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Transgenerational effects of impaired maternal care on behaviour of offspring and grandoffspring

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Cues received from mothers may have important effects on early development in mammals. We examined the behavioural development of genetically wild-type mice, *Mus musculus*, offspring born to wild-type or mutant Peg3$^{+/-}$ (paternally expressed gene 3) mothers who are impaired in various aspects of maternal care during both the pre- and the postnatal periods. We demonstrate that impaired maternal care leads to offspring exhibiting increased neophobia and decreased exploratory behaviour. However, these effects were limited to female offspring only, with male offspring being indistinguishable from wild types in both the open-field and the novel object tests. Due to the breeding design of this study we were able to show that this was due not to the inheritance of a genetic mutation but to an epigenetic inheritance. Consistent with this, we also observed a nongenomic inheritance of impairments in maternal care. Wild-type daughters born to mutant mothers were impaired in their ability to retrieve pups to a nest in a retrieval test. Furthermore, the reduced exploration and neophobic phenotypes were transmitted to a third generation, with the granddaughters of mutant females exhibiting increased neophobia even though they had genetically wild-type mothers. We therefore demonstrate a nongenomic transmission of behavioural traits across successive generations operating via the matriline.

Keywords: developmental plasticity; epigenetics; exploration; genomic imprinting; maternal care; maternal effect; *Mus musculus*; novelty; parent-of-origin effects; transgenerational inheritance

In mammals, females are the primary caregivers and provide developing organisms with cues about how best to develop. Evidence from human and other animal studies shows that aversive events occurring early in life can have long-term effects on offspring phenotype. In humans, neglect and abuse in infancy increase risk of depression and anxiety and lead to metabolic disorders (Susser & Lin 1992; Bateson 2001; Bateson et al. 2004; Gluckman et al. 2005). Infant rhesus monkeys, *Macaca mulatta*, reared in the absence of their mothers, are behaviourally inhibited, show increased responsivity to stress and display impairments in social and reproductive behaviour as adults (Suomi et al. 1971; Ruppenthal et al. 1976). In rodents, daily separations of litters from their mothers during the postnatal period lead to long-lasting changes in cognitive, anxiety, reproductive and social behaviours in offspring, associated with gene expression changes in numerous brain regions (Fleming 1975; Lovic et al. 2001). Even relatively brief separations where pups are handled daily during the first week postpartum for 3–15 min can lead to long-lasting changes in stress reactivity and fearfulness even up to 26 months of age (Levine 1957; Meaney et al. 1988). Moreover, exposure to disruptive events during gestation, such as various stressful episodes or food restriction, induces the development of similar impairments in offspring behaviour (Salas et al. 1984; Champagne & Meaney 2006). Fewer studies have focused on more subtle variations in the mother–infant relationship. Nevertheless, in rodents and primates individual differences in the levels of postnatal contact or tactile stimulation of offspring by mothers may lead to these offspring developing alternative social, cognitive, parental and stress phenotypes (Fairbanks & McGuire

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1988; Champagne et al. 2001). Finally, studies in mice using reciprocal hybrids and embryonic transfer have revealed that differences in the prenatal environment may also shape the offspring's adult phenotype (Calatayud & Belzung 2001; Francis et al. 2003; Calatayud et al. 2004; Caldji et al. 2004; Priebe et al. 2005). In these studies, changes in stress reactivity, exploratory behaviour, response to novelty and maternal behaviour of offspring in response to the maternal environment are observed (Champagne & Curley 2005).

The main focus of the above approaches has been to demonstrate maternal effects on offspring phenotype that occur over one generation. However, life history theory of evolutionary biology strongly suggests transgenerational transmission of phenotypes including behaviour (Jablonka & Lamb 2005). Since grandoffspring may inherit the physical or social environment of their parents and grandparents, it could be beneficial for them to inherit the phenotype and hence epigenetic status of their parents and grandparents also. Evidence supporting this hypothesis comes from the nonmammalian evolutionary biology literature, particularly in *Drosophila* where various environmentally induced morphological variants are able to transmit their phenotype nongenomically via the mother (Rogilds et al. 2005). Such transgenerational maternal effects on offspring morphology and reproductive success have also been reported in vertebrates such as fish (Bashey 2006) and birds (Naguib et al. 2006). In mammals, the possibility for transgenerational maternal inheritance of behavioural phenotypes has not been studied as extensively though examples of these effects have been observed in rats. Meaney and colleagues (Champagne et al. 2003a) have demonstrated that individual differences in the licking and grooming of offspring by rat dams lead to altered behaviour in both male and female offspring and grandoffspring. Through cross-fostering, it has also been demonstrated that the effect of handling pups on their response to novelty as adults can be inherited in a nongenomic fashion across generations (Denenberg & Rosenberg 1967).

