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The Paternally Expressed Gene *Peg3* Regulates Sexual Experience-Dependent Preferences for Estrous Odors

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Abstract

Sexual experience has marked and long-lasting effects on male behavior in mammals, regulating traits such as the anticipation and display of sexual behavior, aggression and olfaction. We conducted urine preference, habituation-dishabituation and partner choice tests with sexually experienced and naïve male mice and found that wild-type males acquire adaptively significant preferences for the odors of receptive, estrous females with sexual experience, and that these preferences are matched by changes in main olfactory system responses involving the piriform cortex, as indicated by c-Fos expression. We also report that these experiential effects are disrupted in male mice carrying a knockout of the imprinted gene *Peg3*. This paternally expressed gene regulates maternal care and offspring development, but we here report that *Peg3* mutant males suffer a complex olfactory deficit that affects estrous odor preferences and the responses of the main olfactory system to such odors. *Peg3* appears to have evolved to regulate the experience-dependent preference for receptive females, an adaptive trait that would enhance male reproductive success and so potentially increase paternal transmission of this paternally expressed gene.

The Paternally Expressed Gene *Peg3* Regulates Sexual Experience-Dependent Preferences for Estrous Odors

Sexual experience is a major modulator of behavior in male mammals, affecting anticipatory and consummatory sexual behavior, olfaction, aggression, and the function of the brain systems that regulate these behaviors. Research with rodents in the laboratory has shown that the motor patterns of male sexual behavior are particularly sensitive to sexual experience and that the latency and frequency of mounting, intromission and ejaculation changes after sexual experience in rats (Dewsbury, 1969; Larsson, 1959), guinea pigs (Valenstein & Goy, 1957); (Valenstein, Riss, & Young, 1955) and hamsters (Pfeiffer & Johnston, 1994). Male-male aggression is sensitive to sexual experience, which increases the incidence of fighting and decreases the latency to initiate aggression (Brain & Al-Maliki, 1979; Goyens & Noirot, 1975) and also increases activation in the brains areas that regulate aggression (Wang, Hulihan, & Insel, 1997). A single ejaculation has been shown to be sufficient to alter behavior, increasing the motivation of male rats to approach receptive females (López, Olster, & Ettenberg, 1999). Sexual experience modifies interest in potential partners, inducing a preference for receptive females which is not exhibited by sexually naïve males (Carr, Loeb, & Dissinger, 1965; Stern, 1970). This preference is likely to involve olfactory cues, as sexually experienced males also develop preferences for the odors of receptive, estrous females (Hayashi & Kimura, 1974; Lydell & Doty, 1972). Olfaction is the most important sensory modality in rodents and sexual experience has significant effects on olfaction-mediated behaviors. When able to investigate non-volatile odors, male mice display a preference for female urine over male urine whether sexually experienced or naïve, however this preference is greatly enhanced in sexually experienced males (Swaney, Curley, Champagne, & Keverne, 2007). Male mice vocalize in

response to female urine, a behavior that is blocked in virgins by removal of the vomeronasal organ, but not in sexually experienced males, who will only cease vocalizations after deafferentation of both the vomeronasal organ and the main olfactory epithelium (Sipos, Wysocki, Nyby, Wysocki, & Nemura, 1995). Sexually experienced male mice also vocalize to odorants in aged female urine. These odorants elicit no response from virgin animals, who will only vocalize in response to freshly voided urine (Sipos, Kerchner, & Nyby, 1992). Sexual experience appears to increase the range of female odorants that males will respond to and also increases the sensitivity of the main olfactory system to these chemosignals.

Changes in brain function due to sexual experience have been described across multiple pathways. Female-induced neural activity in the accessory olfactory system (Hosokawa & Chiba, 2005), the mesolimbic dopamine system (Lopez & Ettenberg, 2002), the medial pre-optic area that regulates male sexual behavior (Lumley & Hull, 1999), and in oxytocinergic neurons of the paraventricular nucleus (Nishitani, Moriya, Kondo, Sakuma, & Shinohara, 2004) increases after sexual experience. Sexual experience reduces the behavioral consequences of lesions to nuclei that control sexual behavior (Arendash & Gorski, 1983; Claro, Segovia, Guilamon, & Del Abril, 1995; Kondo, 1992), as well as moderating the decline in sexual behavior that occurs after castration (Lisk & Heimann, 1980; Phelps, Lydon, O'Malley B, & Crews, 1998).

Although the work discussed above demonstrates the effects of sexual experience on behavior and brain function in male rodents, there has been little research on the adaptive significance of male sexual experience, with the notable exception of two studies reporting that sexually experienced males are both more fecund (Rastogi, Milone, & Chieffi, 1981) and more attractive to females (Galef, Lim, & Gilbert). The broad effects that sexual experience has on male behavior across different species may also have direct consequences on reproductive

success in male mammals, and as such sexual experience should be taken into account when considering adaptive male reproductive behavior and its evolution. One approach to studying the adaptive significance of sexual experience in mammals is to explore the role of genes that have been demonstrated to alter reproductive success broadly and that display expression patterns that are unique to placental species.

Our laboratory has been working on the behavioral and functional role of the paternally expressed gene *Peg3*, one of a subset of mammalian autosomal genes known as imprinted genes which are expressed in a haploid manner, according to the parent-of-origin of each allele. Approximately 100 of these paternally- or maternally-expressed genes have been reported (Morison, Ramsay, & Spencer, 2005), many of which encode developmental proteins such as transcription regulators, growth factors, oncogenes and DNA-binding proteins (Tycko & Morison, 2002). Imprinted genes play essential roles in mammalian development, especially in the brain (Allen *et al.*, 1995; Keverne, Fundele, Narasimha, Barton, & Surani, 1996) and the placenta (Coan, Burton, & Ferguson-Smith, 2005). Although relatively few genes appear to be imprinted, their functions and expression patterns suggest strong links between genomic imprinting and the evolution of placentation in mammals (Kaneko-Ishino, Kohda, & Ishino, 2003; Killian *et al.*, 2001), the evolutionary expansion of the mammalian brain (Keverne, 2001) and the evolution of mammalian reproductive behavior (Keverne, 2007). In humans, abberant expression of imprinted genes causes congenital diseases such as Prader-Willi and Angelman syndromes (Glenn, Driscoll, Yang, & Nicholls, 1997).

