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Title: Genomic imprinting mediates sexual experience-dependent olfactory learning in male mice

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Abbreviations AOB, accessory olfactory bulb; B6, C57BL/6J mouse strain; BNSTp, posterior part of the bed nucleus of the stria terminalis; MeAmgA, anterior medial amygdala; MeAmgPD, posterior-dorsal medial amygdala; MPOA, medial pre-optic area; NAc, nucleus accumbens; p3-SE, sexually experienced *Peg3* knockout mouse; p3-V, virgin *Peg3* knockout mouse; *Peg3*-KO, *Peg3* knockout; VMH, ventromedial hypothalamus; wt-SE, sexually experienced wild-type mouse; wt-V, virgin wild-type mouse.

Abstract

Mammalian imprinted genes are generally thought to have evolved as a result of conflict between parents, however recent knockout studies suggest that coadaptation between mother and offspring may have been a significant factor. Here we present evidence that the same imprinted gene that regulates mammalian maternal care and offspring development also regulates male sexual behavior and olfaction. We have shown that the behavior of male mice carrying a knockout of the imprinted gene *Peg3* does not change with sexual experience and that they are consequently unable to improve their copulatory abilities or olfactory interest in female odor cues after mating experience. Forebrain activation, as indexed by female odor-induced c-Fos protein induction, fails to increase with sexual experience, providing a neural basis for the behavioral deficits they display. The behavioral and neural effects of the *Peg3* knockout show that this imprinted gene has evolved to regulate multiple and varied aspects of reproduction, from male sexual behavior to female maternal care and the development of offspring. Moreover, sexual experience-driven behavioral changes may represent an adaptive response that enables males to increase their reproductive potential over their lifespan, and the effects we have found suggest that the evolution of genomic imprinting has been influenced by coadaptation between males and females, as well as between females and offspring.

Introduction

The expression of certain mammalian autosomal genes in a parent-of-origin fashion was first demonstrated in the 1980's and termed 'genomic imprinting' (1) and since then close to 100 maternally and paternally expressed imprinted genes have been identified (2). Over the last twenty years, the essential roles that imprinted genes play in development, particularly that of the brain, have become clear (3, 4) suggesting that imprinted genes might have enabled the evolutionary expansion of the mammalian brain (5). Most imprinted genes are strongly expressed in the placenta (6) and are involved in regulating fetal development (7). These developmental roles, in particular those of *Igf2*, *Igf2r*, *Igf2AS,* and *H19*, have been cited as evidence to support the conflict theory for the evolution of genomic imprinting (8). This posits that imprinted genes evolved with placentation in mammals as a result of parental conflict over offspring investment. The fetus/placenta is a genetically half-paternal structure which provides the father with an opportunity to manipulate the mother's investment in his offspring, to the possible detriment of her potential future offspring. Thus paternally expressed genes are predicted to enhance offspring growth, while maternally expressed genes are predicted to resist this, as a result of an evolutionary "arms race" over levels of investment in offspring. However, work by our group on the paternally expressed gene *Peg3* has shown that as well as regulating pup development, this gene also regulates mothers' maternal behavior (9), a finding that is not predicted by the conflict theory (10). Targeted deletion of *Peg3* in either pups or independently in mother results in a complementary phenotype affecting pup growth, pup suckling/maternal milk letdown, and pup thermoregulation/maternal nest-building (11), showing that these inter-dependent behaviors are co-regulated by the same imprinted locus. Peg3 is strongly expressed in both the developing embryo and the hypothalamus (9, 12) which controls maternal care in the mouse and in light of these findings, it has been proposed that co-adaptation between mother and offspring may also have been a factor in the evolution of imprinting in mammals.

For a paternally expressed gene to be transmitted down the patriline and become fixed in the population, it would be anticipated to also have adaptive effects on male behavior, enhancing male survival or reproductive success, and so we examined three different aspects of male behavior; sexual behavior, olfaction and sexual experience-dependent changes in behavior in *Peg3* knockout (*Peg3*-KO) animals. Olfaction is the most important sensory faculty in mice and female chemosignals detected by the main and accessory olfactory systems play a vital role in triggering sexual behavior in male mice, as studies involving ablation of the olfactory systems (13) or knockout of olfactory genes (14, 15) have shown. As well as regulating male sexual behavior, the hypothalamus receives input from the accessory olfactory system (16), and so the behavioral and neuronal responses of *Peg3*-KO males to female odors were also investigated to determine how the mutation affects reproduction-directed olfaction and male sexual behavior.

Sexual experience has been shown to have important effects on male behavior in different rodent species, modulating interest in female semiochemicals (17, 18), mating behavior (19, 20), and increasing male fertility (21). Such experience-dependent changes in behavior are likely to be adaptive, enabling males to enhance their sexual behavior throughout life, and so we tested both virgin and sexually experienced males to determine whether sexual experience plays a role in sexual behavior in male mice, and whether it has similar effects in *Peg3*-KO mutants and wild-type males.