In our previous studies we showed that mutant Peg3$^{+/-}$ females are impaired in various components of their maternal care including the supply of nutrients to the developing embryo prenatally and the nursing, retrieval and licking/grooming of pups and nest building postnatally and also that they are neophobic (Li et al. 1999; Curley et al. 2004; Champagne et al. 2005). The *Peg3* gene is imprinted and is expressed in an individual only when it is inherited from the father because the maternal allele is always silenced (Li et al. 1999). It encodes a zinc finger protein that regulates cell survival in the placenta, embryo, and developing and adult brain and therefore has the capacity to shape and remodel offspring development and behaviour (Kuroiwa et al. 1996; Relaix et al. 1998; Li et al. 1999; Deng & Wu 2000; Johnson et al. 2002; Swaney et al. 2007). Mechanistically, the deficits observed in Peg3$^{+/-}$ mutant females appear to be related to impairments in the oxytocinergic system, with mutants possessing fewer oxytocin neurons in the paraventricular and supraoptic nuclei of the hypothalamus and fewer oxytocin receptors in the medial preoptic area (Li et al. 1999; Champagne et al. 2005). Given that mutant Peg3$^{+/-}$ females exhibit these phenotypic differences, we investigated whether their offspring also exhibited changes in exploratory behaviour and maternal behaviour and whether any changes could be transmitted to a third generation. We chose to use two tests (novel object and open field) that investigated both aspects of the approach (exploration) versus withdraw (neophobia) conflict that occurs when an animal is faced with novelty (van Gaalen & Steckler 2000; Tang & Sanford 2005) and the retrieval test to measure maternal care (Champagne et al. 2007). Significantly, as the *Peg3* mutation is expressed only when inherited through the patriline, we were able to investigate the behaviour of genetically wild-type (WT) offspring and grandoffspring that differed only with respect to the quality of the maternal environment during the pre- and postnatal periods (Fig. 1).

METHODS

Subjects

All procedures were undertaken with the relevant ethical approvals, including a review by the University of Cambridge Animal Ethics Committee, a Home Office project licence to E.B.K., as well as Home Office personal licences to E.B.K. and J.P.C. All subjects were laboratory mice, *Mus musculus*. Animals were housed at the Sub-Department of Animal Behaviour (Madingley, Cambridge, U.K.) on a reverse 12:12 h light:dark cycle, with the dark phase commencing at 0800 hours and the light phase at

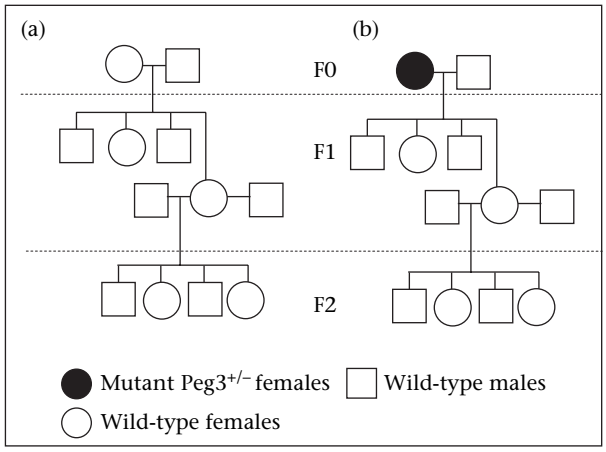

Figure 1. Breeding design. In the parental generation F0, 129Sv wild-type males are mated with either (a) 129Sv wild-type dams or (b) mutant 129SvPeg3$^{+/-}$ dams. Peg3 is an imprinted gene expressed according to parent of origin. Because the heterozygous Peg3$^{+/-}$ female is mated with a wild-type male, none of the F1 or F2 generations expresses this mutation. Hence epigenetic transgenerational effects of reduced maternal care can be determined through these generations. Virgin F1 offspring were behaviourally assessed in the novel object and open-field tests. Twelve F1 females in both groups were not used in these tests but were mated twice with 129Sv wild-type males to assess their maternal behaviour in a retrieval test with their first and second litters. The offspring of the first of these matings were weaned to produce the F2 generation and were tested as virgin animals on the novel object and open-field tests.

2000 hours, under a constant temperature of 21°C and 55% relative humidity. All animals were given ad libitum access to water and the RM1 (E) chow diet supplied by Lillico. Fresh bedding, wood shavings supplied by Lillico, was placed into opaque cages (4200 × 125 × 125 mm) weekly. Behavioural observations took place between 6 and 8 h into the dark period of the light cycle, when the mice were most active. No behavioural trial took place on the day of cage cleaning, and all trials were carried out under dim-red-light illumination.

The original *Peg3* mutation was developed on the 129Sv inbred strain and the generation of the targeted mutation is described elsewhere (Li et al. 1999). Briefly, a 4.8-kb IRES-βgeo-SV40 polyadenylation selection cassette was inserted into the 5′ coding exon of the *Peg3* gene in embryonic stem cells of the 129Sv inbred strain using gene targeting. Heterozygous embryos inheriting the $Peg3^{\beta geo}$ mutation from the paternal germ line showed no detectable wild-type *Peg3* mRNA but did show β-galactosidase (β-Gal) expression. β-Gal is a marker protein inserted into the mutant *Peg3* gene produced by mutant $Peg3^{+/-}$ animals rather than the functional Peg3 protein, allowing mutant animals to be identified. To perform this, cartilage tissue (where there is high expression of the mutation) was removed from a 2-mm-long piece of tail of the subjects. Prior to the removal of the tail a moderate local anaesthetic (lidocaine) was applied to the lower tail area. Postremoval mice were observed for 10 min to ensure that they were unaffected by the procedure and were moving freely in the cage, and no long-term effects of this procedure on the adult behaviour or general functioning have been observed. Tissue was incubated in a small 1-ml Eppendorf tube, in phosphate-buffered saline containing X-gal (1 mg/ml), 5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$ and 0.02% Nonidet-P40 at room temperature and placed on a orbital shaker overnight until colour developed. Although there are differences in the growth and behaviour of wild-type and mutant $Peg3^{+/-}$ mice, there are no significant negative consequences of this mutation on their general health and functioning (Li et al. 1999; Curley et al. 2004; Swaney et al. 2007).