We have been studying the role of *Peg3* in development, behavior and brain function using a *Peg3*-knockout (*Peg3*-KO) mutant mouse. *Peg3* is strongly expressed in the placenta and the developing embryo, particularly in the hypothalamus (Li *et al.*, 1999). *Peg3* 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). *Peg3*-KO females suffer reproductive deficits, giving birth to smaller pups than wild-type females and displaying deficits in maternal behaviors including pup-retrieval, nest-building, nursing and milk-letdown (Champagne, Curley, Swaney, & Keverne, 2005; Li *et al.*, 1999). *Peg3* has also been studied independently in mutant offspring born to wild-type mothers, revealing a pup phenotype that is complimentary to that of the mothers and which affects thermoregulation, suckling and growth rate, suggesting that coadaptation between mother and offspring has played an important role in the evolution of *Peg3* and the behaviors it regulates (Curley, Barton, Surani, & Keverne, 2004). More recently, we have shown that the *Peg3*-KO mutation also affects reproductive behavior in males, particularly their behavioral responses to sexual experience (Swaney *et al.*, 2007). While behavior in wild-type males is altered by sexual experience, neither the motor patterns of sexual behavior nor generalized interest in female urine are changed in *Peg3*-KO males by sexual experience. These behavioral deficits are matched by reduced activation of the vomeronasal system and hypothalamus when males are exposed to female urine, indicating that the vomeronasal responses of *Peg3*-KO males to sexually significant pheromones are attenuated.

As discussed above, male rodents exhibit a preference for estrous female odors once sexually experienced (Hayashi & Kimura, 1974; Lydell & Doty, 1972), a trait which is of particular significance in species, such as rats and mice, in which females are receptive only for brief periods in each estrous cycle and spend most of their lives either pregnant or in non-receptive phases of the estrous cycle. To explore the interaction between sexual experience and *Peg3* and their effects on such adaptive male behavior, we investigated the olfactory interest

of virgin and sexually experienced, wild-type and *Peg3*-KO males in the volatile odors of male and female urine, and estrous and diestrous female urine. We have previously reported that *Peg3*-KO males prefer non-volatile female odors to non-volatile male odors, but that this preference is unaffected by sexual experience (Swaney *et al.*, 2007). We therefore also investigated male preferences for volatile male and female odors to determine whether they are similarly affected by the *Peg3*-KO and by sexual experience. We employed urine choice, habituation-dishabituation and partner choice tests to measure olfactory preferences and discrimination. We also examined whether sexual experience and the *Peg3* mutation affect activation of the main olfactory system by receptive female odors. Using brain tissue from male mice in which we have previously studied accessory olfactory system activity (Swaney *et al.*, 2007), we measured expression of the immediate early gene c-Fos, a commonly used marker of neural activation, in two nuclei of the main olfactory system – the glomeruli of the main olfactory bulb, and the piriform cortex. By measuring changes in adaptive olfactory behavior and the responses of the brain system that regulates it, we hoped to elucidate both how the brain is changed by sexual experience, and the role that genomic imprinting might have played in the evolution of this dynamic behavior.

Method

Subjects

Four groups of mice were used to measure the effects of the *Peg3* mutation on experience-dependent preferences for estrous odors: wild-type virgin (wt-V) males, *Peg3*-KO virgin (p3-V) males, wild-type sexually experienced (wt-SE) males and *Peg3*-KO sexually experienced (p3-SE) males. All mice were of the inbred strain C57BL/6J and were housed on a reversed 12H dark-light cycle at the Sub-Department of Animal Behaviour at the University of

Cambridge, with lights on at 8pm and off at 8am. Males were bred in-house, while females were bought from Harlan UK (Bicester, UK). *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 C57BL/6J strain, homozygous *Peg3*-KO 129sv males were mated with wild-type C57BL/6J females to produce heterozygous *Peg3*-KO hybrid offspring. Males from these litters were backcrossed with wild-type C57BL/6J females, and the subsequent offspring genotyped by tail biopsy to identify the mutant male offspring, and these animals were then mated with further wild-type C57BL/6J females. This backcross was repeated over a total of 20 generations to produce C57BL/6J *Peg3*-KO males. Virgin males were housed in groups of five and sexually experienced males were singly housed to prevent male-male aggression. Virgin mice were singly housed for 24H before all tests, and re-housed in their groups after olfactory tests. RM3E mouse chow (Lillico, Surrey, UK) and water were provided ad libitum, and cages were lined with wood shaving bedding (Lillico) which was changed weekly. To gain sexual experience, males were housed with two to three wild-type C57BL/6J females until at least one female became pregnant, ensuring that sexually experienced males had successfully mated, but also that they had experience of receptive and non-receptive females. In each group, the age of males during experiments was between 3-9 months. All experimental procedures were conducted in accordance with the terms of a project licence issued by the UK Home Office to E.B. Keverne under the Animals (Scientific Procedures) Act 1986.

Experimental Procedures

Olfactory tests

Males were first tested for their preferences for the volatile odors of male and female urine in urine choice tests. Three different tests of males' interest in volatile estrous and diestrous female odors were then conducted: urine choice tests were used to determine urine preferences, habituation-dishabituation tests were used to investigate olfactory discrimination and partner choice tests were used to measure preferences for intact females. Urine for olfactory tests was collected from non-experimental C57BL/6J males and from normally cycling C57BL/6J females. Female estrous state was determined by vaginal smear and urine collected from females in estrus or diestrus. Collected urine was pooled according to type (male, estrous female, diestrous female), aliquoted and then frozen, before being defrosted on the day of use, no more than one hour before olfactory tests were performed. 25mm x 25mm weigh boats with a square of filter paper in the bottom were used as olfactory stimuli, and immediately before use in tests, 10μl of urine was pipetted onto the filter paper. The same C57BL/6J females used for urine collection were used in partner choice tests and were vaginally smeared for three days prior to testing, when an estrous and a diestrous female were selected for use as stimulus females.

Urine choice tests

Two iterations of this test were performed – one using male and estrous female urine, and one using estrous and diestrous female urine. Subject males were tested in a three-chambered 700mm x 200mm x 200mm Plexiglas arena with 80mm x 80mm openings in each partition allowing subjects to move between chambers. The arena was cleaned with water and dried before each test, the floor covered with a mix of clean, wood shaving bedding and soiled bedding from the subject's home cage and then the subject was placed in the arena and allowed to habituate for 30 minutes. At the end of the habituation period the partitions were closed, confining the subject to the central chamber, and two different urine-primed olfactory stimuli

were placed in the end chambers and covered with raised metal grilles which prevented physical contact. The partitions were then removed allowing the subject animal to explore all three chambers, and the time the subject male spent sniffing each stimulus over the following five minutes was recorded. To measure initial interest, each subject male's investigation of the stimuli over the first two minutes of each test was also recorded.