We assessed the neural basis of these behaviors using c-Fos expression as a marker of brain activation in response to female urinary chemosignals in wild-type sexually experienced males (wt-SE), wild-type virgin males (wt-V), *Peg3*-KO sexually experienced males (p3-SE) and *Peg3*-KO virgin males (p3-V).

Results

***Peg3*-KO males' interest in female urine is unaffected by sexual experience.** Adult male C57BL/6J

(B6) mice were tested on their ability to discriminate between male and female urine and their preference

for female urine over male urine in olfactory tests in which they could physically contact the urine and so

use both the accessory and main olfactory systems. Habituation-dishabituation tests were used to measure

the ability of subject males to discriminate between urine and water, and between male and female urine.

Subject males were given three sequential presentations of water, followed by three sequential

presentations of one urine type and finally three sequential presentations of the other urine type. The

ability of the males to discriminate between water and urine and between the two urines was assessed by

analyzing changes in olfactory investigation levels when stimulus type was switched. ANOVA of the

time spent investigating all olfactory stimuli showed no effects of genotype or sexual experience, but did

show a significant interaction between these factors ($F_{1,23}$=8.164, P=0.009). Comparison of the time spent

sniffing the final water presentation and the first urine presentation showed that wt-SE (P=0.005), p3-SE

(P=0.008), wt-V (0.048) and p3-V males (P=0.004) all spent significantly longer sniffing urine than

water. Comparison of investigation levels when the urine stimulus type was changed showed that wt-SE

(P=0.001), wt-V (P=0.033) and p3-V males (P=0.001) all increased their olfactory investigations, but that

p3-SE males did not (figs. 1a&b).

The olfactory interest of wt-SE, wt-V, p3-SE and p3-V males was then investigated in urine preference

tests with simultaneous presentation of male and female urine. The time subject males spent in nasal

contact with male and female urine during 5-minute tests was analyzed to give measures of preferences

for male or female urine. All groups spent significantly longer investigating female urine than male urine

(wt-SE P=0.004, wt-V P=0.012, p3-SE P=0.020, p3-V P=0.023) (fig. 1c), however, comparisons of the

time spent sniffing each urine type showed clear differences between groups. ANOVA of the preference

for female urine, as assessed by the difference in investigation of male and female urine, revealed a

significant effect of sexual experience ($F_{1,33}$=10.541, P=0.003), no effect of genotype ($F_{1,33}$=2.558, P=0.120) and a significant interaction between these factors ($F_{1,33}$=6.791, P=0.014). *Post-hoc* tests showed that wt-SE males had a significantly greater preference for female urine than wt-V males (P=0.004), p3-SE males (P=0.040) and p3-V males (P=0.009), but no other group differences were found

Sexual behavior is enhanced by sexual experience in wild-type males but not in *Peg3*-KO males. To assess whether the *Peg3*-KO has similar effects on male mating behavior to those seen on male olfactory behavior, we tested the sexual behavior of wt-SE, wt-V, p3-SE and p3-V males. Males were paired with receptive virgin B6 females, their sexual behavior over the following hour was recorded and analyzed. Significantly more wt-SE than p3-SE males mounted (P=0.018), intromitted (P<0.001) and ejaculated (P=0.001) during testing, however no other group differences in the proportion of males performing sexual behavior were found. Latency data were analyzed for males that performed each behavior measured, but due to the very low incidence of intromission and ejaculation among p3-SE males, it was only possible to analyze mount latencies. ANOVA revealed an effect of genotype ($F_{1,36}$=13.859, P<0.001) on mount latency, no effect of sexual experience ($F_{1,36}$=2.062, P=0.160) and a significant interaction between the two factors ($F_{1,28}$=7.919, P=0.008). *Post-hoc* tests showed that wt-SE males mounted females significantly faster than wt-V (P=0.004), p3-SE (P<0.001) and p3-V (P<0.001) males, and that there were no other differences between groups (fig. 2a). Frequencies of sexual behavior were compared by ANOVA, revealing a significant effect of genotype ($F_{1,49}$=11.828, P<0.001) on the frequency of mounting, no effect of sexual experience ($F_{1,49}$=2.947, P=0.093) and a significant interaction between the two factors ($F_{1,49}$=9.648, P=0.003). There was also a significant effect of genotype on the frequency of intromission ($F_{1,49}$=25.635, P=0<0.001), a significant effect of sexual experience ($F_{1,49}$=5.978, P=0.018) and a significant interaction between these factors ($F_{1,49}$=9.990, P=0.003). *Post-hoc* tests showed that wt-SE males mounted more frequently than wt-V (P=0.002), p3-SE (P<0.001) and p3-V males (P=0.003),

and that wt-SE males also intromitted more frequently than wt-V ($P<0.001$), p3-SE ($P<0.001$) and p3-V males ($P<0.001$) (fig. 2b&c).