Experimental Design

F0 generation

Twelve nulliparous wild-type and 12 $Peg3^{+/-}$ females aged 90 days were mated with sexually mature 3-month-old wild-type 129Sv males from our colony. We used two 129Sv males from each of six litters. One male from each litter was paired with a wild-type female, and the other was paired with a mutant female. Males were removed immediately after mating, as identified by the presence of a vaginal plug. Twelve wild-type females and nine mutant females gave birth to litters ($Peg3^{+/-}$ dams: 6.1 ± 0.5 pups; wild-type dams: 6.3 ± 0.6 pups; data are means ± SE).

F1 generation

Offspring of these matings remained with their mothers until day 28 postnatally (pn) when they were individually ear punched and housed in same-sex groups of five mice, with four receiving one punch to either the top or the bottom of either ear and one receiving no punch. As before, mice were observed post ear punching for 10 min to ensure that they were unaffected and were moving freely in the cage. We have found no long-term effects of ear punching on the general functioning or adult behaviour of mice. Animals remained in these groups throughout the duration of the experiment, having their bedding changed once a week. Sexually naïve animals aged 90–120 days were used in behavioural tests and females were always tested while in diestrus as determined by vaginal smears. The novel object test was conducted first with open-field testing taking place 1 week later. Twelve wild-type daughters of each maternal type (wild-type and $Peg3^{+/-}$ mother, one daughter from each dam) were randomly selected and placed at day 30 pn with virgin 129Sv wild-type males selected as in the previous generation to produce the third-generation offspring. Males were removed 1 day prior to parturition but were allowed to remate the females following a retrieval test on the day of birth so that the maternal behaviour with a second litter could be assessed.

F2 generation

Offspring of these matings were housed in the same manner as the F1 generation. Their behaviour was then assessed in novel object and open-field tests also as in the previous generation.

Genotyping

All subjects were genotyped prior to weaning, with no detectable β-Gal expression in any offspring or grandoffspring, indicating that all were genetically wild-type and expressing a functional copy of their paternal *Peg3* gene (see above). All mutant $Peg3^{+/-}$ mothers in the parental generation were shown to express the paternal $Peg3^{+/-}$ mutation by the same staining procedure.

Novel Object Test

This test procedure was adapted from that used in a previous study (Bateson & D'Udine 1986) and was developed as a voluntary exploration paradigm to identify genetic influences on exploratory behaviour. Animals were housed singly for 24 h before testing in a smaller cage (3600 × 125 × 125 mm) with their own bedding. At the beginning of each test, the mouse's cage was positioned at eye level 80 cm in front of the observer. The top metal grid of the cage was carefully lifted and a 6 × 4 × 2 cm empty cardboard matchbox was gently dropped in front of the animal. Each animal received a new matchbox that had not previously been used in any test. In a 300-s test, the latency to perform and the frequency of the following behaviours were recorded manually: (1) sniff: the animal approached the box at a distance of less than 1 cm and sniffed, (2) nasal touch: the animal approached the box and gently touched it with the tip of its nose, (3) paws on: the animal placed one or more paws on the box and (4) bite: the animal bit a part of the box. For the

purpose of analysis a general category, 'contacts' was used for all those activities that included contact with the box (nasal touch, paws on, bite). Additionally, the total time spent in investigation or contact with the box was recorded.

Open-Field Test

The open-field test is a standard tool for measuring exploratory behaviour in rodents (for reviews see Archer 1973; Crawley 1985; Belzung & Griebel 2001; Takahashi et al. 2006). The open field used was a $60 \times 60 \times 40$ cm plastic box. The sides of this box were black and the floor consisted of white tiles. The behaviour 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 areas of the field as well as the overall activity of the animal. 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. All testing was conducted under red (dark phase) lighting conditions. During analysis of the recordings, the field was divided into a grid of 10×10 squares. We assessed three behavioural indicators of exploratory behaviour: (1) inner field exploration: time spent in the inner 9×9 squares, (2) activity: number of square crossings and (3) freezing: time spent immobile. For statistical analysis, behavioural data were analysed over the full 10 min and separately for the first minute because the initial behavioural reactivity to the open field is representative of the response to novelty and is distinct from habituation effects (Vadasz et al. 1992; Kalueff et al. 2006; Takahashi et al. 2006).

Maternal Retrieval Test

Twelve daughters of each dam type were mated with wild-type males at 30 days pn as described under Experimental design. Males were removed 1 day prior to birth and the dams' maternal behaviour with her first litter was examined. The retrieval behaviour of females on the day of the parturition of their second litter was also measured. All 12 daughters of wild-type dams gave birth to one litter (mean age of dams = 61.1 days), and 10 gave birth to a second litter (mean age of dams = 91.1 days). Nine of 12 daughters of mutant dams gave birth to one litter (mean age of dams = 63.2 days), and eight gave birth to a second litter (mean age of dams = 92.3 days). Females were tested in their home cage on the day of birth of their litters. Three pups were randomly placed away from the nest end of the home cage, and the latency to retrieve each of the three pups, nestbuild and crouch over the pups was recorded manually. If a female had not completed this within 15 min the test was terminated, and the female was allocated a latency of 900 s for those behaviours that were not completed. Any pups not used during the test were huddled together in a separate cage and kept warm under a lamp. All pups were returned to the mother after she had completed each behaviour or at the end of the 15 min and were observed for the following hour to ensure that the dam took care of all of the pups. After this period, we observed that all mothers nursed and licked/groomed their pups.