Habituation-dishabituation tests

Habituation-dishabituation tests were conducted in subject animals' home cages, and involved nine sequential presentations of olfactory stimuli; three of water, followed by three of one female urine type (estrous or diestrous) and finally three of the other female urine type. Olfactory stimuli were placed on top of the cage lid, and each stimulus presentation lasted two minutes with a one-minute interval between presentations. Each animal was tested twice, on successive days, with the order of presentation of the female urines reversed in each test. The mean investigation time for each stimulus presentation over the two tests was calculated to control for any effects of presentation order on investigation times. Subjects were scored as being engaged in investigation when they were sniffing the air directly beneath the stimuli.

Partner choice tests

The test apparatus consisted of three cages each measuring 300mm x 125mm x 125mm, linked by a Plexiglas T-tube 50mm in diameter, with a long arm measuring 390mm and a short arm measuring 90mm. All three cages could be sealed from the tube with metal grilles which allowed air to pass in and out. Cages were lined with fresh, wood shaving bedding before each test, and the tube was cleaned with water and dried. At the start of each test, the two cages at each end of the long arm of the T-tube were closed with metal grilles, and a subject male placed in the cage at the base of the T-tube and allowed to explore the apparatus and habituate to it for

30 minutes. After 30 minutes, males were confined to the start cage at the base of the T by means of a metal grille, and an estrous and a diestrous female were placed in the two cages at each end of the long arm of the T-tube. They were then given five minutes to habituate to their novel cages before the grille confining the male to his start cage was removed, allowing him to enter the tubes and approach the two cages containing the stimulus female animals. The time spent by the subject male sniffing at the grille of each female cage was recorded over the following five minutes.

Exposure to estrous urine and c-Fos immunohistochemistry

To examine the effects of the *Peg3* mutation and sexual experience on main olfactory responses to estrous female urine, males from the four experimental groups were either exposed to estrous female urine or handled only, giving a total of eight groups: urine-exposed and control wt-V, p3-V, wt-SE and p3-SE males. Urine exposed males were scruffed and 50µl of estrous female urine was applied to the nose, while control males were scruffed but not exposed to urine. Two hours after this treatment, subjects were given an overdose of ketamine/xylazine anesthetic followed by transcardial perfusion with 20ml of phosphate-buffered saline (PBS) and then 20ml of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde in PBS for three hours, incubated in 30% sucrose in PBS overnight at 4°C and then cut at -15°C on a freezing microtome into 40µm sections for c-Fos immunohistochemistry. Olfactory bulbs were bisected and each bulb was sectioned sagitally, while forebrains were sectioned coronally. Sections encompassing the main olfactory bulb and the piriform cortex were selected and stained for expression of c-Fos. Sections were washed in PBS, incubated overnight at 4°C in PBS containing 0.3% Triton X-100, 1.5% normal goat serum (Vector Laboratories, Peterborough, UK) and 1:1000 polyclonal anti-c-Fos primary antibody (Santa Cruz Biotechnology, Santa Cruz,

CA), then washed in PBS before being incubated in 4% H_2O_2, 10% methanol and 0.3% Triton X-100 in PBS for 15 minutes. Sections were then washed in PBS before incubation in 1.5% normal goat serum, 0.3% Triton X-100 and 1:2000 biotinylated goat anti-rabbit secondary antibody (ABC elite kit; Vector Laboratories) in PBS for 30 minutes. After further washing in PBS, sections were incubated for 30 minutes in avidin-biotin-peroxidase complex solution (ABC elite kit; Vector Laboratories), and washed in PBS before being stained in Vector SG peroxidase substrate solution (Vector Laboratories) for 4 minutes. Sections were washed twice in PBS, mounted on gelatin-coated slides, cleared and dehydrated before being coverslipped with DePeX (BDH Chemicals, Poole, UK). Sequential, anatomically-matched sections were selected using the mouse brain atlas of Paxinos and Franklin (2001) and c-Fos positive neurons were counted. In the main olfactory bulb, three sections in each hemisphere of every subject were selected at approximately lateral 1.20mm (see Figure 1a) and c-Fos-positive periglomerular cells were counted within a 600μm x 350μm box inside the ventral portion of the olfactory bulb. Activity mapping studies have shown that glomeruli within this area of the olfactory bulb are most responsive to urinary odors (Schaefer, Yamazaki, Osada, Restrepo, & Beauchamp, 2002; Xu *et al.*, 2005), and that c-Fos expression in associated periglomerular cells in this part of the olfactory bulb is increased by urine exposure. For each animal, four sections at approximately Bregma 1.70mm (see Figure 1a) were selected for the anterior piriform cortex and c-Fos neurons were bilaterally counted within a 600μm x 450μm box laid over this area. The anterior piriform cortex was selected as it receives a major input from the main olfactory bulb, has been shown to respond strongly to acute odor exposure (Illig & Haberly, 2003) and is also a site of strong *Peg3* expression. c-Fos neurons were counted using MCID Basic software (Interfocus, Linton, UK)

and mean counts of c-Fos neurons per mm^2 per section per animal were calculated for each nucleus.

Staining for β-galactosidase expression

The βgeo cassette inserted into the *Peg3* gene contained the LacZ marker gene, allowing *Peg3*-KO mutants to be identified by expression of β-galactosidase. To identify *Peg3*-KO mice in mixed litters, 5mm of tail was removed from all pups at weaning, skinned and incubated overnight in 0.4 mg/ml X-Gal dissolved in staining buffer. Tail cartilage from mutant animals stained blue, allowing them to be discriminated from their wild-type littermates.

Statistical analysis

All statistical analysis was conducted using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). Female preference scores in urine choice tests were calculated by subtracting time spent sniffing male urine from time spent sniffing female urine, and estrous preference scores by subtracting time spent sniffing diestrous urine from that spent sniffing estrous urine in the respective tests. Between-group differences in preference scores were analyzed by two-way ANOVA, with genotype and sexual experience as factors, and by post-hoc Tukey-Kramer tests where interactions were found. For within-group analysis of olfactory interest, time spent sniffing each urine type was analyzed by three-way ANOVA, with urine type, genotype and sexual experience as factors. When significant interactions were found, post-hoc LSD tests were used to analyze within-group differences in time spent sniffing the two urine types. In habituation-dishabituation tests, the mean duration of investigation of each of the nine stimulus presentations was calculated from the two habituation-dishabituation tests that each animal underwent. To determine whether there were differences between groups in investigation behavior, mean investigation times were analyzed by two-way repeated-measures ANOVA with

genotype and sexual experience as factors. Bonferroni-corrected paired *t*-tests were used to determine whether there was a significant change in each group's mean investigation times when the olfactory stimulus type was changed. Mean counts of c-Fos neurons per mm^2 per section in the main olfactory bulb and the piriform cortex were analyzed by three-way ANOVA, with genotype, sexual experience and urine exposure as factors. When there were significant interactions, counts were compared groups using post-hoc Tukey-Kramer tests.

