Peg3* mutants have deficits in sexual behavior and olfactory recognition of sexually receptive mates (Swaney et al., 2007; Swaney et al., 2008) and *Peg3* mutant females show longer latencies to retrieve pups after hormonal priming and have reduced hypothalamic oxytocin neurons (Curley et al., 2004; Li et al., 1999). Interestingly, genetically wild type offspring reared by these *Peg3* females also display impairments in growth and behavior suggesting that there is a transmission of the effects of the mutation in the absence of the transmission of the mutant *Peg3* gene (Curley, Champagne, Bateson, & Keverne, 2008).

Previous studies of *Peg3* function in female mice have been limited to the analysis of pup retrieval in females generated on a 129Sv strain. However, our previous studies have demonstrated that this behavior does not predict postpartum maternal care of pups and thus may not be critical for shaping offspring development (Champagne, Curley, Keverne, & Bateson, 2007). In the present study, we describe the behavioral effects of the *Peg3* gene on both the original 129Sv lineage and on a C57Bl/6J (B6) genetic background. This design allows us to address two important questions: (1) the stability of the effects of the *Peg3* mutation over multiple generations (32 generations in the case of 129Sv females) and (2) the effect of *Peg3* in the context of two phenotypically divergent genetic backgrounds, allowing for the analysis of the interaction between background genotype, phenotype and the effects of *Peg3*. Conclusions regarding the functional role of genes often come from knockout models in which the gene is studied in one genetic context (i.e., background strain). However, to fully understand gene function, converging evidence from multiple models should be used to account for gene-environment and gene-gene interactions. The more active and exploratory patterns of behavior exhibited by B6 mice also permits a more thorough examination of the behavioral consequences of the *Peg3* mutation. In addition to comparison of pup retrieval across these strains, here we present the first investigation of the effects of *Peg3* on locomotor activity, response to novelty, olfaction, postpartum maternal care of pups and aggression toward intruders. Given the importance of specific forms of maternal care in shaping offspring development (Champagne & Curley, 2005; Meaney, 2001), we have further examined postpartum maternal behavior in response to own and cross-fostered pups and the effects of the *Peg3* mutation on the density of oxytocin receptors (OTR) in those brain regions previously shown to mediate individual differences in postpartum care.

Method

Subjects

Adult Day 70 female wild type (WT) and *Peg3*-KO mice tested in these studies were of the inbred 129Sv and C57BL/6J (B6) strain and were housed on a reversed 12H dark-light cycle at the Sub-Department of Animal Behavior at the University of Cambridge, with lights on at 8 p.m. and off at 8 a.m. *Peg3*-KO mice were originally generated on the 129Sv inbred strain at the Wellcome CRC Institute at the University of Cambridge by insertion of a 4.8kb βgeo cassette into the 5′ coding exon of the *Peg3* gene (a detailed description of the methods can be found in Li et al., 1999). To generate *Peg3*-KO mice on the B6 strain, homozygous *Peg3*-KO 129sv males were mated with WT B6 females to produce heterozygous *Peg3*-KO hybrid offspring. Males from these litters were backcrossed with WT B6 females and the subsequent offspring genotyped by tail biopsy to identify the mutant male offspring. Males carrying the *Peg3* mutation were then bred with WT B6 females. This backcross was repeated over a total of 20 generations to produce B6 *Peg3*-KO females. Expression of the *Peg3* gene in the B6 adult female brain was found to be consistent with the pattern previously described in 129Sv mice (Li et al., 1999; Figure 1). Females were weaned at postnatal Day 28 (PND 28) at which time genotype was confirmed using cartilage from a 2 mm tail biopsy. Tissue was incubated in a 1 ml volume Eppendorf tube, in PBS containing X-gal (1 mg/ml), 5 mM $K_4Fe(CN)_6$-$3H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, and 0.02% NP-40 at room temperature on an orbital shaker overnight until color developed. Female pups were weaned into (16” × 5” × 5”) cages of up to five

Figure 1. LacZ staining of coronal brain sections of an adult female B6 mouse indicating the expression pattern of *Peg3.*

individuals with same-sex, same-age, same-strain, and same-genotype cage-mates (typically 2–3 littermates and 2–3 nonlittermates) and provided with ad libitum access to water and the RM1(E) chow diet (Lillico). All behavioral observations took place during the dark period of the light cycle, with the onset of testing occurring at least 1 hour after the onset of the dark cycle. No behavioral trial took place on the day of cage cleaning, and all trials were carried out under dim red light illumination. All experimental procedures were conducted in accordance with the terms of a project license issued by the U.K. Home Office to E.B. Keverne under the Animals (Scientific Procedures) Act, 1986.

Procedure

Behavioral assessment of WT and *Peg3*-KO mice commenced when these females were 70 days of age. Fourteen WT and 14 *Peg3*-KO females of each strain were first tested for exploratory behavior in the open-field apparatus. After a 4-day interval, females were tested for olfactory discrimination and habituation and then mated. After testing, females were housed 2 to 3 per cage with an adult male (purchased from Harlan, U.K.) for a period of 14 days. Males were then removed and females monitored daily. Within 1 to 2 days before parturition females were singly housed. On the day of birth (PND 0), the dam was weighed, pups were counted, and weighed and retrieval behavior was tested. Only litters containing four or more pups were included in subsequent analyses. From PND 1 to PND 6 mother-pup interactions in the home-cage were observed. Litters were left undisturbed until PND 28 at which time pups were weaned. Two weeks postweaning, five females per strain and genotype were sacrificed by cervical dislocation and whole brains extracted for oxytocin receptor binding analysis.

A second cohort of 8 WT and 11 *Peg3*-KO B6 adult females was also mated to determine group differences in maternal behavior toward cross-fostered pups and to measure levels of maternal aggression. Females were housed and mated as in the previous cohort. On the day of birth (PND 0), pups were removed and replaced with a litter of 129Sv-B6 hybrid pups provided by a donor female. From PND 1 to PND 6, mother-pup interactions in the home-cage were observed. On PND 7, maternal aggression testing was performed followed by a test of pup retrieval.