Sexual experience enhances forebrain activation by female chemosignals in wild-type but not *Peg3*-KO males. *Peg3* is expressed in the developing and adult hypothalamus (9, 12) and expression of the LacZ insertional mutation marker in adult *Peg3*-KO males indicates that it is particularly strongly expressed in the accessory olfactory pathway (fig. 3). The effects of the knockout on male behavior may thus be a consequence of a lack of *Peg3* expression in this pathway. To investigate the neural basis for the behavioral changes associated with sexual experience and the *Peg3* mutation, brain activation in response female odor cues was measured in wt-SE, wt-V, p3-SE and p3-V males by immunohistochemistry for c-Fos, a marker of neuronal activity that is commonly used in olfaction studies (22, 23). Subject males were exposed to estrus female urine or handled only, giving 8 experimental groups – exposed and control wt-SE, wt-V, p3-SE and p3-V males. c-Fos-positive neurons were counted in different nuclei of the accessory olfactory system including the accessory olfactory bulb (AOB), the anterior and posterior-dorsal medial amygdala (MeAmgA and MeAmgPD), the posterior bed nucleus of the stria terminalis (BNSTp), the medial pre-optic area (MPOA) and the ventromedial hypothalamus (VMH). While the accessory olfactory system primarily processes input from the vomeronasal organ, there is convergence in the forebrain with the main olfactory system, allowing the integration of volatile and non-volatile signals detected by both systems (24). c-Fos expression was also examined in the core and shell of the nucleus accumbens (NAc), a key nucleus in the brain's mesolimbic reward system which is activated by sexual activity (25). Moreover, activation of the nucleus accumbens by female chemosignals is affected by sexual experience in the rat (26) and the NAc receives a projection from the olfactory medial amygdala (27), suggesting that it may be involved in the sexual experience-dependent behavioral changes seen in male rodents.

ANOVA of mean c-Fos neuron counts revealed significant effects of sexual experience, genotype and urine exposure in each nucleus, as well as significant interactions (these data are presented in Table 1) and when appropriate, *post-hoc* tests were carried out (fig. 4).

In the AOB, urine exposure increased c-Fos neuron numbers in all groups except p3-V males. Moreover, there were significantly more AOB c-Fos neurons in urine-exposed wt-SE males than in any other urine-exposed male group. In the MeAmgA there were significantly more c-Fos neurons in urine-exposed wt-SE and p3-SE males than in control wt-SE and p3-SE males, however no differences due to urine exposure were seen in wt-V and p3-V males. Comparing only urine-exposed groups showed that the number of MeAmgA c-Fos neurons was significantly greater in wt-SE than either p3-SE or wt-V males, while no differences were found between p3-SE and p3-V males, or between wt-V and p3-V males. In the MeAmgPD there was a significant increase in c-Fos neurons associated with urine exposure in wt-SE and p3-SE males, but not in wt-V and p3-V males and no significant differences between any of the urine-exposed groups. In the BNSTp, there was a significant increase in c-Fos neurons associated with urine exposure in wt-SE and p3-SE males, but not wt-V or p3-V males. Comparing the urine-exposed males showed that while no differences were found between wt-V and p3-V males, BNSTp c-Fos neurons were more abundant in wt-SE than p3-SE males. Sexual experience also had significant effects on the BNSTp responses of urine-exposed males, with more c-Fos neurons in wt-SE than wt-V males, and in p3-SE than p3-V males. In the MPOA, urine exposure induced an increase in c-Fos in wt-SE males only, and c-Fos neurons were significantly more numerous in urine-exposed wt-SE males than urine-exposed wt-V, p3-SE or p3-V males. No other MPOA c-Fos differences were seen. c-Fos expression in the VMH was unaffected by either urine exposure, genotype or sexual experience. In the NAc core there were significant increases in c-Fos expression associated with urine exposure in wt-SE and wt-V males, but not in p3-SE or p3-V males. Among urine-exposed males, NAc core c-Fos expression did not differ between wt-V and wt-SE males, nor between p3-SE and p3-V males, however more c-Fos neurons were present in

wt-SE than p3-SE males and in wt-V than p3-V males. In the NAc shell, exposure to female urine produced an increase in c-Fos in wt-SE and wt-V males, but not in p3-SE or p3-V males. Among urine-exposed males, there were significantly more c-Fos neurons in the NAc shell in wt-SE males than p3-SE males, and in wt-V males than p3-V males.

Discussion

The data presented here show that as in other rodents, sexual experience has a powerful effect on male behavior in wild-type mice, improving copulatory abilities and sharpening olfactory interest in sexually-relevant female chemosignals. Mirroring these behavioral changes, we also found significant changes in the activation of the accessory olfactory pathway, with enhanced neuronal responses to female odors in wild-type sexually experienced males.

Both wt-SE and wt-V males were able to discriminate between male and female urine in habituation-dishabituation tests, and in urine preference tests both groups spent significantly longer sniffing female urine than male urine. Since both sexually experienced and naïve males preferred female urine, sexual experience appears not to be a prerequisite for this olfactory preference. However wt-SE males exhibited a far stronger preference for female urine than wt-V males. This increased interest in female chemosignals was matched by the shorter latencies of wt-SE males to mount females, as well as more frequent mounting and intromission. Male mice have previously been shown to prefer the volatile odors of estrus females (17) and to vocalize to aged female urine (28), but only when sexually experienced. Exposure to female urine also induces testosterone release in sexually experienced males, a pheromonal effect that appears to facilitate the initiation of sexual behavior in male mice (29). The increased interest in female odors exhibited by wt-SE males suggests that sexual experience increases the behavioral significance of female odors, reinforcing their signal value. Rodent studies have shown that latencies to mount, intromit and ejaculate with females decrease in male rats when sexually experienced (30) and that male hamsters

are faster to mount females when sexually experienced (31). Our findings match this previous work and suggest that increased investigation of female chemosignals may be a component of the increase in sexual interest exhibited by sexually experienced males.