Results

Olfactory Tests

Male-female urine choice tests

Comparison of the preference scores by two-way ANOVA revealed similar results in the first two minutes and the full five minutes of testing: there were no effects of genotype (0-2 minutes: $F_{1,35}$=0.261, p=0.613; 0-5 minutes: $F_{1,35}$=0.215, p=0.646) on preferences for female urine, significant main effects of sexual experience (0-2 minutes: $F_{1,35}$=4.733, p=0.036; 0-5 minutes $F_{1,35}$=5.605, p=0.024) but no interactions between these factors (0-2 minutes: $F_{1,35}$=1.676, p=0.204; 0-5 minutes $F_{1,35}$=1.112, p=0.299). Within-group analysis was done by three-way ANOVA of sniffing times over the first two minutes and the full five minutes of testing. This showed main effects of sexual experience (0-2 minutes: $F_{1,70}$=6.578, p=0.012; 0-5 minutes: $F_{1,70}$=5.249, p=0.025), a main effect of urine type in the five minute test ($F_{1,70}$=6.888, p=0.011) but not in the first two minutes ($F_{1,70}$=2.480, p=0.120), no effects of genotype (0-2 minutes: $F_{1,70}$=1.619, p=0.207; 0-5 minutes: $F_{1,70}$=2.591, p=0.112) and significant interactions between sexual experience and urine type (0-2 minutes: $F_{1,70}$=4.111, p=0.046; 0-5 minutes: $F_{1,70}$=4.375, p=0.033). Post-hoc tests of within-group comparisons showed that wt-SE males spent longer sniffing female urine over the five minute test duration (P=0.049) and that p3-SE

males spent longer sniffing female urine in both the first two minutes (p=0.008) and the full five minutes of testing (p=0.002), but that there were no differences in the time wt-V and p3-V males spent sniffing male and female urine (see Figure 4a & b).

Estrous-diestrous urine choice tests

Comparison of urine preference scores over the full five minutes of testing by ANOVA found no effect of genotype ($F_{1,26}$=0.857, p=0.363) or of sexual experience ($F_{1,26}$=3,179, p=0.086) and no interaction between these factors ($F_{1,26}$=1.131, p=0.297). However ANOVA of the preference scores from the first two minutes of testing demonstrated a significant effect of genotype ($F_{1,26}$=7.497, p=0.011), no effect of sexual experience ($F_{1,26}$=2.990, p=0.096) and a significant interaction between these factors ($F_{1,26}$=7.998, p=0.009). Post-hoc analysis showed that wt-SE males had a significantly greater preference for estrous urine than wt-V (p=0.017), p3-V (p=0.020) and p3-SE males (p=0.004) (see Figure 3c). Three-way ANOVA of time spent sniffing either urine type over the full five minutes showed a main effect of sexual experience ($F_{1,52}$=75.342,p<0.001) but no effect of either genotype ($F_{1,52}$=0.257,p=0.614) or urine type ($F_{1,52}$=2.805,p=0.100). There was also a significant interaction between genotype and sexual experience ($F_{1,52}$=4.249,p=0.044). Post-hoc within-group analysis showed that only wt-SE males spent longer sniffing estrous urine than diestrous urine over the five minute test (p=0.018) (see Figure 3a). Three-way ANOVA of time spent sniffing either urine type over the first two minutes showed a main effect of sexual experience ($F_{1,52}$=42.424,p<0.001) but no effect of genotype ($F_{1,52}$=1.958,p=0.168) or urine type ($F_{1,52}$=3.579,p=0.064). There were also significant interactions between genotype and urine type ($F_{1,52}$=5.848,p=0.019) and between genotype, sexual experience and urine type ($F_{1,52}$=6.231,p=0.016). Post-hoc within-group analysis showed

that only wt-SE males spent longer sniffing estrous urine than diestrous urine over the first two minutes of the test ($p<0.001$) (see Figure 3b).

Estrous-diestrous female urine habituation-dishabituation tests

Repeated measures ANOVA showed that there was a significant effect of genotype ($F_{8,208}=2.455$, $p=0.015$) on investigation patterns over the course of the test, a significant effect of sexual experience ($F_{8,208}=9.914$, $p<0.001$) and a significant interaction between the two factors ($F_{8,208}=2.825$, $p=0.005$). Analyzing the responses of each group to the change in olfactory stimulus showed that all groups investigated more when the stimulus type was changed from water to urine (wt-V – $p=0.026$; p3-V – $p=0.014$; wt-SE – $p=0.011$; p3-SE – $p=0.008$). However, when the female urine type was switched, there was no change in the interest of wt-V ($p=0.713$), p3-V ($p=0.989$) or p3-SE ($p=0.322$) males, but wt-SE males ($p=0.001$) significantly increased their investigation (see Figures 4a and 4b).

Estrous-diestrous partner choice tests

In the partner choice tests, none of the groups spent longer sniffing at the cage of either the estrous or diestrous female (wt-V – t=0.434, df=5, p=0.683; p3-V – t=1.666, df=4, p=0.171; wt-SE – t=2.068, df=8, p=0.073, t=-0.466, df=8, p=0.654) (see Figure 5), although there was a trend towards greater investigation of the estrous female among the wt-SE males. Analysis of estrous preference scores from the partner choice tests showed no effect of genotype (F1,25=0.007, p=0.936) or of sexual experience (F1,25=0.304, p=0.586), but did show a significant interaction between these factors (F1,25=4.974, p=0.035), however post-hoc analysis did not demonstrate any significant differences between the groups.

Female Urine Exposure and c-Fos Expression

c-Fos-positive neurons were counted in the glomeruli of the main olfactory bulb and in the piriform cortex, two important nuclei within the main olfactory system. Comparison of counts in the main olfactory bulb glomeruli found no effects of the experimental treatments on numbers of c-Fos neurons. There was no effect of genotype ($F_{1,38}$=0.007, p=0.932), sexual experience ($F_{1,38}$=0.105, p=0.748) or exposure to estrous urine ($F_{1,38}$=0.409, p=0.526) on glomerular c-Fos counts, nor were there any interactions between of these factors (see Figure 6a).