Statistical Analysis

All behavioural tests and decoding were performed blind. All statistical tests were undertaken using SPSS v13.0, Chicago, IL, U.S.A. Parametric statistics were used because assumptions of these tests were met. All animals that comprised each of the eight groups (sons, daughters, grandsons or granddaughters of wild-type or mutant dams) for the novel object and open-field tests came from at least six separate litters and a maximum of 12 litters and no more than two animals per litter per sex were used. In the assessment of maternal behaviour, each of the 12 females mated had originated from separate litters. Group sizes were unequal due to a number of factors, mainly because of variation in the litter size and sex ratio of litters. There were also fewer females used in the F1 novel object and open-field tests because at least one daughter per mother was removed to be mated for pup retrieval analysis and because mutant dams gave birth to more male-biased litters. Group sizes were also smaller in the F2 generation because of smaller litters produced by the F1 females. To correct for any contribution to the effects of using multiple individuals from a single litter, 'litter-of-origin' was used as a covariate in the analysis of the novel object and open-field tests. This was not necessary in the maternal behaviour assessment because each female originated from a separate dam.

Novel object test

For each subject animal, the latency to perform each behaviour, the frequency of each behaviour and the total time spent investigating were analysed in separate ANCOVAs using a GLM with maternal type and sex as fixed factors and litter-of-origin as a covariate.

Open-field test

For each subject animal, the time spent in the inner area, the number of square crossings and the time spent freezing in the first minute and over the full 10 min were analysed in separate ANCOVAs using a GLM with maternal type and sex as fixed factors and litter-of-origin as a covariate. In both the novel object and the open-field analyses, in cases where the model yielded a significant interaction between the two fixed factors, Tukey's post hoc test was conducted to determine the significant contrasts.

Maternal retrieval test

For each dam, the latency to complete each behavioural outcome measure was analysed in separate ANOVAs using a GLM with maternal type as a fixed factor. The behaviour of each dam with her first and second litters was also analysed in separate ANOVAs. All data reported in Results, figures and tables are means $\pm$ SE.

RESULTS

F1 Generation: Behaviour of Offspring of Wild-type and Mutant Peg3$^{+/-}$ Dams

Exploration of a novel object

The latency to perform and the frequency of each behaviour (Table 1) were analysed in the four groups (sons of wild-type dams, sons of mutant dams, daughters of wild-type dams, daughters of mutant dams). GLMs indicated a significant sex by maternal genotype interaction on latency to bite the object ($F_{1,59} = 7.66$, $P < 0.01$), number of nasal touches ($F_{1,59} = 3.78$, $P < 0.05$), number of bites ($F_{1,59} = 7.10$, $P < 0.01$), total number of contacts ($F_{1,59} = 6.83$, $P < 0.01$) and total time spent investigating the object ($F_{1,59} = 6.40$, $P < 0.05$). Post hoc tests revealed that daughters of wild-type dams were quicker to bite the novel object ($P < 0.001$) and made more nasal touches ($P < 0.001$), bites ($P < 0.001$) and total contacts ($P < 0.001$; Fig. 2a) with the object than daughters of mutant dams. The total investigation time of the novel object was also higher in daughters born to control than to Peg3$^{+/-}$ mothers ($P < 0.001$; Fig. 2b). There were no differences between daughters of either maternal type in the latency to sniff or in the total number of sniffs. Moreover, sons of wild-type dams did not differ from males born to Peg3$^{+/-}$ mothers during the novel object test in any aspect of behaviour (Table 1).

Among offspring born to WT mothers, sexually dimorphic behaviours were observed in response to the novel object. Post hoc tests revealed that females were significantly faster than males to bite the novel object ($P < 0.001$) and made more bites ($P < 0.001$), nasal touches ($P < 0.001$) and total contacts ($P < 0.001$) of the object. Control females also investigated the novel object for significantly longer ($P < 0.001$) than control males. However, none of these behaviours was sexually dimorphic in the offspring born to Peg3$^{+/-}$ mothers.

Open-field activity and exploration

The activity (as measured by the number of squares crossed) observed in the first minute of the open-field test is referred to as the initial reactivity (Table 2). GLMs indicated a significant interaction between sex and maternal genotype in initial reactivity ($F_{1,59} = 4.85$, $P < 0.05$). Post hoc analysis indicated no significant difference in the initial reactivity of sons born to WT or Peg3$^{+/-}$ mothers; however, daughters of Peg3$^{+/-}$ mothers showed significantly less initial reactivity than daughters born to wild-type dams ($P < 0.01$). A significant interaction between sex and maternal genotype was found in time spent immobile ($F_{1,59} = 4.25$, $P < 0.05$), with daughters of wild-type dams spending significantly less time inactive than daughters of mutant dams ($P < 0.05$) and the sons of wild-type dams ($P < 0.01$). No differences were found in time spent exploring the inner area of the open field as a function of sex or maternal phenotype.

Maternal behaviour

There were no significant differences between daughters of each maternal type in their latency to investigate a pup, nestbuild or crouch over pups with either their first or their second litters. However, there were significant differences in the latency to retrieve the first ($F_{1,19} = 4.933$, $P < 0.05$), second ($F_{1,19} = 4.593$, $P < 0.05$) and third ($F_{1,19} = 4.528$, $P < 0.05$) pups, with daughters of wild-type dams being quicker to retrieve than daughters of mutant dams with their first litters (Fig. 3). There were no differences in the latency to retrieve pups in daughters of wild-type and mutant Peg3$^{+/-}$ mothers with their second litters.