Sexual experience had little effect however on the mating and olfactory behavior of *Peg3*-KO males. Although both p3-V males were able to discriminate between male and female urine in habituation-dishabituation tests and both p3-V and p3-SE males spent longer sniffing female urine in urine preference tests, there was no effect of sexual experience on their interest in female urine. p3-V and p3-SE males displayed similar levels of interest in female urine to wt-V males and spent less than half the time investigating female urine that wt-SE males did. This insensitivity to sexual experience also affected their sexual behavior, as p3-V and p3-SE males did not differ in their mount latency nor in their frequency of mounting or intromission. Furthermore, significantly fewer p3-SE than wt-SE males mounted, intromitted and ejaculated. Sexual experience had no effect on any aspect of male behavior in *Peg3*-KO males: both p3-V and p3-SE males resembled wt-V males in their olfactory interest in female urine and their sexual behavior.

The reinforcement of the behavioral significance of female odors seen in wild-type males is likely to be an adaptive response to their mating experiences, enabling them to improve and refine their responses to females. Sexual experience has been shown to increase the fertility of mating males (21) and these behavioral changes may be a part of this. Increased interest in female chemosignals might increase the probability of mating opportunities for sexually experienced males due to greater motivation to follow female odor trails, while changes in the latency and frequency of sexual behavior suggest that sexually experienced males are more motivated to copulate than naïve males. The absence of such experiential changes in *Peg3*-KO males suggests that this gene facilitated the evolution of such adaptive traits.

Peg3 is a large zinc-finger protein that is strongly expressed in the hypothalamus during development (12, 32), and analysis of expression in the adult has shown that it is expressed particularly strongly in areas of

the hypothalamus involved in olfaction and the control of male sexual behavior (fig 3). *Peg3* is thought to

be involved in p53-mediated apoptosis (33) and the normal pattern of apoptosis may be disrupted in the

developing hypothalamus of *Peg3*-KO mice, leading to aberrant forebrain function that produces the

behavioral deficits we observed. Our analysis of forebrain c-Fos expression showed significant sexual

experience-dependent changes in neuronal responses to female chemosignals in wild-type males but far

smaller changes in activation in *Peg3*-KO males, matching the behavioral findings. Exposure to female

urine induced little change in neuronal activation in wt-V males, except in the AOB and the core and shell

of the NAc. In wt-SE males however, there was a large increase across almost all brain regions examined,

with significantly more c-Fos-positive neurons in all nuclei of the accessory olfactory projection except

the VMH. In p3-V males, there was no increase in c-Fos expression associated with exposure to female

urine in any forebrain nuclei whatsoever, but sexual experience did have an effect on the activation of the

accessory olfactory system in *Peg3*-KO males, as significant activation of the MeAmgA, MeAmgPD and

BNSTp was seen in urine-exposed p3-SE males, although it was far smaller than in wt-SE males in both

the MeAmgA and BNSTp. The absence of any MPOA c-Fos response to urine exposure in both mutant

groups is of particular note, as it is a crucial site for the control of male sexual behavior (34, 35) and is

very active during copulation (36). In wild-type mice, the MPOA was strongly activated by female urine,

but only in sexually experienced males, suggesting that simultaneous exposure to female odors and

activation of the MPOA during mating associates the odor cues with copulation and that female

chemosignals are subsequently able to induce MPOA activation themselves. The ability of *Peg3*-KO

males to successfully mate indicates that the mutation does not totally compromise the function of the

MPOA, however the absence of any increased MPOA response to female urine in p3-SE males suggests

that the association between mating and female odors does not occur in the mutant animals and that the

MPOA may be an important site for the sexual experience-dependent changes in behavior seen in the

wild-type animals.

The NAc is part of the brain's mesolimbic reward system which mediates reinforcement (37) and is activated both by mating (38) and exposure to female odors (39). The NAc receives a projection from the accessory olfactory system via the medial amygdala (27), suggesting links with the accessory olfactory system. Furthermore, sexual experience has been shown to increase c-Fos responses in the NAc shell of male rats exposed to females (40). The lack of female odor-induced NAc activation in either group of *Peg3*-KO males suggests that they are unable to activate this part of the mesolimbic reward system in response to female pheromones and that the NAc and mesolimbic reward system may be important for reinforcing the behavioral significance of the cues encountered during mating. The effect of the mutation on the NAc may thus be a key part of the mutant males' inability to modify their behavior in response to sexual experience. A reduction in the responses of the mesolimbic system in *Peg3*-KO males would be anticipated to affect the general reinforcement of rewarding behavior and may explain the lack of female urine-induced activation of the MPOA and its failure to respond to sexual experience.