In the anterior piriform cortex, there were significant main effects of genotype ($F_{1,40}$=6.551, p=0.014), of sexual experience ($F_{1,40}$=11.511, p=0.002) and of urine exposure ($F_{1,40}$=20.189, p<0.001) on counts of c-Fos neurons. There was also a significant interaction between sexual experience and urine exposure ($F_{1,40}$=10.695, p=0.002). Post-hoc analysis showed that exposure to estrous urine increased piriform cortex c-Fos neuron numbers in wt-SE males (p<0.001) but not in any other group (see Figures 1b and 6b). Moreover, when urine-exposed males were compared, wt-SE males had significantly more piriform cortex c-Fos neurons than both urine-exposed wt-V (p=0.001) and p3-V males (p<0.001). There were no other differences in piriform cortex c-Fos neuron counts between the urine-exposed groups.

Discussion

Previous work by our group has shown that the *Peg3*-KO mouse exhibits deficits in responses to sexual experience which affect sexual behavior and the function of the vomeronasal system (Swaney *et al.*, 2007). Frequency of mounting and intromission do not increase in *Peg3*-KO males and generalized interest in female non-volatile cues is unchanged by sexual experience in these animals. However the data we report here show that the *Peg3*-KO mutation has a more complex effect on behavior. The results of the male-female urine choice tests show

that both wt-SE and p3-SE males exhibited a preference for the volatile odors of female mice when presented alongside male odors, while virgin males of neither genotype showed any preference for male or female urine. In our previously published work, we reported that sexual experience had no effect on the preferences of *Peg3*-KO males for female urine, but this apparent inconsistency between the two sets of results may actually be due to methodological differences. In the earlier study, subjects were able to physically contact the olfactory stimuli and investigate both volatile and non-volatile chemosignals in male and female urine, however in the urine choice tests described here, subjects were unable to contact olfactory stimuli, restricting their investigations to volatile odors. This distinction is important as pheromones in mice have generally been thought to be non-volatile, high molecular weight proteins (Brennan & Zufall, 2006) which communicate complex information (Nevison, Armstrong, Beynon, Humphries, & Hurst, 2003), and such cues in urine have been shown to be unconditionally attractive when volatile odors alone are not (Moncho-Bogani, Lanuza, Hernandez, Novejarque, & Martinez-Garcia, 2002). Indeed, when able to physically contact male and female urinary stimuli in our previous study, all males exhibited a significant preference for female urine, albeit one which was unaffected by sexual experience in *Peg3*-KO males. In the male-female urine choice tests we describe here however, neither wt-V nor p3-V males displayed any preference, but both wt-SE and p3-SE males spent significantly longer investigating female urine over the full five minute tests. Thus, sexual experience induced a preference for volatile female odors which was not present in virgin mice of either genotype. Wild-type virgin mice have been shown to be able to discriminate between volatile odors in male and female urine (Baum & Keverne, 2002) and to vocalize in response to non-volatile chemosignals in female urine (Sipos *et al.*, 1992) but these data suggest that preferences for female volatile odors are dependent on experience of successful

mating. Moreover, knocking out *Peg3* does not disrupt the acquisition of this preference, indicating that *Peg3*-KO males do not suffer a gross deficit in sexual experience-dependent behavioral plasticity, but rather a more specific one.

The choice tests with estrous and diestrous female urine replicated previous findings (Hayashi & Kimura, 1974) indicating that a preference for estrous odors is induced by sexual experience in wild-type male mice. wt-V, p3-V and p3-SE males exhibited no preference for estrous urine but wt-SE males spent significantly longer investigating estrous urine than diestrous urine, particularly over the first two minutes of testing. In this initial phase, the preference of the wt-SE males for estrous urine was significantly different to the other groups' preferences. The results from the two different urine choice tests show that the *Peg3*-KO mutation has a complex effect on the responses of male mice to volatile odors and does not simply block the behavioral changes triggered by sexual experience nor render these animals anosmic to volatile odors. Mutant males are able to mate and acquire a generalized preference for the volatile odors of females from such mating experience, suggesting that they are able to associate some general female odors with the reward of successful copulation. However sexual experience does not cause them to prefer the odors of receptive females relative to those of unreceptive females, indicating that they are unable to discern the specific olfactory cues of female receptivity and associate them with mating. The lack of an experiential preference for estrous odors might be due to a failure to associate the odors of receptive females with successful mating, or it may be due to a simple inability to distinguish the odors of estrous and diestrous female mice.

To further investigate these possibilities, we conducted habituation-dishabituation tests with estrous and diestrous female urine to determine whether subject animals were able to

discriminate between the two female urine types. During repeated presentation of the same olfactory stimulus type, subject animals' interest is expected to decline as they habituate to the stimulus odor. When the stimulus type is switched, their interest and investigation behavior should increase if they discern a difference between the odors. After three presentations of water, all male mice increased their olfactory investigation when urine was first presented in habituation-dishabituation tests, showing that they could discriminate urine from water and were not anosmic. Of the four different groups, only wt-SE males then increased their investigation when the urine type was switched. The lack of dishabituation seen in wt-V males suggests that these males were unable to distinguish the two urine types from each other and that this discriminatory ability in wild-type males is dependent on sexual experience. The lack of any dishabituation response in either p3-V or p3-SE males suggests that they could not discriminate between the two urine types, and that sexual experience did not change the mutant males' perception of the signal value of these odors. It should be noted however that olfactory behavior in tests such as these is affected by motivation, and thus it is not a pure test of sensory ability, but rather one of both discriminatory ability as well as sustained motivation to investigate female odors. The behavior of the wt-SE males in this test indicates that wild-type males have the olfactory potential to discriminate between estrous and diestrous odors as well as the motivation, but that sexual experience is necessary for it to emerge in such tests.

In the partner choice tests, none of the males displayed a preference for the estrous female, despite being able to see and hear the intact females as well as sniff their volatile odors. This was surprising given the greater sensory information that can presumably be derived from intact females relative to female urine alone, however it is worth noting that although none of the

groups exhibited a significant preference for estrous females, there was a strong trend among the wt-SE males ($p=0.073$) to spend longer investigating the estrous females.