F2 Generation: Behaviour of the Grandoffspring of Wild-type and Mutant Peg3$^{+/-}$ Dams

Exploration of a novel object

Significant grandmaternal genotype by sex interactions were found in the number of nasal ($F_{1,50} = 2.918$, $P < 0.05$) and paw ($F_{1,50} = 4.127$, $P < 0.01$) touches as well as the total number of contacts with the novel object ($F_{1,50} = 3.722$, $P < 0.05$; Table 3). Granddaughters of WT

Table 1. Behaviour of F1 offspring in the novel object test

	Maternal type, sex & (*N*)			
	WT F (15)	Mutant F (15)	WT M (24)	Mutant M (9)
Latency to sniff (s)	59±16	66±12	60±17	62±31
Latency to nasal touch (s)	160±29	253±19	227±21	255±31
Latency to paws on (s)	189±28	269±1	223±20	216±35
Latency to bite (s)	169±29^{a}	285±12^{d}	264±16^{d}	255±32^{b}
Latency to 1st contact (s)	154±30	245±21	199±22	204±36
Sniff total	12.1±1.5	10.8±1.9	8.8±1.0	11.4±2.4
Nasal touch total	4.0±0.9^{a}	0.5±0.2^{d}	0.9±0.4^{d}	0.4±0.3^{d}
Paws on total	4.0±1.3^{d}	0.7±0.5	1.5±0.4	1.4±1.0
Bite total	4.7±1.3^{a}	0.5±0.5^{d}	0.9±0.4^{d}	1.2±1.1^{b}
All contacts total	12.7±3.2^{a}	1.7±1.1^{d}	3.3±1.1^{d}	3.2±2.6^{c}
Total time investigating (s)	71.8±13.7^{a}	22.9±5.8^{d}	30.4±5.9^{d}	30.5±12.3^{c}

In Tables 1–4, superscript letters refer to results of post hoc tests where group differences were identified by the significant interaction: a versus b, $P < 0.05$; a versus c, $P < 0.01$; a versus d, $P < 0.005$; b versus c, NS; b versus d, NS; c versus d, NS. All data are means ± SE.

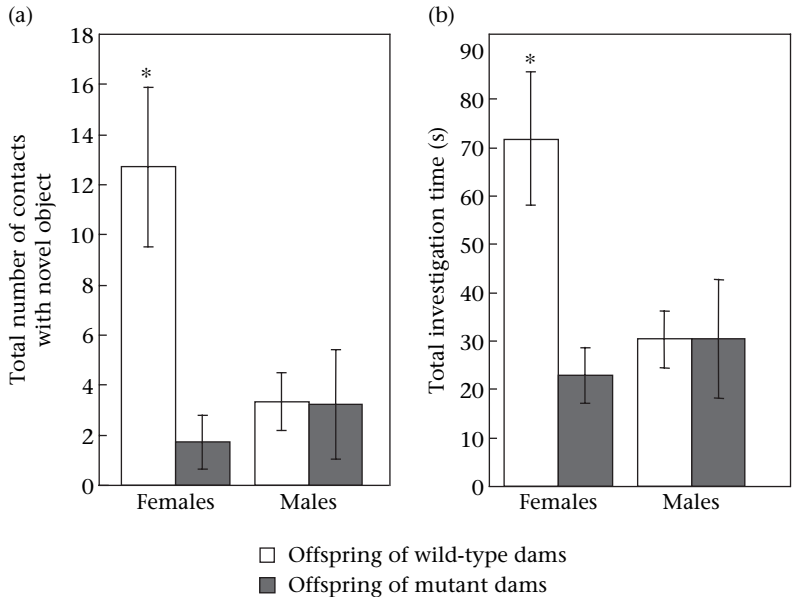

Figure 2. F1 Generation investigation of a novel object. (a) Total number of contacts (nasal touches, bites and paw contacts) made with the novel object in a 5-min test by F1 offspring of both sexes. (b) Total time spent investigating the novel object in a 5-min test by F1 offspring of both sexes. See Table 1 for *N*s. Data are means ± SE.

dams made more paw and nasal touches to the object than granddaughters of mutant dams ($P < 0.05$). Likewise, granddaughters of wild-type dams made more total contacts with the object than granddaughters of mutant dams ($P < 0.05$; Fig. 4a). Sexually dimorphic effects were found, with the granddaughters of wild-type females making more paw contacts and total contacts than grandsons of wild-type dams ($P < 0.05$). No significant differences were found in grandsons of wild-type and mutant mothers in the number of contacts with the object nor in grandsons and granddaughters of mutant dams. Moreover, the latency to sniff, nasal touch, bite or put paws on or make a first contact or the total number of sniffs or bites with the object did not differ significantly between any groups.

Figure 4b shows the total time spent investigating the novel object and the GLM revealed significant interaction effects between grandmaternal genotype and sex ($F_{1,50} = 3.448$, $P < 0.05$). Granddaughters of wild-type dams spent longer investigating the object than both granddaughters of mutant dams ($P < 0.05$) and grandsons of wild-type dams ($P < 0.05$). No significant differences were found between grandsons of wild-type and mutant dams nor between grandsons and granddaughters of mutant dams in the total time spent investigating the object.