Peg3 has previously been shown to regulate offspring development, as well as the behavioral interactions between adult females and their offspring (11), and our results represent the first example of a role for this imprinted gene in male-specific behavior. *Peg3*'s role in complementary maternal and offspring behavior has led to coadaptation between mother and offspring being proposed as an important factor in the evolution of genomic imprinting (41). The same allele inherited down the patriline regulates both offspring development and also the maternal behavior of daughters that receive it, and the fact that this gene must therefore have evolved under maternal selection pressures raises the question as to why it is maternally silenced. When monoallelic expression is established at a locus, the speed with which a gene conferring advantages would spread through a population is significantly greater (due to the lack of allelic competition). However when a gene is expressed in a gender-specific fashion as *Peg3* is, it must confer advantages on that gender or it will drift out of the population. Sexual experience-dependent changes in sexual behavior and interest in females may represent such an adaptive male trait. The ability to refine

sexual behavior with experience is likely to increase the mating success of males and the role of this gene in male sexual behavior may have been crucial in enabling its expression from the paternal allele to persist.

Moreover, we have shown that *Peg3* is an imprinted gene that is involved in every aspect of the reproductive cycle – from conception, when it is heavily expressed in the placenta and regulates offspring growth and development, through to both adult male sexual behavior and female maternal behavior. That these different behaviors are regulated by *Peg3* suggests that as well as having evolved through coadaptation between mother and offspring, its function has also been driven by coadaptive selection pressure between males and females, as its role in hypothalamic development regulates both the ability of males to mate with females and the ability of females to successfully nurture and raise the resulting offspring. The regulation of behaviors such as mating and gestation that require careful coordination between two genetically distinct individuals by a single imprinted gene adds further weight to the idea that coadaptation between individuals has been a key driver in the evolution of imprinting and its major role in mammalian brain evolution.

Materials and Methods

Animals. All mice used were of the inbred strain C57BL/6J (B6) and were bred at the Sub-Department of Animal Behaviour at the University of Cambridge except for virgin females, which were purchased from Harlan UK (Bicester, UK). *Peg3*-KO males were originally generated on the 129sv inbred strain at the Wellcome CRC Institute of Cancer and Developmental Biology at the University of Cambridge by insertion of a 4.8kb βgeo cassette into the 5' coding axon of the *Peg3* gene (9). To produce B6 strain *Peg3*-KO mice, mutant 129sv males were crossed with wild-type B6 females, their mutant male offspring were backcrossed with wild-type B6 females, and this backcross was then repeated over 20 successive

generations. All *Peg3*-KO mice were heterozygous mutants produced from crosses of *Peg3*$^{+/-}$ heterozygous mutant males and wild-type females, identified by tail biopsy and screening for expression of the LacZ gene carried in the inserted βgeo cassette. Mice were weaned at 28 days of age and males were housed in groups of 5 as virgins or singly housed when sexually experienced, while females were group housed. Food and water was available *ad libitum*. Virgin males were singly housed for 24 hours before olfactory or sexual behavior testing, and group housed again after olfactory tests only. Mice were housed under a reversed 12-hour dark/light cycle, all experiments being carried out in the dark phase when animals were active. To gain sexual experience, male mice were housed with up to 4 post-pubertal wild-type B6 females until at least one female was pregnant, at which point males were singly housed again. This ensured that all sexually experienced males had mated, but also had experience of both receptive, estrus females and non-receptive, diestrus females. Urine for olfactory experiments was collected from adult, wild-type B6 male and estrus female mice (determined by vaginal smear). Urine was collected and aliquoted before being frozen at -80°C, and defrosted one hour before experimental use. All mice used were aged between 3 and 12 months old, and all experiments were carried out in accordance with UK Home Office regulations and a project license issued under the Animal (Scientific Procedures) Act of 1986.

Habituation-dishabituation. Habituation-dishabituation tests with male and female urine were conducted in the home cage of subject males and involved the sequential presentation of nine olfactory stimuli. Each presentation lasted two minutes with an interval of one minute between presentations. The first three presentations were of water, the next three were of one urine type, and the final three were of the second urine type. Each animal was tested twice, with the presentation order of male and female urine being reversed in each test and the two tests being carried out on successive days. Olfactory stimuli consisted of 40mm x 10mm polyethylene plastic strips with a 10mm x 10mm square of filter paper at the base. Immediately prior to presentation, 10µl of either water, male urine or female urine was pipetted onto

the filter paper, the strip was placed in the male's home cage against the cage wall and the time subject males spent in direct nasal contact with the olfactory stimuli was recorded. Mean investigation times were calculated from both tests.

Urine preference. Urine preference tests were conducted in a 700mm x 200mm x 200mm clear Perspex cage, divided into three equal sized chambers by clear Perspex partitions with 80mm x 80mm openings, allowing subject males to pass between each chamber. The cage was cleaned before testing and a mixture of clean bedding and home cage bedding was added before the subject male was placed in the center chamber and allowed to explore the apparatus for 20 minutes. The partitions were then closed, isolating the test male in the center chamber, olfactory stimuli inoculated with 10μl of male or female urine were placed in each end chamber before the partitions were opened and the time subject males spent sniffing each urine type over the next five minutes was recorded. Olfactory stimuli consisted of 20mm x 20mm plastic weigh boats lined with filter paper and stuck to a Petri dish.