We have previously shown that virgin males are attracted to non-volatile chemosignals in female urine (Swaney *et al.*, 2007), and these data collectively show that sexual experience is required for the volatile odors of receptive females to acquire greater salience than those of non-receptive females. *Peg3*-KO males do not appear able to make this association and so fail to exhibit a preference for estrous odors or even discriminate between estrous and diestrous female odors, despite being capable of acquiring a preference for female odors relative to male odors. The distinction between volatile and non-volatile odors is an important one in such behavioral tests. Like most mammals, rodents have two olfactory systems, the main olfactory system and the vomeronasal system, and they have traditionally been viewed as functionally distinct, volatile odors being processed by the main olfactory system and non-volatile odors by the vomeronasal system. Although the two systems are no longer viewed as mutually exclusive (Brennan & Zufall, 2006), the two systems are not redundant but appear rather to have some overlapping function (Xu *et al.*, 2005). Manipulations of a single olfactory system can nevertheless have severe consequences, and the main olfactory system has been shown to play an essential role in male sexual behavior in both gene knockout (Mandiyan, Coats, & Shah, 2005) and lesion (Keller, Douhard, Baum, & Bakker, 2006) studies. We have previously demonstrated that the *Peg3*-KO mutation compromises the function of the vomeronasal system and in particular blocks the changes in neural activity which are normally triggered by sexual experience (Swaney *et al.*, 2007).

The data we report here show that the main olfactory system is also affected by the *Peg3* mutation, specifically at the level of the piriform cortex. *Peg3* is strongly expressed in the

developing and adult hypothalamus (Li *et al.*, 1999), but is also expressed throughout the olfactory brain, including the piriform cortex. Exposure to female urine failed to induce a significant increase in expression of the immediate early gene c-Fos in the piriform cortex of either p3-V or p3-SE males and while wt-V males also did not show a significant change in c-Fos expression in response to female urine, wt-SE males had significantly increased piriform cortex c-Fos after exposure to estrous female urine. It should be noted that c-Fos was measured in the main olfactory system of males that were exposed to estrous female urine by direct application to the nose. While this ensured consistent levels of stimulus exposure among animals that displayed different levels of interest in estrous odors in behavioral tests, it also meant that subjects in the c-Fos study were exposed to both volatile and non-volatile urinary chemosignals. This methodology would have led to stimulation of the vomeronasal organ as well as the main olfactory epithelium, however the piriform cortex is not known to receive innervation from the accessory olfactory system (Pro-Sistiaga *et al.*, 2007). The stimulus intensity would also have been greater than in the behavioral tests with volatile odors alone, but despite this greater level of odor exposure for all groups, enhanced main olfactory activation was only observed in the wt-SE males.

The piriform cortex is an important downstream processing nucleus within the main olfactory system which receives a major connection from the main olfactory bulb and plays a significant role in the discrimination of complex odors, as indicated by lesion studies (Staubli, Schottler, & Nejat-Bina, 1987). It sends output to the cortical and medial amygdala (Coolen & Wood, 1998; Majak, Ronkko, Kemppainen, & Pitkanen, 2004), the latter being a key node in the control of male sexual behavior (Newman, 1999). Expression of androgen and estrogen receptors (Kritzer, 2004; Shughrue, Lane, & Merchenthaler, 1997; Simerly, Chang, Muramatsu, &

Swanson, 1990) has been reported in the piriform cortex, suggesting it may be responsive to the key hormones that regulate male sexual behavior. It has also been implicated in olfactory learning (Brennan & Keverne, 1997) and such plasticity suggests this nucleus may have the capacity to play a role in experience-dependent changes in olfactory preferences.

This experience-driven change in c-Fos expression in the piriform cortex of wild-type males suggests that the changes in olfactory behavior and odor preferences may be regulated by changes in the main olfactory system, as well as in the vomeronasal system. The reduced activation of the piriform cortex in p3-SE males suggests that it may be involved in the previously reported absence of any experience-dependent changes in sexual behavior in *Peg3*-KO males (Swaney *et al.*, 2007). The undifferentiated levels of c-Fos expression seen in the piriform cortex in all *Peg3*-KO male groups indicate that sexual experience does not enhance the responsivity of the main olfactory system to estrous female odors in these mice, and consequently that sexual experience does not increase the signal value of these volatile odors. This effect of the knockout may be due to a breakdown in signal detection at the level of the olfactory epithelium, or due to an inability to integrate olfactory information with feedback from downstream forebrain nuclei that control sexual behavior. We are unable to tell from these results whether there is a primary deficit in main olfactory signal detection in *Peg3*-KO males, as we found no effect of urine exposure on c-Fos expression in the olfactory bulb of either wild-type or *Peg3*-KO males and no significant differences in olfactory bulb activation in any of the group comparisons. However the behavioral data from both the male-female choice tests and the habituation-dishabituation tests show that the mutant males were able to detect urine and were not anosmic. The lack of piriform cortex response in the p3-SE males indicates that despite acquiring a preference for volatile female odors in general, sexual experience did not alter

activation of the main olfactory system by estrous female urine. This then suggests that the experience-driven changes in activation of the piriform cortex may be involved in the estrous preferences and the increase in olfactory acuity seen in wild-type males and that the absence of such neural and behavioral changes in *Peg3*-KO males may be associated.

We have presented data here that shows that the paternally expressed gene *Peg3* regulates the effects of sexual experience on main olfactory responses to female odors and preferences for the volatile odors of receptive females. The involvement of *Peg3* in sexual experience-driven changes in sexual behavior and the function of both olfactory systems indicates that this gene has evolved to adaptively regulate male reproductive behavior in general, rather than in a specific system. *Peg3* is involved in multiple aspects of female-directed behavior in males, from detection of receptive females at a distance based on volatile odors, to proximal investigation of females and the production of sexual behavior itself. Knocking out *Peg3* does not abolish any of these behaviors, but disrupts the shaping of adaptive behaviors by sexual experience. These effects of the knockout on sexual experience-driven changes suggest that *Peg3* plays a developmental role linking the different systems that control sexual behavior and the sensory systems that provide relevant information. We suggest that as a purported apoptotic protein (Relaix *et al.*, 2000) that is developmentally expressed in the hypothalamus and olfactory systems (Li *et al.*, 1999), *Peg3* may be important in sculpting connections between these areas. Knocking out *Peg3* may prevent the accurate formation of these links and so disrupt the behavioral tuning that occurs with sexual experience in wild-type males.