Open-field reactivity and exploration

GLMs revealed no significant effect of sex or grandmaternal genotype on the number of squares crossed during the first minute of testing. Furthermore, no significant interaction existed between sex and grandmaternal genotype in the number of squares crossed during this period (Table 4). Congruent with this finding, there was no significant effect of sex or grandmaternal genotype on the time spent immobile in the first minute. In the first minute of testing there was a main effect of sex on exploratory behaviour ($F_{1,50} = 11.952$, $P < 0.001$), with males spending longer in the inner area than females. A main effect of grandmaternal genotype was found on initial exploratory behaviour ($F_{1,50} = 4.181$, $P < 0.05$), with grandoffspring of mutant *Peg3*$^{+/-}$ dams spending less time in the inner area than the grandoffspring of wild-type dams during the first minute of testing. Over the full 10 min of the test, a significant interaction was found between sex and grandmaternal genotype in the amount

Table 2. Behaviour of F1 offspring in the open field

	Maternal type, sex & (*N*)			
	WT F (15)	Mutant F (15)	WT M (24)	Mutant M (9)
1st min, time immobile (s)	8.6±2.5[a]	20.0±2.8[b]	17.0±2.6[c]	15.8±2.9[a]
1st min, time in (s)	4.8±1.0	4.7±0.8	3.8±1.0	4.1±0.9
1st min, total squares crossed	36.3±4.9[a]	19.4±2.1[c]	21.7±2.7[d]	20.2±3.7[c]
10 min, time immobile (s)	322.0±33.7	408.2±29.4	384.8±25.1	334.4±36.8
10 min, time in (s)	17.4±4.2	14.2±4.4	14.8±3.6	20.2±6.1
10 min, total squares crossed	168.1±40.8	96.2±24.7	97.8±19.7	115.2±24.7

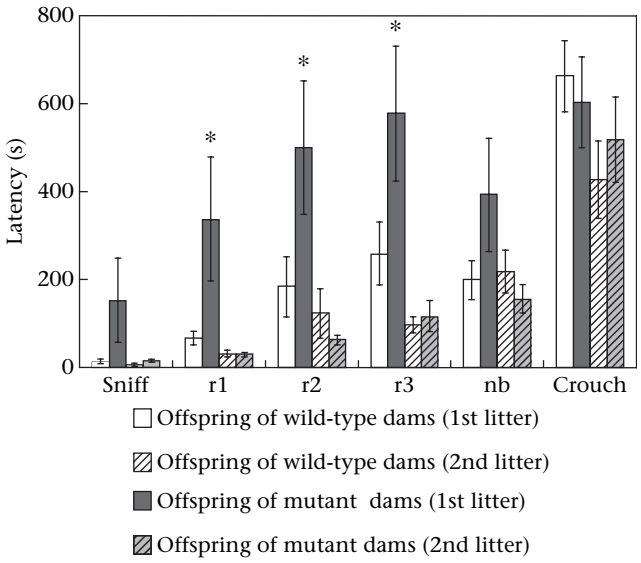

Figure 3. Maternal behaviour of F1 females in a retrieval test. Latency to sniff a pup, retrieve first pup (r1), retrieve second pup (r2), retrieve third pup (r3), nestbuild (nb) and crouch over pups. See Methods for *N*s. Data are means ± SE.

of time spent in the inner area ($F_{1,50} = 6.489$, $P < 0.05$). Grandsons of wild-type dams spent significantly longer in the inner area of the open field than granddaughters of wild-type dams ($P < 0.001$). The grandsons and granddaughters of mutant females did not differ in their investigation of the inner area. Moreover, grandsons of wild-type dams spent longer in the inner area than grandsons of mutant dams ($P < 0.01$), but no significant difference was found between the granddaughters of wild-type and mutant females in time spent in the inner area.

DISCUSSION

Impaired Maternal Care and Offspring Behavioural Development

Our findings demonstrate that the adult female offspring of Peg3$^{+/-}$ mothers show distinct phenotypes. Daughters of mutant mothers were inhibited towards a novel object, exhibiting significantly less contact than control daughters, whereas male offspring were not affected. Female offspring of mutant mothers were slower to make contact and had a lower frequency of contacts with the object on all measures of contact (nasal touch, paws on, bites). However, daughters from both maternal groups had equivalent latencies to sniff the object and made the same number of olfactory investigations, suggesting that the behavioural changes in daughters of mutant mothers are related specifically to overcoming the neophobia of initiating and sustaining contact with the object (Bateson & D'Udine 1986). Similar increases in neophobia were observed during the open-field test in daughters but not in sons of mutant dams. In the first minute of the test, daughters of mutant mothers were frequently immobile and less exploratory than the daughters of wild-type dams. The same results were found over the full 10-min trial, with the female offspring of control mothers being more active than the female offspring of mutant mothers. Such decreased activity in mice of various 129Sv background strains is consistent with increased fearfulness and decreased exploration (Tang & Sanford 2005; van Bogaert et al. 2006). Overall, the open-field behaviour observed is consistent with the findings of the novel object test, indicating a loss of sexually dimorphic exploratory behaviour in the offspring of mutant mothers related to decreased exploration in daughters.

That increased neophobia and decreased exploratory behaviour of offspring can be induced by variations in the maternal environment is consistent with findings from previous studies. In rodents and primates, perturbation or variation in the quality of the prenatal and/or postnatal maternal environment has been demonstrated to modify offspring development with particularly profound effects on the hypothalamic–pituitary–adrenal response to stress (Liu et al. 1997; Champagne & Curley 2005). Embryo transfer and postnatal cross-fostering studies confirm the importance of mother–infant interactions in mediating these effects (Champagne et al. 2003a; Francis et al. 2003; Priebe et al. 2005). The present study is unique in that we have been able to examine the behaviour of wild-type offspring that have received reduced pre- and postnatal maternal care in a transgenic model without