Pup Retrieval Test

On the day of birth, the lactating female and pups were removed from the home cage briefly (approximately 10 seconds) and bedding was disturbed throughout the cage. Three pups from the litter were then randomly placed away from the nest end of the home cage, and the mother was then reintroduced to the cage. The latency (in seconds) to sniff a pup, retrieve each of three pups, nestbuild, and crouch over pups was recorded. If a female had not completed this response within 15 minutes the test was terminated, resulting in a latency of 900 seconds for any behaviors not yet observed. After testing, all pups were returned to the home cage. All testing occurred within the colony room.

Maternal Observations

The procedure for assessing maternal behavior in mice was adapted from previous work examining natural variations in maternal care in rats (Champagne, Francis, Mar, & Meaney, 2003). Maternal behavior was scored from PND 1 to PND 6. Observers were trained to a high level of interrater reliability (i.e., >0.90). Dams were observed in their home cage during the dark-phase of the light cycle under dim red light (<5 lux) and not disturbed for the duration of the 6-day observation period. Each day consisted of four observation periods, two within the first 5 hours after the onset of the dark cycle (0800 to 1300) and two within 7 hours of the end of the dark cycle (1300 to 2000). Each observation was 60 minutes in duration and no observation sessions took place within the 1-hr period before or after the transition from the light to dark cycle. Within each observation period, the behavior of each mother was scored every 3 minutes (20 observations/period $\times$ 4 periods per day = 80 observations/mother/day = 480 observations per mother over the 6 days). The following behaviors were scored: mother off pups, mother licking and grooming any pup (both body and anogenital licking were included), mother in nursing posture over pups, nest-building (while in contact with pups, nursing pups, or not in contact with pups), self-grooming, eating, and drinking. Variations in nursing posture previously observed in lactating rat dams were not evident in the 6 days postpartum and nursing thus describes a crouched arch posture over pups. We have used this method in previous studies of natural variations in maternal care in 129Sv and B6 mice (Champagne et al., 2007).

Open-Field Test

The open field test is a standard tool for measuring exploratory and anxiety-like behavior in rodents (for reviews see Archer, 1973; Belzung & Griebel, 2001; Crawley, 1985). There is also evidence from several rodent models that illustrate the relationship between open-field measures and the indices of maternal care to be explored in these females (Clinton et al., 2007; Neumann, Kromer, & Bosch, 2005). The open-field used was a 24" $\times$ 24" $\times$ 16" plastic box. The sides of this box were black and the floor consisted of white tiles. The behavior of the animal in this field was recorded with a video camera mounted on a tripod adjacent to the field. Coding of these video recordings was completed using a DOS-based program designed to give summaries of the amount of time spent in the inner and outer area of the field, as well as the overall activity of the animal. Females were tested in the open-field at approximately 70 days of age and confirmed to be in diestrus on the day of testing after analysis of the cytology of a vaginal smear. On the day of testing, the mouse was removed from its home cage and placed directly into one corner of the open field. After a 10-min session, the mouse was removed and returned to its home cage. Counts of fecal boli were assessed at this time. All testing was conducted under red (dark phase) lighting conditions. During analysis of the recordings, the field was divided into a grid of 10 $\times$ 10 squares. For the purposes of analysis, inner field exploration was defined as the time spent in the inner 9 $\times$ 9 squares, activity was defined as the number of square crossings and pauses in movement within the field were defined as the duration of time spent immobile. The length of time spent immobile is a measure created from a log starting 5 seconds after the rodent stops engaging in grid crossings during the test and indicates the duration of time during which there are no grid crossings. Though there may be some aspects of "freezing" included in this measure, it is a more general indicator of immobility.

Habituation-Dishabituation Olfactory Testing

As is the case for open-field exploration, there is strong empirical support for the role of olfactory ability in maternal care, with gross disruptions of olfaction leading to enhanced maternal responsivity in virgins and decreased maternal responsivity in lactating females (Fleming & Rosenblatt, 1974). The habituation-dishabituation test examines olfactory discriminatory ability by measuring the frequency and duration of approach to repeated presentations of two olfactory stimuli (Baum & Keverne, 2002). This test determines the ability to detect differences between two distinct olfactory stimuli by examining habituation (reduced olfactory investigation) to repeated presentations of the same stimuli and dishabituation (increased olfactory investigation) when a new stimuli is introduced. Females were tested 4 days after completion of the open-field test and confirmed to be in diestrus. Females were singly housed 30 minutes before testing. At the start of testing, the cage lid (containing food and water bottle) was removed and replaced with a clean cage lid. The first three stimuli presentations determined baseline levels of approach to the stimuli and habituated the animals to the testing procedure. There was 30 μl of water placed on a piece of filter paper that was then positioned on the cage lid. Number of approaches toward the stimuli and the duration of these approaches were recorded using a stopwatch. After a 2-min exposure and a 1-min interval, the procedure was repeated twice, such that there were a total of 3 × 2-min presentations of the water. The olfactory testing phase was then initiated using two stimuli: urine pooled from unfamiliar group-housed dietrus 129Sv females or urine pooled from unfamiliar group-housed diestrus B6 females. The ability to distinguish these volatile urinary cues likely requires main rather that accessory olfactory function. Though the ability to distinguish between 129Sv and B6 urine may not be relevant to reproductive success of females in an ecological context, the inability to distinguish these two urine types would indicate a gross rather than subtle impairment in olfactory processes, which would have reproductive consequences (Fleming & Rosenblatt, 1974). The first stimulus was presented for 3 × 2-min exposure periods, each separated by a 1-min interval. In each case, 30 μl of urine was placed on the filter paper and placed on the lid of the cage. After a 1-min interval, the second urine stimulus was presented for 3 × 2-min exposure periods each separated by a 1-min interval. The order of presentation of the two urine types (B6 then 129Sv or 129SV then B6) was counterbalanced. After testing females were returned to the home cage.

In the analysis of the data obtained from this testing, overall investigation (frequency and duration) of the stimuli was compared with a repeated measures ANOVA. In addition, the habituation to the first urine type was determined as the frequency/duration of approach to the first presentation subtracting the frequency/duration of approach to the third presentation, with higher values indicating increased habituation. Dishabituation was determined to be the frequency/duration of approach to the first presentation of the second urine type subtracting the frequency/duration of approach to the third presentation of the first urine type. These values were calculated for each animal and compared as a function of genotype using a *t* test.