These data paint a picture of a more complex phenotype in Peg3-KO mice than initially understood. We are able to discount the possibility that Peg3-KO males suffer a general learning deficit, as they acquired a preference for female volatiles after sexual experience. However their

failure to acquire a preference for estrous volatiles is of particular interest when considering how this gene evolved, and the involvement of imprinted genes in the evolution of mammalian reproduction in general. For paternally expressed genes to spread through a population, they would be expected to regulate the expression of adaptive male traits, as it is only when these genes are transmitted down the patriline that they are then expressed in offspring and so are functionally active to regulate traits in that next generation. The acquisition of a preference for estrous volatiles is such an adaptive trait, and in small-brained mammals like rodents this ability can have considerable impact on male reproductive success. An estrous preference would enable males to discriminate potential mates from females that are not receptive and offer no chance of sexual success. In rodents such as mice with life expectancies in the wild of less than 4 months, females spend the large part of their adult lives either pregnant or in a non-receptive phase of the estrus cycle. Thus, reproductively-capable females are likely to be at a premium, and any males that can find estrous females based on airborne chemosignals would be at a distinct reproductive advantage. *Peg3* appears to have evolved to regulate responses to sexual experience, which induces such a preference, across different brain areas including the main olfactory system, the vomeronasal system, the hypothalamus and the mesolimbic dopamine system, suggesting that it has been involved in the evolution of the behavior, rather than the evolution of a particular system. In the context of the evolution of genomic imprinting, the small advantage conferred on males that were able to learn to distinguish receptive females may have been essential for fixing this paternally expressed gene in the genome. The other reported roles of this gene on maternal behavior and offspring development (Curley *et al.*, 2004) could only evolve if Peg3 was also regulating the reproductive behavior of the males that actively transmitted it to their offspring.

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b)

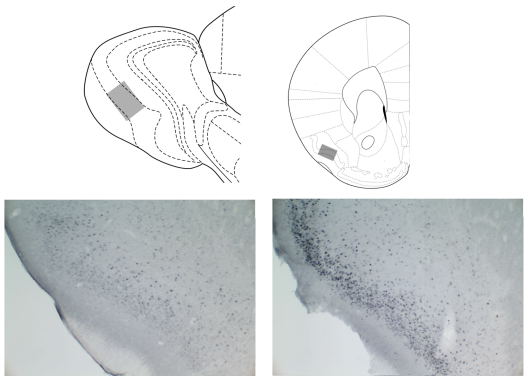

Figure 1

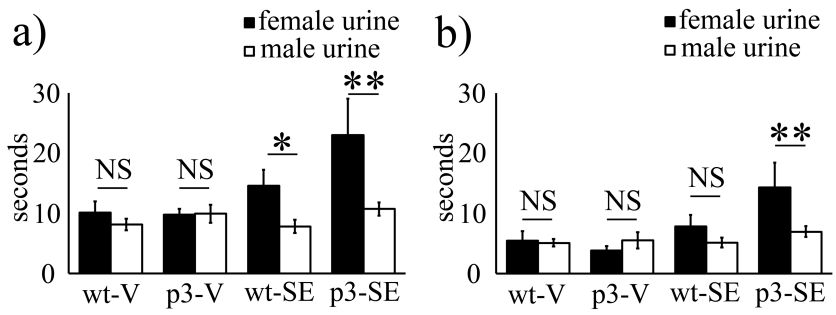
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Figure 2

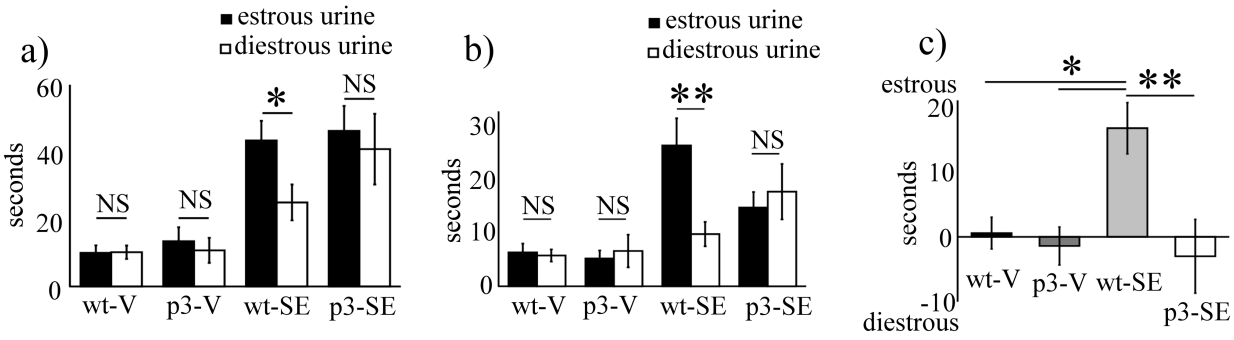
a)
estrous urine
diestrous urine
seconds
60
40
20
0
NS
NS
*
NS
wt-V
p3-V
wt-SE
p3-SE
b)
estrous urine
diestrous urine
seconds
30
20
10
0
NS
NS
**
NS
wt-V
p3-V
wt-SE
p3-SE
c)
estrous
diestrous
seconds
20
10
0
-10
*
**
wt-V
p3-V
wt-SE
p3-SE

Figure 3

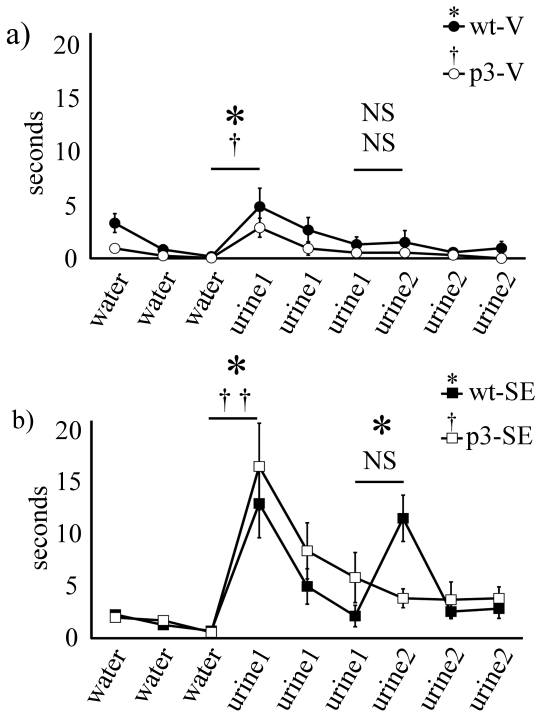
a)
seconds
20
15
10
5
0
*
†
NS
NS
*
wt-V
†
p3-V
water
water
water
urine1
urine1
urine1
urine2
urine2
urine2
b)
seconds
20
15
10
5
0
*
† †
*
NS
*
wt-SE
†
p3-SE
water
water
water
urine1
urine1
urine1
urine2
urine2
urine2