Sexual behavior. wt-SE, wt-V, p3-SE and p3-V males were paired with receptive, virgin B6 females and their behavior over an hour was recorded. Females were hormonally primed with subcutaneous injections of 50μg of estradiol benzoate in 50μl of peanut oil 72 hours before testing, and 400μg of progesterone in 50μl of peanut oil 3 hours before testing. Males were singly housed and at test a female was placed in each male's home cage and their behavior over one hour was recorded by video camera and then analyzed.

c-Fos immunohistochemistry. The expression of the immediate early gene c-Fos was examined in wt-SE, wt-V, p3-SE and p3-V males that were either exposed to estrus female urine or handled only. Urine-exposed males were scruffed and 30μl of estrus female urine was smeared on the nose using a clean cotton swab, while control males were scruffed only. Two hours later, males were sacrificed by ketamine/xylazine overdose before transcardial perfusion with 20ml of PBS followed by 20ml of 4%

paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde in PBS for three hours before overnight incubation at 4°C in 30% sucrose in PBS, and coronal sectioning on a freeze microtome at 40μm. Sections were washed twice in PBS, incubated overnight at 4°C in PBS containing 0.3% Triton X-100 (PBST), 1.5% normal goat serum (Vector Laboratories) and polyclonal anti c-Fos antibody (Santa Cruz Biotechnology, 1:1000), washed in PBS, incubated for 15 minutes in 4% H_2O_2 and 10% methanol in PBST, washed in PBS then incubated for 30 minutes in biotinylated goat anti-rabbit secondary antibody (ABC elite kit, Vector Laboratories, 1:2000) and 1.5% normal goat serum in PBST. After a wash in PBS, sections were incubated for 30 minutes in avidin-biotin-peroxidase complex solution (ABC elite kit, Vector Laboratories), washed in PBS, then stained in Vector SG peroxidase substrate solution (Vector Laboratories) for 4 minutes, washed in PBS, mounted on gelatin-coated slides, dehydrated and cleared before being coverslipped with DePeX (BDH).

To assess c-Fos neuron numbers in the AOB, MeAmgA, MeAmgPD, BNSTp, MPOA, VMH and NAc, anatomically-matched, sequential sections that contained these nuclei were selected using the mouse brain atlas of Paxinos & Franklin (42) – 8 sections in each hemisphere of the AOB, 4 sections in the VMH and 6 sections for all other nuclei. c-Fos-positive neurons were counted within fixed sampling areas for each nucleus bar the AOB: 250μm diameter circles for the MeAmgA, MeAmgPD and VMH; a 340μm x 200μm oval for the BNSTp; a 420μm x 280μm oval for the MPOA; a 650μm diameter circle for the NAc core, and a 650μm x 200μm rectangle for the NAc shell. In the AOB, c-Fos neurons were counted in the whole nucleus. The mean number of c-Fos positive neurons per section per animal was calculated for each nucleus. c-Fos neurons were counted using MCID Basic software (Imaging Research, Ontario, Canada).

Staining for LacZ expression. A sexually experienced, adult *Peg3*-KO male was sacrificed, perfused and the brain removed to be sectioned as described in the c-Fos immunohistochemistry methods. Brain sections were washed twice in PBS before overnight incubation at 4°C in 5mg/ml X-gal, 25% dimethyl

formamide, 0.38mg/ml EGTA, 1mg/ml MgCl, 1.88mg/ml $K_4Fe(CN)_6 \cdot 3H_2O$ and 2mg/ml $K_3Fe(CN)_6$ in PBS. Sections were washed twice in PBS before being mounted on gelatin-coated glass slides, dehydrated, cleared and coverslipped with DePeX mounting medium (BDH). All *Peg3*-KO mice were born in mixed litters and identified by tail biopsy and staining for LacZ expression in vertebral cartilage (12). A 5mm piece of tail was removed, skinned and incubated overnight at room temperature in 0.4mg/ml X-Gal (BDH), 2% methyl formamide, 0.38mg/ml EGTA, 1mg/ml MgCl, 1.88mg/ml $K_4Fe(CN)_6.3H_2O$ and 2mg/ml $K_3Fe(CN)_6$ in PBS. *Peg3*-KO mice were identified by blue-staining cartilage.