Maternal Aggression

On postpartum Day 7, each B6 female was exposed to an intruder male for 7 minutes in her home-cage. All tests took place between 4 and 7 hours after the onset of the dark light cycle. Immediately before the behavioral test, pups were removed from the home-cage. Previous work has demonstrated that removing pups does not diminish the expression of maternal aggression in mice (Svare, Betteridge, Katz, & Samuels, 1981). Postpartum Day 7 was chosen as this is within the period of high levels of maternal aggression in mice that occurs between postpartum Days 4 and 10 (Svare, 1990). At the commencement of the test, an adult, group-housed, virgin B6 intruder male was placed in the dam's home cage at the opposite end from the nest-site. Each test was recorded on videotape and reviewed posttest to analyze maternal aggression. No male was used more than twice, with WT and *Peg3* mutant females receiving males with equal levels of previous test experience. Males were given at least 1 day of rest between tests. The latency, frequency and total duration of the following behaviors were quantified by individuals blind to the genotype of females—sniff the male, lunge at the male without contact, chase the male, or bite the male (aggressive behaviors); freeze when approached by the male, run away from the male, or establish a subordinate posture—the female stands on her hind legs with her paws upright and nape displayed (subordinate behaviors); self-groom, climb on the cage, or bury themselves in bedding (distractive behaviors). Tests were to be immediately terminated if overt aggression was observed, although this was not the case with any females tested here. Females who did not display a given behavior during the 7-min test were assigned a latency of 420 seconds for that behavior.

Oxytocin Receptor Binding Assay

Mice were sacrificed through rapid decapitation 2 weeks post-weaning and brains were extracted, placed briefly in isopentane, and kept at −80 °C until processed. Females were confirmed to be in diestrus at the time of sacrifice through cytological analysis of vaginal swabs. Brains were sectioned in the coronal plane at 16 μm, and sections thaw mounted onto poly-L-lysine coated slides that were stored at −80 °C until the assay was performed. Slide-mounted coronal brain sections were processed for receptor autoradiography using ^{125}I-d(CH$_2$)$_5$[Tyr-Me)$_2$,Tyr-NH$_2^9$] OVT (New England Nuclear, Boston) as previously described (Francis, Champagne, & Meaney, 2000). ^{125}I-OVT has a very high affinity for OT receptors (K_d = 0.048 ± 0.008 nM) and a 10-fold greater affinity for oxytocin receptors than OT (Elands et al., 1988). The affinity of this compound for OT receptors is approximately 300-fold higher than for V1 and V2 vasopressin receptors (Elands et al., 1988). After a prewash in Tris-HC1 (50 mM, pH 7.4), slides were exposed to a 75-min incubation (at room temperature) of 60 pM ^{125}I-OVT in Tris with MgCl (10 mM), BSA (0.1%), and bacitracin (0.05%). Nonspecific binding was defined in adjacent sections by adding 50 nM Thr^{4}Gly^7-oxytocin (a concentration previously found to completely displace 60 pM ^{125}I-OVT; Elands et al., 1988) to the incubation buffer. The final 35-min wash was performed at room temperature in 50 mM Tris (pH 7.4), 10 mM MgCl to reduce background radiation. After air-drying, the slides were exposed to BioMax MR film (Kodak) for 48 hours. ^{125}I autoradiographic standards (Amersham) were included in the cassette for quantifi-

cation. All autoradiograms were analyzed using an image-analysis system (MC1D-4, Interfocus Imaging, Cambridge, U.K.). Three sections were analyzed bilaterally for each brain region. For each animal, total and nonspecific binding was measured for each region and the difference taken to yield specific binding. Specific binding was greater than 90% of total binding. The statistical analysis was performed on the mean of these values for each animal by brain region according to the mouse brain atlas (Paxinos & Franklin, 2003). Receptor density was measured in the medial preoptic area (MPOA), lateral septum (LS), bed nucleus of the stria terminalis (BNST), the basolateral (BL), medial (MD), and central nuclei (CN) of the amygdala and the ventral medial hypothalamus (VMH).

Results

Open-Field Exploration

Among 129Sv mice, the *Peg3* mutation significantly increased the time spent immobile [$t(26) = 4.16$, $p < .001$] and decreased the number of grid crossings [$t(26) = 4.34$, $p < .001$] as well as the amount of time spent in the inner area of the field [$t(26) = 2.86$, $p < .01$; Figure 2A]. The number of grid crossings in both the outer [$t(26) = 4.14$, $p < .001$] and inner [$t(26) = 2.96$, $p < .01$] area of the field were lower among the *Peg3*-KO females (Figure 2A). In contrast, the *Peg3* mutation on the B6 genetic background had the effect of increasing levels of activity as evidenced by the elevated number of total squares crossed by B6 *Peg3*-KO females [$t(26) = 2.86$, $p < .05$]. Though there was a trend toward WT females spending more time immobile than *Peg3*-KO females during the test [$t(26) = 1.82$, $p = .08$] this effect was not statistically significant. Consistent with the effect found in 129Sv females, B6 *Peg3*-KO females spent significantly less time in the inner area of the open-field [$t(26) = 2.86$, $p < .05$; Figure 2B]. Number of squares crossed in the inner area versus outer area of the field was not significantly different as a function of genotype among B6 mice though there was a trend toward *Peg3*-KO females exhibiting higher numbers of crossings in the inner area of the field [$t(26) = 1.88$, $p = .07$]. These behavioral differences were apparent throughout the 10-min test. Interestingly, despite significant strain differences in behavior, *Peg3*-KO females on both backgrounds had higher rates of defecation during testing [WT-129Sv = 1.0 ± .23, 129Sv *Peg3*-KO = 2.5 ± .44, $t(26) = 3.90$, $p < .01$; WT-B6 = 1.14 ± .25, B6 *Peg3*-KO = 2.29 ± .43, $t(26) = 4.26$, $p < .05$].