Figure 4

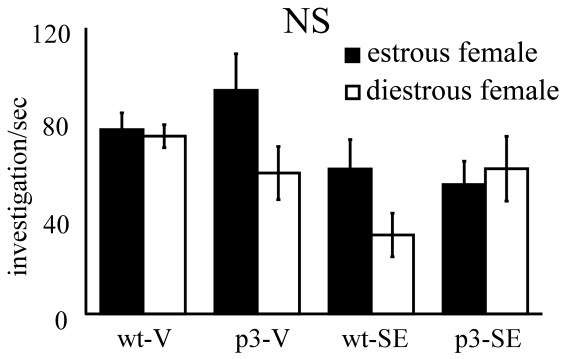
NS
120
80
40
0
investigation/sec
estrous female
diestrous female
wt-V
p3-V
wt-SE
p3-SE

Figure 5

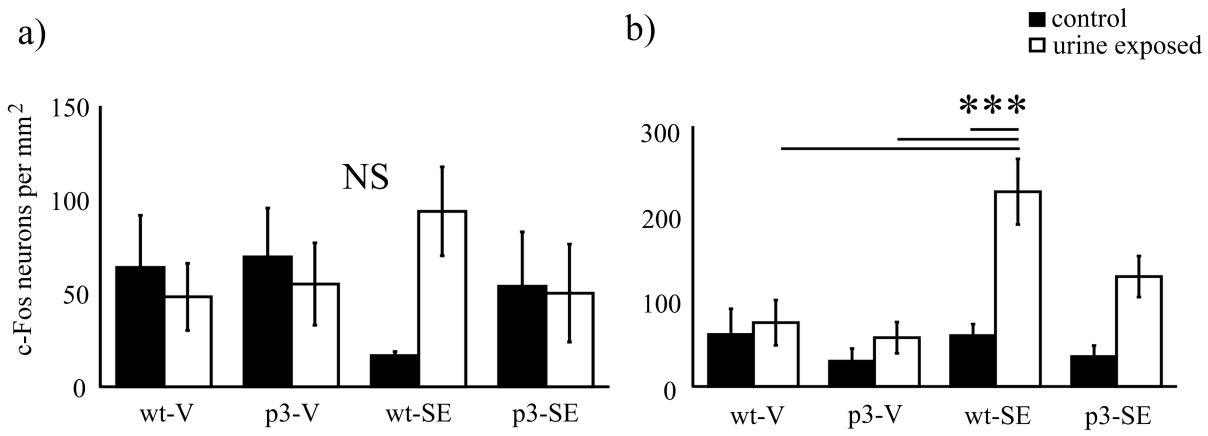
a)
b)
control
urine exposed
c-Fos neurons per mm2
150
100
50
0
NS
300
200
100
0
***
wt-V
p3-V
wt-SE
p3-SE
wt-V
p3-V
wt-SE
p3-SE

Figure 6

Figure Captions

Figure 1. a) Representative illustrations of brain areas sampled for c-Fos counts in the main olfactory system. Images are adapted from *The Mouse Brain in Stereotaxic Coordinates* (2nd ed) Figures 17 and 111, Paxinos & Franklin (2001). Copyright by Elsevier, adapted with permission. Neurons were counted inside sampling areas covering the glomeruli of the main olfactory bulb at approximately 1.20mm lateral (left) and the piriform cortex at approximately 1.70mm Bregma (right). b) Photomicrographs of c-Fos positive neurons in the anterior piriform cortex of control (left) and urine exposed (right) wt-SE males.

Figure 2. Mean investigation (±SEM) of male and female urinary volatiles by wt-V males (n=8), p3-V males (n=8), wt-SE males (n=13) and p3-SE males (n=11) in a) the full five-minutes and b) the first two minutes of choice tests with male and female urinary volatiles. Sexually experienced wild-type males exhibited a preference for female odors in the full five minute test, and sexually experienced *Peg3*-KO males in both the first two minutes and the full five minutes of testing. * = $p \leq 0.05$; ** = $p \leq 0.01$.

Figure 3. Behavior of wt-V males (n=8), p3-V males (n=8), wt-SE males (n=7) and p3-SE males (n=7) in five-minute choice tests with estrous and diestrous female urinary volatiles. a) Mean investigation (±SEM) over the full five-minute urine choice test. b) Mean investigation (±SEM) over the first two minutes of testing. c) Preference for estrous urine (±SEM) over the first two minutes of testing, as calculated by subtracting diestrous investigation time from estrous investigation time. wt-SE males exhibited a significant preference for estrous urine over both the full test duration and the first two minutes of testing. wt-SE males' preference in the first two minutes was significantly greater than those of the other groups. * = $p \leq 0.05$, ** = $p \leq 0.01$.

Figure 4. Mean investigation (±SEM) of volatile odors by a) wt-V males (n=8) & p3-V males (n=8) and by b) wt-SE males (n=7) and p3-SE males (n=7) in habituation-dishabituation tests with water, estrous urine and diestrous urine. All groups increased their investigation when the first urine type was presented, but only wt-SE males increased their investigation when the second urine type was presented. */† = $p \leq 0.05$, †† = $p \leq 0.01$.

Figure 5. Mean investigation (±SEM) of estrous and diestrous females by wt-V males (n=6), p3-V males (n=5), wt-SE males (n=9) and p3-SE males (n=9) in five-minute partner choice tests. None of the male groups displayed a preference for estrous females, although there was a trend among wt-SE males (p=0.073) to investigate estrous females more than diestrous females.

Figure 6. a) Mean counts of c-Fos-positive neurons (±SEM) in the main olfactory bulb glomeruli of control and estrous urine-exposed wt-V males, p3-V males, wt-SE males and p3-SE males (n=5 for control p3-V and control wt-SE males, n=6 for all other groups). There were no significant differences between groups in counts of glomerular c-Fos neurons. b) Mean counts of c-Fos-positive neurons (±SEM) in the anterior piriform cortex of control and estrous female urine-exposed wt-V males, p3-V males, wt-SE males and p3-SE males (n=6 for all groups). In the anterior piriform cortex, exposure to urine significantly increased the number of c-Fos neurons in wt-SE males alone, and urine-exposed wt-SE males had significantly more piriform cortex c-Fos neurons than either urine-exposed wt-V or p3-V males. No other differences were seen among the urine-exposed male groups. *** = $p \leq 0.001$.