Statistics. Statistical tests were carried out using SPSS 13.0 (SPSS Inc.) for Windows. All 2-way ANOVA were conducted with genotype and sexual experience as factors. Investigation in habituation-dishabituation tests was compared by 2-way repeated-measures ANOVA, and changes in olfactory investigation across presentations were analyzed by Bonferroni-corrected paired T-tests for multiple comparisons. Investigation of male and female urine in preference tests was compared by paired *T*-tests, and urine preference between groups was compared by 2-way ANOVA, followed by *post-hoc* Bonferroni tests where appropriate. In the analysis of sexual behavior, the incidence of each sexual behavior was compared by Fisher's exact test. Mount latency data were not normally distributed, as indicated by 1-way Kolmogorov-Smirnov tests, and were transformed to the normal distribution using $\ln(x + 1)$ before 2-way ANOVA and *post-hoc* Bonferroni tests. Mount and intromission frequency data were analyzed by 2-way ANOVA and *post-hoc* Bonferroni tests. c-Fos data for each brain region were analyzed by 3-way ANOVA with genotype, sexual experience and urine exposure as factors, followed by *post-hoc* Tukey-Kramer HSD tests. In figures, */†/‡ = P<0.05, **/††/‡‡ = P<0.01, ***/†††/‡‡‡ = P<0.001, and error bars indicate ± SEM.

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Fig. 1. Mean time spent sniffing water, male urine and female urine by wt-SE (N=7), p3-SE (N=7), wt-V (N=6) and p3-V (N=7) males in olfactory tests. *(a)* & *(b)*: habituation-dishabituation tests. All groups sniffed the first urine stimulus more than the final water stimulus and wt-SE, wt-V and p3-V males increased their olfactory investigations when the urine types were switched. *(c)*: urine preference tests. All groups spent longer sniffing female urine than male urine but wt-SE males had a significantly greater preference for female urine than all other groups. Significance is represented by * for comparison of interest male and female urine, and by † for comparison with the preference of wt-SE males.

Fig. 2. Sexual behavior of wt-SE (N=16), p3-SE (N=10), wt-V (N=14) and p3-V (N=10) males in one-hour sexual behavior tests. *(a)* Mean latencies to mount females. wt-SE males mounted females faster than all other groups. Frequency of *(b)* mounting and *(c)* intromission during tests. wt-SE males mounted and intromitted more frequently than all other groups. Comparisons are with wt-SE males.

Fig. 3. 40μm sections through the brain of a *Peg3*-KO male mice, stained for expression of the LacZ gene that is carried in the inserted βgeo cassette and expressed in tissues where *Peg3* is normally expressed. *(a)* AOB, *(b)* MeAmgA , *(c)* BNSTp, *(d)* MPOA, *(e)* VMH, *(f)* NAc. Black bars = 100μm.
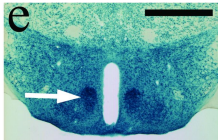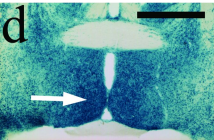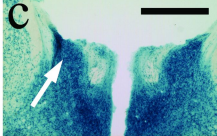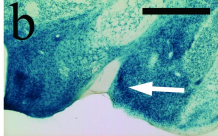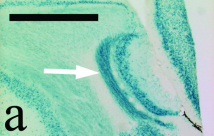
Fig. 4. Mean counts of c-Fos-positive nuclei in forebrain nuclei in wt-SE, p3-SE, wt-V and p3-V males that were exposed to estrus female urine or handled only. Significance is represented by * for comparison of control and exposed males, by † for comparison with exposed wt-SE males and by ‡ for comparison with exposed wt-V males. **AOB** (N=6 for each group) – urine exposure increased AOB c-Fos neuron number in wt-SE (P<0.001), p3-SE (P=0.015) and wt-V (P=0.008) males. Urine-exposed wt-SE males also had significantly more AOB c-Fos neurons than urine-exposed wt-V (P=0.041), p3-SE (P<0.001)

and p3-V (P=0.016) males. **MeAmgA** (N=7 for urine-exposed wt-SE & p3-SE males, N=6 for other groups) – urine exposure increased MeAmgA c-Fos neuron number in wt-SE (P<0.001) and p3-SE (P=0.002) males, but not in wt-V or p3-V males. There were significantly more MeAmgA c-Fos neurons in urine exposed wt-SE males than in all other urine-exposed males (P<0.001 for all groups) . **MeAmgPD** (N=7 for urine-exposed wt-SE & p3-SE males, N=6 for other groups) – urine exposure increased MeAmgPD c-Fos neuron numbers in wt-SE (P=0.001) and p3-SE males (P=0.023), but not in wt-V or p3-V males. There were no MeAmgPD differences between urine-exposed males. **BNSTp** (N=8 for urine-exposed wt-SE & p3-SE males, N=6 for other groups) – urine exposure increased the number of c-Fos neurons in the BNSTp of wt-SE (P<0.001) and p3-SE males (P<0.001), but not wt-V or p3-V males. Among urine-exposed males, c-Fos neurons were more numerous in wt-SE than wt-V (P<0.001), p3-SE (P=0.016) and p3-V males (P<0.001), and in p3-SE than p3-V males (P<0.001). **MPOA** (N=9 for urine-exposed wt-SE & p3-SE males, N=6 for other groups) – there were more MPOA c-Fos neurons in urine exposed wt-SE males than in any other group (P<0.001 for all groups), no other group differences were found. **VMH** (N=7 for urine-exposed wt-SE & p3-SE males, N=6 for other groups) – no significant differences in c-Fos neurons were found. **NAc core** (N=7 for virgin groups, N=7 for sexually experienced groups) – urine exposure increased NAc core c-Fos neurons in wt-SE (P=0.008) and wt-V males (P=0.001) but not in p3-SE or p3-V males. Among urine exposed males, wt-SE males had more NAc core c-Fos neurons than p3-SE males (P=0.006) and wt-V males had more than p3-V males (P=0.004). **NAc shell** (N=7 in virgin groups, N=7 in sexually experienced groups) – urine exposure increased NAc shell c-Fos neurons in wt-SE (P=0.001) and wt-V males (P=0.006) but not in p3-SE or p3-V males. Among urine exposed males, wt-SE males had more NAc core c-Fos neurons than p3-SE males (P=0.022) and wt-V males had more than p3-V males (P=0.044).
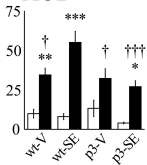
Table 1. Statistical analysis of c-Fos-positive neuron counts in brain areas of male mice: F- and P-values were obtained from 3-way ANONA with genotype, urine exposure and sexual experience as factors. Significant P-values are in bold.
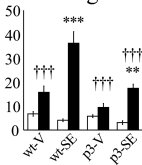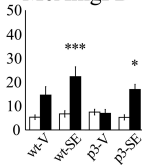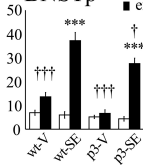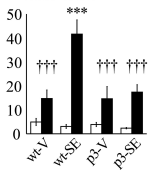
a