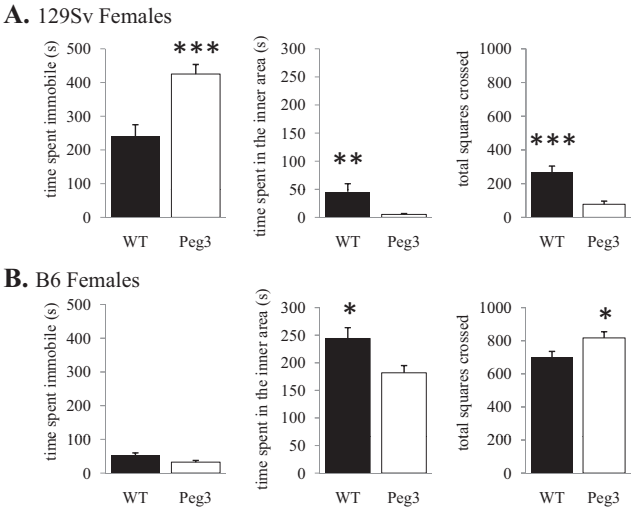

Figure 2. Open-field behavior of WT and *Peg3* mutant females. Time spent immobile, time spent in the inner area, and total activity observed among (A) 129Sv females as a function of genotype and (B) B6 females as a function of genotype. $^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$.

Olfactory Discrimination

Repeated measures analysis of the behavior of 129Sv WT and *Peg3* mutant females indicated no significant effect of genotype on the number of approaches or the time spent sniffing the stimuli, though there was a main effect of exposure time/type on these behaviors [approaches: $F(8, 26) = 4.49$, $p < .001$; time spent sniffing: $F(8, 26) = 4.29$, $p < .001$; Figure 3A] indicating the change in behavior occurring during successive presentations of the stimuli. More relevant to the question of habituation and dishabituation is the comparison between the behavior toward the first urine presentation compared to the third urine presentation (habituation; a1 vs. a3 on Figure 3) and the change in behavior from the third presentation of the first urine type and the first presentation of the second urine type (dishabituation; a3 vs. b1 on Figure 3). However, there was no significant effect of genotype on either of these behavioral dimensions among 129Sv mice. Repeated measures analysis of the olfactory behavior of B6 females indicated a main effect of genotype on the number of approaches to the stimulus [$F(1, 26) = 10.98$, $p < .01$] and an interaction between genotype and exposure time/type on the number of approaches [$F(8, 26) = 1.98$, $p < .05$]. Similar effects were found for duration of time spent sniffing the stimulus [genotype: $F(1, 26) = 6.5$, $p < .05$; genotype X exposure $F(8, 26) = 2.1$, $p < .05$; Figure 3B]. Though B6 females with the *Peg3* mutation did not differ significantly in the number of approaches and time spent sniffing the water or the first urine stimulus, there was a significant effect of genotype on these variables when the second urine stimulus was presented ($p < .01$). *Peg3* females were significantly less exploratory of the second urine type regardless of whether it was B6 or 129Sv urine. Both WT and *Peg3*-KO females exhibited habituation to the urine stimulus, showing reduced exploration of this stimulus over successive presentations. Behavioral dishabituation in response to the second urine type differed significantly as a function of genotype, with WT females showing increased approach ($p < .01$) and time spent sniffing ($p < .05$) the novel stimulus and *Peg3*-KO females showing no significant change in behavior in response to the new olfactory stimulus.

Reproductive Success

After olfactory testing all females were mated with a same strain male for a 2-week period. Females were monitored daily to determine the date of birth of the litter and to assess litter size and weight. Among the 129Sv females, 12 of the 14 WT females gave birth to litters; 2 of those females were excluded from further testing because of a litter size of fewer than 4 pups. Ten of the 14 *Peg3*-KO females gave birth; 2 of those females were excluded

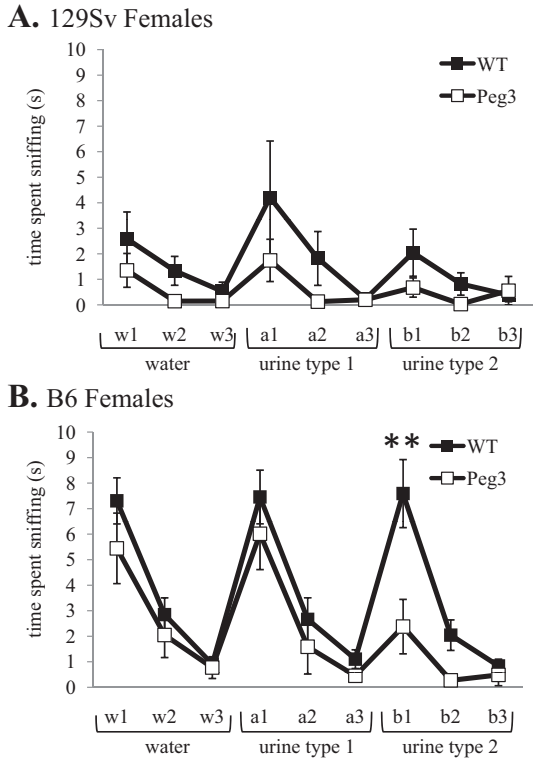

Figure 3. Time spent investigating an olfactory stimulus during a habituation-dishabituation task by (A) 129Sv WT and *Peg3* mutant females and (B) B6 WT and *Peg3* mutant females both. ** $p < .01$; water stimuli: w1, w2, w3; urine stimulus 1 (129Sv or B6 urine): a1, a2, a3; urine stimulus 2 (129Sv or B6 urine): b1, b2, b3.

from testing as the litters died within 48 hours of birth. Litter size, pup weight, and average pup weight on PND 0 did not differ between WT and *Peg3*-KO 129Sv females (Table 1). Among B6 WT females, 13 of the 14 females gave birth; 2 of those litters had fewer than four pups and were excluded from analysis. One additional female was also excluded as the pups died within 48 hours of birth. There were nine litters born to *Peg3*-KO B6 females however, four of those females were excluded from the maternal analysis as the litters died within 48 hours of birth. Litter size and weight were significantly higher among B6 WT females compared to B6 *Peg3*-KO females [$t(13) = 2.41$, $p < .05$; $t(13) = 2.67$, $p < .05$]; however, average pup weight was not significantly different as a function of maternal genotype (Table 1).