Table 3. Behaviour of F2 offspring in novel object test

	Grandmaternal type, sex & (*N*)			
	WT F (15)	Mutant F (13)	WT M (14)	Mutant M (12)
Latency to sniff (s)	40±19	77±26	98±31	81±29
Latency to nasal touch (s)	201±32	264±21	251±27	286±14
Latency to paws on (s)	213±29	265±20	260±24	289±11
Latency to bite (s)	230±29	267±21	276±22	295±5
Latency to 1st contact (s)	179±34	228±27	231±27	282±14
Sniff total	9.6±1.5	9.1±1.8	8.0±1.3	8.9±1.7
Nasal touch total	1.5±0.6^{a}	0.4±0.2^{b}	0.5±0.3^{b}	0.1±0.1^{b}
Paws on total	3±1.1^{a}	0.7±0.3^{c}	0.5±0.3^{b}	0.2±0.2^{b}
Bite total	1.9±0.9	0.5±0.3	0.5±0.4	0.2±0.2
All contacts total	6.6±2.4^{a}	1.6±0.6^{b}	1.5±1.0^{b}	0.4±0.3^{b}
Total time investigating (s)	47.6±14.3^{a}	22.2±5.5^{b}	16.3±4.2^{b}	12.2±2.4^{b}

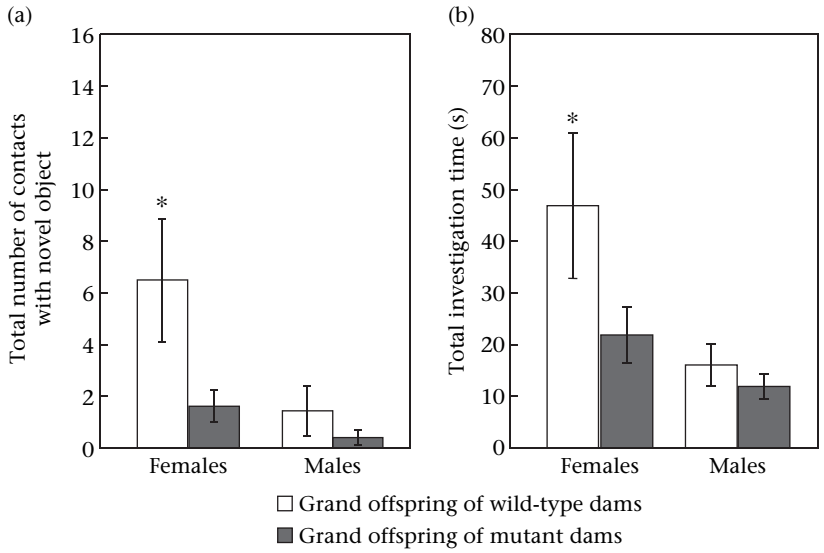

Figure 4. F2 Generation investigation of a novel object. (a) Total number of contacts (nasal touches, bites and paw contacts) made with the novel object in a 5-min test by F2 offspring of both sexes. (b) Total time spent investigating the novel object in a 5-min test by F2 offspring of both sexes. See Table 3 for *N*s. Data are means ± SE.

disturbing the mother–infant relationship postmating. It remains to be seen what neural or hormonal mechanisms underlie the behavioural differences that we have observed, though these changes are likely to be induced via changes in gene expression occurring during the pre- and postnatal phases. Because the female offspring of mutant dams appear to be more male-like in their phenotype, one potential mechanism is the masculinization of offspring related to changes in androgen or estrogen levels and responsiveness. Similar masculinization effects related to altered steroid hormone regulation have been found in the female offspring of gestationally stressed guinea pig dams, as these daughters develop decreased exploration and increased anxiety in adulthood (Kaiser et al. 2003; Kaiser & Sachser 2005). Nevertheless, regardless of the precise mechanism, the increased neophobia and decreased exploration exhibited by wild-type daughters show an inheritance of this phenotype from mutant mother to daughter via a non-Mendelian epigenetic mechanism.

It is also significant to note that we have found striking sex differences in the effect of reduced maternal care on offspring behaviour. Many studies investigating other types of disruption to the mother–infant relationship have not examined sex-specific effects because often only one sex (usually males) is tested. Nevertheless, several studies have reported that female offspring are more responsive to deterioration in the quality of care received than are male offspring. For instance, daughters but not sons of undernourished rat dams have altered feeding behaviour (Bellinger et al. 2004), and daughters but not sons of stressed dams show increased adult anxiety levels (Ladd et al. 2000). However, it is likely that the response of offspring to maternal environment may be dependent upon the exact type of disruption to the mother–infant relationship (Wigger & Neumann 1999).

Transmission of Maternal Behaviour

We observed an inheritance of the maternal behaviour phenotype in F1 females, even without an inheritance of genotype. Primiparous wild-type offspring of mutant dams were slower to retrieve pups back to a nest than wild-type daughters of wild-type dams. The daughters of mutant dams therefore exhibited the same deficits in maternal care that have been previously described for

Table 4. Behaviour of F2 offspring in the open field

	Maternal type, sex & (*N*)			
	WT F (15)	Mutant F (13)	WT M (14)	Mutant M (12)
1st min, time immobile (s)	21.4±3.6	23.2±3.7	17.5±4.0	23.4±5.1
1st min, time in (s)	3.1±0.8	2.1±0.6	14.2±4.2	6.1±1.4
1st min, total squares crossed	18.3±2.6	18.4±3.3	23.0±5.1	20.8±4.8
10 min, time immobile (s)	484.5±17.0^{b}	416.8±30.0^{a}	335.5±38.6^{a}	445.7±34.2^{b}
10 min, time in (s)	5.3±1.3^{d}	9.0±2.2^{d}	29.6±7.1^{a}	12.8±3.8^{c}
10 min, total squares crossed	43.5±6.8	81.4±24.8	148.3±48.3	79.8±30.5

the mutant dams themselves (Li et al. 1999; Curley et al. 2004). The transmission of maternal deficits observed in our study could be due to prenatal or postnatal factors or a combination of both affecting the central and peripheral nervous system development of offspring. As mutant Peg3$^{+/-}$ dams exhibit reduced oxytocin neurons and receptors (Li et al. 1999; Champagne et al. 2005), it may be possible that this is also the neural mechanism through which offspring are impaired. Furthermore, the reduction in exploration exhibited by female offspring of mutant dams may be a contributing factor to their impairments in maternal behaviour because these deficits are observed in primiparous but not multiparous dams. For primiparous mothers, newborn pups are novel stimuli and overcoming the neophobia of pups is an important prerequisite to successful maternal care (Leckman & Herman 2002). Indeed, female offspring of mutant mothers are impaired specifically in their ability to retrieve pups and not in other aspects of maternal behaviour such as crouching or nest building.