b

c

a — seconds; b — mounts per min; c — intromissions per min. Groups: wt-V, wt-SE, p3-V, p3-SE.
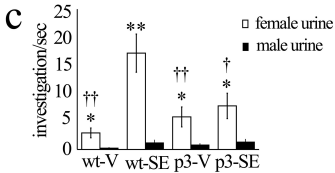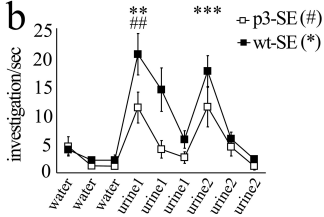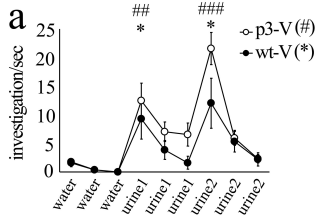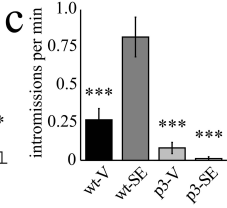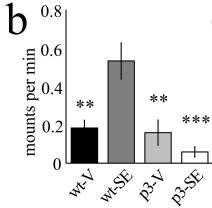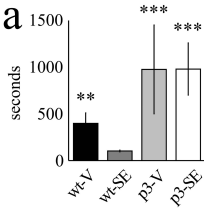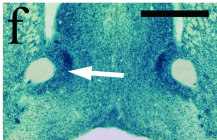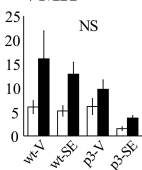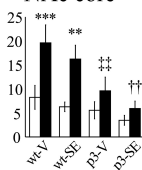
EFFECT	AOB		MeAmgA		MeAmgPD		BNSTp		MPOA		VMH		NAc core		NAc shell	
	$F_{(1,40)}$	P	$F_{(1,42)}$	P	$F_{(1,42)}$	P	$F_{(1,44)}$	P	$F_{(1,46)}$	P	$F_{(1,42)}$	P	$F_{(1,44)}$	P	$F_{(1,44)}$	P
Genotype	6.062	**0.018**	16.61	**<0.001**	3.291	0.077	11.38	**0.002**	5.704	**0.021**	7.551	**0.009**	13.87	**0.001**	4.863	**0.033**
Urine exposure	81.37	**<0.001**	79.44	**<0.001**	27.97	**<0.001**	111.47	**<0.001**	46.14	**<0.001**	11.43	**0.002**	16.09	**<0.001**	17.43	**<0.001**
Sexual experience	0.112	0.740	12.20	**0.001**	6.098	**0.018**	51.94	**<0.001**	5.780	**0.020**	4.433	**0.041**	2.571	0.116	2.505	0.121
Genotype/ Urine exposure	5.537	**0.024**	12.21	**0.001**	4.070	**0.050**	4.953	**0.031**	4.267	**0.045**	2.946	0.093	4.551	**0.039**	5.009	**0.030**
Genotype/ Sexual experience	6.985	**0.012**	3.577	0.065	0.038	0.846	0.184	0.670	4.730	**0.035**	0.900	0.348	0.004	0.952	0.108	0.743
Urine exposure/ Sexual experience	4.469	**0.041**	25.96	**<0.001**	7.367	**0.010**	60.71	**<0.001**	9.139	**0.004**	0.297	0.589	0.189	0.666	0.574	0.453
Genotype/ Urine exposure/ Sexual experience	2.139	0.151	3.558	0.066	0.739	0.395	0.212	0.647	5.009	**0.030**	0.021	0.886	0.001	0.981	0.019	0.891