Pup Retrieval

On the 129Sv background, the *Peg3* mutation significantly increased the latency to sniff/approach the pups [$t(16) = 2.61$, $p < .05$] and to nestbuild [$t(16) = 2.68$, $p < .05$] during the 15-min observation period (Figure 4A). No significant differences were found on measures of pup retrieval or latency to crouch over pups among 129Sv females. In contrast, the *Peg3* mutation in B6 females had a pronounced effect on latency to retrieve pups [$t(13) = 2.1$, $p < .05$] with no significant effect on the latency to approach/sniff pups. Consistent with the effect of the mutation in 129Sv females, B6 *Peg3*-KO females displayed longer latencies to nestbuild [$t(13) = 5.13$, $p < .001$] but did not differ significantly from WT females in latency to crouch over pups (Figure 4B).

Postpartum Maternal Care

Maternal behavior toward pups was observed in the home cage from PND 1 to PND 6. 129Sv WT females were observed to engage in a higher frequency of licking/grooming [$t(16) = 2.22$, $p < .05$] and nursing of pups [$t(16) = 2.34$, $p < .05$] as well as overall increases in mother-pup contact [$t(16) = 2.41$, $p < .05$] compared to 129Sv *Peg3*-KO females (Table 2). No significant differences were observed in frequency of nestbuilding, self-grooming, eating, or drinking during the first week postpartum. Among B6 females, the *Peg3* mutation was associated with decreased levels of maternal licking/grooming [$t(13) = 2.20$, $p < .05$] and frequency of nursing [$t(13) = 2.25$, $p < .05$] as well as increases in frequency of drinking [$t(13) = 3.61$, $p < .01$]. B6 WT and *Peg3*-KO females did not differ in frequency of nestbuilding, self-grooming, eating, or overall contact with pups (Table 2).

Maternal Behavior of Females With Cross-Fostered Pups

To assess whether the deficits in maternal behavior observed in B6 *Peg3* dams were independent of pup phenotype, an additional cohort of 8 WT and 11 *Peg3* B6 females were mated and assessed for postpartum behavior, maternal aggression toward an intruder male, and pup retrieval at postpartum Day 7. From this cohort, 7 of the 8 WT females gave birth and were included in the study whereas only 6 of the 11 *Peg3*-KO females gave birth to live litters and were included in subsequent analyses. Biological offspring were removed at birth and replaced with a litter of six 129Sv-B6 hybrid pups provided by a donor female. Observations of postpartum care of these foster litters indicated that compared to WT females, *Peg3*-KO females engaged in less frequent licking/grooming of pups [$t(11) = 2.92$, $p < .05$], nursing [$t(11) = 2.83$, $p < .05$], and overall contact with pups [$t(11) = 2.89$, $p < .05$].

Table 1
Reproductive Success Amongst WT and Peg3-KO Female Mice

Strain	Genotype	% gave birth	Litter size	Litter weight	Average pup weight
129Sv	WT	86%	4.90 ± .58	7.03 ± .72	1.47 ± .04
	Peg3-KO	71%	5.62 ± .53	8.02 ± .85	1.43 ± .04
B6	WT	93%	6.90 ± .67	8.98 ± .73	1.33 ± .04
	Peg3-KO	64%	4.00 ± 1.04*	5.32 ± 1.28*	1.37 ± .06

* $p < .05$.

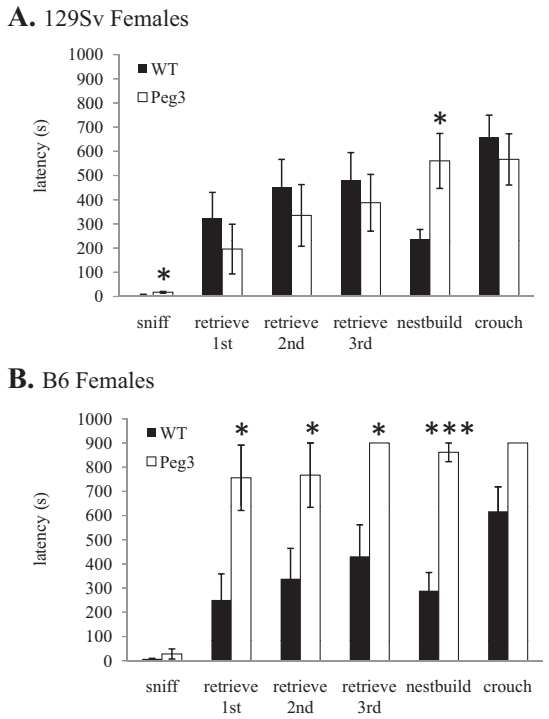

Figure 4. Pup retrieval on the day of parturition by (A) 129Sv WT and *Peg3* mutant females and (B) B6 WT and *Peg3*-KO females. * $p < .05$. *** $p < .001$.

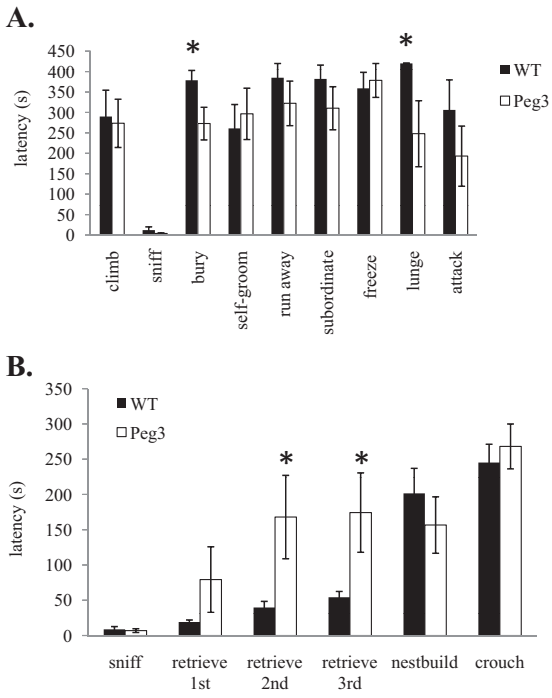

Figure 5. Maternal aggression and pup retrieval among B6 WT and *Peg3*-KO females caring for cross-fostered 129Sv-B6 hybrid pups. (A) Latency to display behavior during a 7-min exposure of lactating females to a male intruder. (B) Latency to retrieve foster pups on postpartum Day 7 by *Peg3*-KO compared to WT females. * $p < .05$.

Peg3-KO females spent significantly more time self-grooming [$t(11) = 3.08$, $p < .01$] but did not differ from WT females in frequency of nestbuilding, eating, or drinking. Thus, despite overall increases in maternal behavior among B6 females caring for 129Sv-B6 hybrid foster pups compared to their own biological offspring (Table 2), the deficits in maternal behavior as a function of genotype are still observed.