Several studies have demonstrated a transmission of maternal care related to the postnatal environment (Champagne & Curley 2005). In rats, nonhuman primates and humans, individual differences in maternal behaviour can be passed down the matriline. For instance, in humans the attachment style of an infant to its mother has been shown to be transmitted down three generations (Benoit & Parker 1994). In vervet monkeys, *Chlorocebus aethiops*, and rhesus monkeys the amount of time spent in contact with offspring is also inherited maternally (Fairbanks 1989). In rats, natural variations in licking and grooming by dams lead to the epigenetic modification of the oestrogen receptor alpha gene promoter in the medial preoptic area of female offspring, resulting in differential receptor expression which in turn leads to variations in licking and grooming by these offspring towards their own pups (Champagne et al. 2003a, b, 2006). Moreover, disruptions to the mother–infant relationship can also lead to the inheritance of impaired maternal care. Reducing the normal exposure of female mouse pups to maternal interactions through early weaning is associated with female offspring displaying lower levels of licking, grooming and nursing towards their own pups (Kikusui et al. 2005). Female rat pups that are artificially separated from their mothers for short repeated periods (Lovic et al. 2001) or that experience complete maternal deprivation (Gonzalez & Fleming 2002) exhibit impaired maternal care, retrieving fewer pups during a retrieval test and exhibiting reduced licking, grooming and crouching behaviours (Fleming et al. 2002). The accumulating evidence that maternal care can be inherited nongenomically by offspring from their mothers has important implications for the inheritance of other patterns of behaviour across generations.

Transmission of Exploratory Behaviour and Neophobia Phenotypes Across Generations

We found a remarkable transmission of the behavioural phenotype across generations, with the female grandoffspring of mutant dams also showing increased neophobia and decreased exploration. Female offspring and grandoffspring of Peg3$^{+/-}$ mutant mothers show elevated levels of neophobia and decreased levels of exploration when exposed to a novel object. As with the previous generation, no significant differences existed in any aspects of the male behaviour in this test. In the open-field test, however, less evidence existed for a transmission of phenotype from offspring to grandoffspring. This appears to be due primarily to the lower levels of exploration exhibited by the female control offspring (i.e. the female grandoffspring of wild-type dams) in this generation, which may be related to the younger age of their mothers (Crusio & Schmitt 1996). The mothers of the grandoffspring were mated immediately after puberty to measure their reproductive success and were therefore much younger dams than the 4-month-old wild-type dams in the previous generation. Another explanation for the discrepancy in transmission may be, as already outlined, that the phenotypes that these two tests measure are slightly different (Tang & Sanford 2005; van Bogaert et al. 2006); the open-field test is a forced exploration of a novel environment whereas the novel object test is a voluntary exploration of a novel object and is less anxiogenic. It may be that it is the exploratory component of the phenotype, rather than the anxiety component, that can be transmitted transgenerationally.

The pathways via which the environmentally induced transgenerational inheritance of phenotypes operates are an exciting area of epigenetic research. We suggest that there are two broad possibilities for our observed nongenomic inheritance of exploration of novelty. One is that the decreased levels of maternal care received by female offspring in the F1 generation could have led to the altered epigenetic regulation of genes in both the germ line and the somatic tissues. This would be consistent with the observed phenotypes occurring in both the F1 and the F2 generations. Such germ line transmission of environmentally induced epigenetic modifications has been reported in individuals suffering some form of disruption in their in utero environment (Anway et al. 2005). Another potential mechanism for these transgenerational maternal effects is that the decreased care received by wild-type daughters of mutant dams leads to these offspring having altered neural or hormonal regulation of either or both the pre- and the postnatal environments. Prenatally, these F1 females may be less able to provide nutrition to offspring, for example due to a smaller uterine size; alternatively, they may have altered secretion of hormones during pregnancy that again disrupts development of the next generation. Such prenatal mechanisms have been observed in relation to the transmission of metabolic phenotypes (Zamenhof et al. 1971; McLeod et al. 1972). During the postnatal phase, maternal behaviour deficits in F1 females could lead to F2 offspring also demonstrating increased neophobia. This is akin to the model of nongenomic transmission of licking/grooming maternal behaviour down the matriline of rat dams (Champagne et al. 2006). Because this maternal behaviour also regulates the epigenetic modifications of the glucocorticoid receptor gene promoter, a nongenomic inheritance of anxiety and exploration

phenotypes is also observed (Weaver et al. 2004). Our finding that F1 primiparous females show impairments in one aspect of their maternal care (performance on the retrieval test) is tentative support for this hypothesis, though investigating the nursing and tactile stimulation given to the F2 pups by the F1 females and the cross-fostering of litters will be necessary to substantiate this. In future studies we shall investigate which of these mechanisms may be responsible for the transgenerational transmission of phenotypes.

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