On postpartum Day 7, pups were removed from the home cage and an intruder male was placed in the cage. Fewer than 50% of the females showed any aggression toward the male (6 out of 13). However, a higher percentage of *Peg3*-KO females displayed aggressive behavior (67%; 4 out of 6) compared to WT females (28%; 2 out of 7). Latency to lunge at the male intruder was significantly shorter among *Peg3* mutant females [$t(11) = 2.32$, $p < .05$] as was the latency to bury themselves in the bedding material [$t(11) = 2.35$, $p < .05$; Figure 5A]. No genotype differences were found in the frequency or duration of the behaviors observed during the 7-min test.

The latency to engage in maternal behavior on postpartum Day 7 was assessed in a retrieval test after the maternal aggression test. *Peg3*-KO females displayed longer latencies to retrieve all three pups during the 15-min observation [$t(11) = 2.29$, $p < .05$] but did not differ in latency to sniff/approach pups, nestbuild, or crouch over pups (Figure 5B).

Oxytocin Receptor Density

Repeated measures ANOVA with OTR density measures from 129Sv mice indicated a main effect of region [$F(6, 54) = 7.5$, $p < .001$] but no significant main effect of genotype or interactions between genotype and region despite a substantial mean difference

Table 2
Postpartum Maternal Behavior of WT and Peg3-KO Female Mice

Strain	Genotype	Lick/groom	Nursing	Contact	Nest-build	Self-groom	Eat	Drink
129Sv	WT	5.40 ± .42	84.42 ± 4.54	85.50 ± 4.36	2.15 ± .52	1.33 ± .38	9.93 ± 3.08	0.98 ± .41
	Peg3-KO	3.99 ± 0.47*	70.19 ± 3.70*	71.34 ± 3.65*	4.19 ± 1.32	1.13 ± .42	16.39 ± 2.62	1.22 ± .50
B6	WT	10.18 ± 1.53	55.05 ± 3.58	58.52 ± 10.35	6.01 ± 1.72	2.06 ± .53	23.49 ± 2.08	2.08 ± .21
	Peg3-KO	5.20 ± .51*	41.70 ± 9.72*	50.79 ± 6.67	5.77 ± .78	1.40 ± .44	21.10 ± 2.53	.80 ± .26**
B6 (foster pups)	WT	15.26 ± 2.1	61.42 ± 2.53	72.52 ± 2.84	9.25 ± 1.70	6.07 ± .66	15.72 ± 1.96	1.09 ± .34
	Peg3-KO	8.14 ± 1.06*	47.75 ± 4.33*	58.90 ± 3.87*	10.67 ± 2.30	9.16 ± .76**	20.21 ± 2.57	2.59 ± .67

* $p < .05$. ** $p < .01$.

in binding in WT compared to *Peg3*-KO females within the MPOA (Table 3). Based on an a priori hypothesis that the MPOA would be a region in which genotype differences would likely be observed, we analyzed OTR binding in this region and found significantly reduced levels of OTR density in the MPOA of 129Sv *Peg3*-KO females [$t(8) = 2.38$, $p < .05$]. The analysis of density of OTRs in B6 female mice did indicate a main effect of region [$F(6, 54) = 7.28$, $p < .001$] and a genotype by region interaction [$F(6, 54) = 5.01$, $p < .05$]. Post hoc analysis indicated significantly reduced levels of OTR binding in the MPOA ($p < .05$) and LS ($p < .05$) of *Peg3* mutant compared to WT females (Table 3).

Discussion

The paternally expressed/maternally imprinted gene *Peg3* provides a mechanism for paternal influence on offspring development achieved through the interplay of germline epigenetic and genetic factors. Our results indicate a pervasive effect of disruption of the *Peg3* gene on the behavior and reproductive success of female mice. Mutation of the *Peg3* gene has previously been shown to alter growth at early stages of development (Curley et al., 2004; Li et al., 1999) and here we report on the consequences of this disruption for adult behavior either as a consequence of the developmental effects of *Peg3* or the continued absence of the *Peg3* gene in later life. Interestingly, the effects of disruption of this paternally expressed gene on adult behavior are dependent on the background genetic strain of the mouse model being used, with some consistency of effect across strains and some strain-specific effects that illustrate the interaction between the mutation and the genetic context in which the mutation is embedded.

In rodents, the initial approach of a female toward pups is influenced by reproductive state and trait levels of anxiety-like behaviors (Champagne et al., 2003; Fleming & Luebke, 1981). Thus, females who exhibit high levels of neophobia or anxiety-like behavior may be inhibited from exploring pups and thus be less likely to engage in a maternal response. We have observed that regardless of mouse strain, the *Peg3* mutation significantly reduces the level of exploration of a virgin female within a novel environment; a finding that is consistent with the effects of mutation another imprinted gene *Nesp55* (Plagge et al., 2005). The inner area of an open-field apparatus has been found to be anxiety-inducing as evidenced by the increased exploration of this area by rodents treated with anxiolytic drugs (Prut & Belzung, 2003). Both 129Sv and B6 females with a mutation of the *Peg3* gene spend less time in this inner area. Conversely, the direction of the effect of *Peg3* on activity levels is highly strain dependent. Among 129Sv females, we find that the *Peg3* mutation further reduces activity levels and increases time spent immobile whereas B6 *Peg3*-KO females display increased activity levels compared to B6 WT females. Though the strain effects on *Peg3*-induced changes in activity can be interpreted in many ways, it is plausible to suggest that fear responses in these two mouse strains may manifest themselves very differently at a behavioral level. In the inhibited 129Sv mice, fear-evoking stimuli, such as a novel environment, may exacerbate this inhibition leading to decreased mobility. In contrast, among more active mouse strains, the fear response may stimulate hyperactivity. In support of the anxiety-like phenotype of *Peg3*-KO females on both the 129Sv and B6 strains, rates of defecation during the test were elevated in mutants compared to WT females. This physiological indicator of stress reactivity has previously been demonstrated to differentiate individuals characterized as high versus low in emotionality and HPA reactivity (Ferre et al., 1995).

In previous studies of male sexual behavior, we have found that the *Peg3* gene plays a significant role in olfactory recognition of female reproductive state (Swaney et al., 2008). The inability to differentiate sexually receptive versus nonreceptive females and the reduced improvement in sexual behavior as a consequence of sexual experience that is exhibited by *Peg3*-KO males provides evidence for the importance of this imprinted gene in male reproductive success. Here, we report that *Peg3*-KO female mice on a B6 background have diminished ability to discriminate strain differences in a urine olfactory stimulus. This effect was not observed on the 129Sv background, though this may be because of the very low levels of olfactory exploration exhibited by this strain. The habituation-dishabutuation paradigm has been used to measure discrimination ability in a number of sensory domains. Though we do not observe an effect of *Peg3* on the process of habituation, there is a pronounced effect of the mutation of this gene on the ability of a B6 female to dishabituate to a novel olfactory stimulus. One interpretation of this finding is that *Peg3*-KO females have diminished capacity to differentiate the olfactory stimuli used in this study and consequently do not exhibit renewed interest when the stimulus type changes. The inability to distinguish the two olfactory stimuli used in this study (129Sv vs. B6 urine) suggests that though not anosmic, these females have a profound olfactory deficit that may lead to the disruptions in reproductive behavior we have observed. However, it should be noted that these data may also reflect a reduced motivation to approach and explore the olfactory stimuli that would also be consistent with the behavioral patterns observed in *Peg3*-KO females.

Previous studies of 129Sv *Peg3*-KO mice have indicated deficits in pup retrieval among these females and our findings are

Table 3
Oxytocin Receptor Density in the Brain of WT and Peg3-KO Female Mice

Strain	Genotype	MPOA	BNST	LS	BL AMYG	MD AMYG	CN AMYG	VMH
129Sv	WT	13.64 ± 2.36*	24.15 ± 2.30	35.85 ± 5.37	19.73 ± 3.51	18.23 ± 2.02	18.84 ± 1.74	28.00 ± 10.25
	Peg3-KO	6.23 ± 1.84	24.47 ± 3.19	46.52 ± 8.60	22.67 ± 5.91	18.97 ± 2.32	22.63 ± 4.88	35.80 ± 12.76
B6	WT	5.45 ± 1.65	19.12 ± 2.33	33.89 ± 2.64	17.13 ± 4.77	12.96 ± 2.44	15.46 ± 3.27	35.32 ± 10.97
	Peg3-KO	.44 ± .31*	16.73 ± 2.11	18.60 ± 3.99*	12.52 ± 2.48	11.87 ± 2.48	16.15 ± 2.81	28.99 ± 11.30

* $p < .05$.

consistent with these earlier reports. However, the magnitude of the effect on behavior during the retrieval test appears to be diminished among 129Sv compared to the previous cohort of 129Sv females (Curley et al., 2004; Li et al., 1999) and in comparison to the current cohort of B6 mice. The same is true of litter size/weight that does not differ significantly among the 129Sv females included in the current study. The *Peg3* mutation was originally generated on the 129Sv strain and there has been 32 generations of mutant offspring in the interval between the initial publication (Li et al., 1999) and the more recent behavioral work. This lengthy time period, in which only offspring who can survive the prenatal and postnatal deficits associated with this mutation are selected for mating, may lead to the introduction of compensatory mechanisms that permit successful reproductive behavior in adulthood. Data on average pup weight of *Peg3*-KO dams as a percentage of pup weight of WT dams and percentage of pup mortality as a function of generation supports this notion of compensation over time. In the first generation after mutation of the *Peg3* gene, litters born to 129Sv *Peg3*-KO females were 85% of the average weight of WT litters (Li et al., 1999) with this percentage increasing to 89% after 20 generations (Curley et al., 2004) and 97% following 32 generations (Table 1). Mortality rates of litters of *Peg3*-KO dams were 92% in the first generation (Li et al., 1999), 37% after 20 generations (Curley et al., 2004) and 29% after 32 generations. Successful reproduction involves multiple neuroendocrine factors and each of these factors can be regulated through multiple pathways (Bridges, 1996; Fleming, 1986). When one system is compromised, such as in the case of the *Peg3* mutation, these other factors may be up-regulated to compensate for the genetically induced deficit. This recovery of function over successive generations is likely to be common to knockout models in which the growth, survival, and reproduction are impaired and may be especially notable for maternal care phenotypes as those compensatory mechanisms that are involved in supporting infant survival may also be those that regulate maternal care (Curley et al., 2004; Kolliker, Brodie, & Moore, 2005; Peripato et al., 2002).

Here we provide the first report of the frequency of postpartum care of pups among *Peg3*-KO females, illustrating that 129Sv and B6 *Peg3*-mutant females provide less licking/grooming and engage less frequently in nursing contact with pups. In the case of B6 females, we have used cross-fostering to verify that these deficits emerge even when females are caring for foster pups who have not been exposed to disruptions in the in utero environment of *Peg3* females. In the cross-fostering study, females caring for 129Sv-B6 hybrid pups were observed to engage in a higher frequency of maternal licking/grooming suggesting a role for either the fostering procedure itself or pup genotype in stimulating maternal care. Though individual differences in this form of mother-infant contact have been observed to be a stable trait among lactating females, there is certainly a role for pup phenotype/genotype in soliciting maternal care (Ressler, 1962; van der Veen, Abrous, de Kloet, Piazza, & Koehl, 2008). However, even when caring for pups that are very vigorous in suckling behavior, the *Peg3*-KO females provide care less frequently than WT females.

Natural variations in maternal licking/grooming behavior have been found to be critical in shaping offspring development (Champagne et al., 2003; Meaney, 2001). Low levels of licking/grooming received in infancy are associated with increased HPA response to stress, decreased exploratory behavior and reduced maternal care by female offspring toward their own litters (Caldji et al., 1998; Francis, Diorio, Liu, & Meaney, 1999; Liu et al., 1997). Though these previous studies have all been conducted in a rat model, there is more recent evidence that these effects can also be observed in mice (Coutellier, Friedrich, Failing, Marashi, & Wurbel, 2008; Coutellier, Friedrich, Failing, & Wurbel, 2008). Thus, mutation of the *Peg3* gene induces a reduction in a form of maternal care that can exert long-term effects on offspring development. Though the *Peg3* mutation is not transmitted from mother to offspring because of the epigenetic silencing of the maternal allele, the effects of the mutation may be transmitted indirectly through reductions in the level of maternal care. Recently, we have demonstrated that the offspring and grand-offspring of *Peg3* mutant females display increased neophobia and reduced maternal responsivity (Curley et al., 2008), suggesting a maternally mediated nongenomic transmission of behavior. Natural variations in maternal licking/grooming are transmitted across generations through epigenetic modification to hypothalamic estrogen receptors that regulate neuroendocrine response to circulating hormones (Champagne, Diorio, Sharma, & Meaney, 2001; Champagne et al., 2006). The molecular mechanism through which the effects of the *Peg3* mutation are transmitted have yet to be elucidated but may likewise involve the epigenetic regulation of gene expression in somatic cells. In this case, a genetic effect would be propagated through epigenetic mechanisms leading to the stable inheritance of phenotype (Keverne & Curley, 2008).

The postpartum period is characterized by an elevated level of oxytocin receptors in both the mammary gland and brain which are essential for lactation and maternal care (Gimpl & Fahrenholz, 2001; Russell, Douglas, & Ingram, 2001). Here we demonstrate that among *Peg3*-KO females there is reduced density of oxytocin receptor binding in both the medial preoptic area (MPOA) and lateral septum (LS). Though we have not examined peripheral OTR density, reductions in receptor levels in the mammary gland and uterus may account for the reduced reproductive success among *Peg3*-KO females. Density of hypothalamic oxytocin receptors in the MPOA and LS have been found to differ between High and Low licking/grooming rat dams (Champagne et al., 2001; Francis et al., 2000) and administration of a selective oxytocin receptor antagonist reduces the frequency of licking/grooming behavior during the postpartum period (Champagne et al., 2001). Thus, disruption of *Peg3* may impair maternal behavior through reduction in oxytocin receptor levels. Though OT receptor densities have previously been compared in studies of within strain variation in rats (Champagne et al., 2001) and between species variation in voles (Insel & Shapiro, 1992), this is the first examination of between strain variations in oxytocin receptors in mice. It is interesting to note that despite significant reductions in OT receptor density in the hypothalamus of *Peg3*-KO females, the levels of binding in WT B6 females are comparable to those of 129Sv *Peg3* mutant females, consistent with the compensation in reproduction observed in the 129SV strain. Overall, these data indicate that *Peg3* may influence multiple aspects of the development of the hypothalamus, including the number of oxytocin releasing neurons (Li et al., 1999) and the density of oxytocin receptors. The mechanism through which this occurs is unclear, however, the *Peg3* gene encodes for a transcription factor implicated in p53-mediated apoptosis (Deng & Wu, 2000; Relaix et al.,

2000) and mutation of this gene may induce abnormalities in cell survival during the development of this neuroendocrine pathway.

In addition to regulating care of offspring, hypothalamic oxytocin is implicated in maternal aggression toward intruders (Bosch, Meddle, Beiderbeck, Douglas, & Neumann, 2005) and this protective behavior is thought to be an important aspect of the maternal response. However, despite the reduced density of oxytocin receptors in B6 *Peg3*-KO females, there are no reductions in maternal aggression. Mutation of the *Peg3* gene results in a more rapid onset of aggressive lunges toward an intruder introduced into the female's home cage. Though this behavior is typically attributed to elevated maternal responsivity, this interpretation would be inconsistent with the other measures of maternal behavior in which *Peg3* females have been assessed. Taken together with the reductions in maternal care, the persistence of maternal aggression in the B6 *Peg3*-KO dams suggests distinct neuroendocrine mechanisms mediating these different aspects of maternal care. However, the low levels of maternal aggression in our sample of WT females limit the ability to draw any conclusions from this data.

We have demonstrated that the expression of the paternally expressed gene *Peg3* in the adult maternal hypothalamus is critical for regulating several aspects of maternal care including prenatal food-intake, in utero nutrient supply, pup retrieval, nest-building, and milk let-down (Curley et al., 2004). The same gene, when expressed in the developing placenta and fetal and pup hypothalamus, regulates several complementary aspects of offspring physiology and behavior such as growth, suckling, and thermogenesis, that ensure pup survival and adequate priming of postnatal maternal care (Curley et al., 2004; Li et al., 1999). Consequently, we have argued that this gene and perhaps other paternally expressed genes such as *Peg1* that are coexpressed in the hypothalamus and placenta, have coadaptively evolved to ensure successful mammalian reproduction (Keverne & Curley, 2008). Furthermore, this same gene when expressed in the adult male brain enhances the ability of these males to identify sexually receptive females and mate with them (Swaney et al., 2008). Thus, as these genes are monoallelically expressed only when inherited down the patriline, fathers who are more likely to find mating opportunities are guaranteed to pass these physiological and behavioral enhancements to both their male and female offspring. Moreover, these paternal effects are augmented by maternal genetic background illustrating the interaction between maternal and paternal influences.

In this paper, we provide evidence for the role of *Peg3* in regulating critical aspects of maternal behavior. Moreover, the positive impact of the *Peg3* gene on nursing and licking/grooming of pups suggests that benefits because of a genetic effect in one generation (albeit *via* an epigenetic silencing of the imprinted gene) may have nongenomically, epigenetically inherited reproductive and phenotypic benefits for several successive generations. There is accumulating evidence that such individual differences in maternal care in rodents may shape changes in fear response, exploration, social and reproductive behavior not only in offspring but also in subsequent generations (Champagne, 2008). Indeed, we have previously found that the wild-type offspring of 129Sv-*Peg3* mutant dams exhibit impaired maternal care and neophobia that is transmitted to their own wild-type offspring, who are themselves two generations removed from the original mutation (Curley et al., 2008). Thus, even though wild-type females pass on a silenced copy of the functioning *Peg3* allele, they still transmit epigenetic benefits to their offspring of both sexes and their matrilineal grand-offspring by altering their daughter's maternal care, whereas grand-offspring born to their sons will inherit benefits through reexpression of the imprinted gene. There are multiple genetic and epigenetic routes at play across generations in the development of phenotypes. Behavioral traits exhibited by an individual are not simply the product of alterations in a singular aspect of the environment or genome, but a consequence of the interplay of multiple environments and genetic background factors from previous generations during development.

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