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Sex Differences in Social Investigation: Effects of Androgen Receptors, Hormones and Test Partner

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Abstract

We examined the role of the androgen receptor (AR) in the investigatory behaviour of conspecifics using mice carrying the testicular feminisation mutation ($X^{Tfm}Y$). Responses to members of the same and opposite sex were evaluated in a habituation / dishabituation task. Adult mice were gonadectomised and treated with oestradiol (E_2) or testosterone. After E_2 treatment, regardless of the sex of the stimulus mouse, wild-type (WT) males engaged in significantly more investigation than WT females. $X^{Tfm}Y$ males treated with E_2 showed 'male-like' behaviour in response to a male but behaved 'female-like' when the stimulus was a female. Because WT and $X^{Tfm}Y$ males behaved the same in response to another male, we used two additional mouse models to ask whether sex chromosomes were responsible for this phenomenon. Regardless of sex chromosome complement, gonadal males displayed high levels of investigation. When mice were treated with testosterone, investigation by WT females was enhanced, which eliminated the sex differences. Most strikingly, $X^{Tfm}Y$ males receiving testosterone-treatment increased the investigation of females to levels equal to those shown by WT mice. Given that testosterone, but not its metabolite E_2 , caused $X^{Tfm}Y$ males to investigate female conspecifics at high levels, it is plausible that nonclassical actions of AR, and / or activation of a novel AR, may be involved in this behaviour. Taken together, our data show that AR activation during adulthood is not required for males to investigate mice of either sex. However, 'male-like' levels of investigation of a female stimulus may depend on neonatal activation of the classic nuclear AR.

Keywords

androgen receptor; testicular feminisation mutation; CAIS; sex differences; social investigation; complete androgen insensitivity

Sexual differentiation of the nervous system occurs during critical periods of development, through an array of mechanisms, associated with the action of steroid hormones and receptors. One critical organisational period when hormones affect the brain occurs at the end of gestation and just after birth. In male mice and rats, the neonatal rise in testosterone levels (1, 2) mediates the differentiation of neural structures and pathways responsible for the expression of sex-specific (i.e. 'male-like' or 'female-like') behaviours (3). The effect of testosterone is mediated via the androgen receptor (AR) or after aromatisation to oestradiol

(E₂) via one of the oestrogen receptors (ER), which are expressed in neural regions that undergo sexual differentiation and regulate sexually dimorphic behaviours (4).

Several studies using different animal models support a role for gonadal hormones in sex differences of social behaviour. In mice, activational E₂ during adulthood appears to be sufficient for sex-specific partner preference in gonadectomised males and females (5). In addition, studies with knockout mice for ER, aromatase enzyme, α -foetoprotein and androgen receptor (AR) suggest that both neonatal oestrogens and non-aromatisable androgens are important for the masculinisation of partner preferences (5–8). Interestingly, sex differences in social behaviours involving nonvolatile cues appear to be mediated primarily by non-aromatisable androgens (5–7, 9, 10). Taken together, these findings suggest that the role of hormones in sexual differentiation of social discrimination varies according to the type of behaviour measured and the behavioural paradigm used.

Previous studies in our laboratory using mice carrying the testicular feminisation mutation (X^{Tfm}Y), which renders a nonfunctional AR, found that AR plays a critical role in the masculinisation of social discrimination of cues from awake stimulus mice and soiled-bedding from conspecifics, during simultaneous-choice tasks (5, 10). In the present study, we used X^{Tfm}Y mice to directly assess the role of AR and activational hormones (testosterone versus E₂) with respect to sex differences of the social investigation of conspecifics. We employed a habituation / dishabituation task to assess two measures of social interaction: social interest in a familiar conspecific and the ability to discriminate between a familiar and novel stimulus (social recognition). Two additional mouse models, the Four Core Genotype (FCG) and the Y*, were used to explore the contribution of sex chromosome complement in males investigating another male.

Materials and methods

Animals

Animal care and surgery were conducted in accordance with the University of Virginia Animal Care and Use Committee guidelines. Three lines of mice were used in the present study. First, we used mice carrying the testicular feminisation mutation (X^{Tfm}). X^{Tfm}X carrier dams were originally purchased from the Jackson Laboratories (stock #000569) (Bar Harbor, ME, USA). This mutation is a spontaneous point mutation in the AR gene, the deletion of C at the 1112 position, which causes a premature stop codon and a shortened transcript, generating a truncated and thus nonfunctional receptor. X^{Tfm}Y mutants have been described in many species, including rats, mice and humans; and, in all cases, if the mutation is complete and the AR is not functional, XY individuals appear externally female with small abdominal testes (11). Given that the AR is located on the X chromosome, X^{Tfm}Y males are infertile, although heterozygous females (with only one mutated copy) can be used as carriers. We produced X^{Tfm}Y and normal XY and XX offspring by mating female carriers with normal C57BL / 6J males. We genotyped all the offspring with real-time polymerase chain reaction (PCR) for the AR mutation and only used homozygous XX wild-type (WT) females in these studies, along with their normal and mutant male littermates.

In the second line of mice, FCG, testis determination and chromosomal sex are disassociated. In these mice, the Y chromosomal testis-determining gene (*Sry*) is mutated (producing the Y⁻ chromosome) and rescued by a fully penetrant transgenic copy of *Sry* on an autosome (12). As a result, all individuals possessing the *Sry* transgene develop testes and have a male external phenotype, regardless of their sex chromosome complement, whereas individuals lacking the transgene have external female secondary sex characteristics and ovaries. XY⁻ *Sry* males are mated with C57BL / 6J XX females to produce the four genotypes: XX females (XXF), XY⁻ females (XYF), XY⁻ *Sry* (XYM) and XX *Sry* (XXM)

males. In this study, only XYM and XXM mice (testis-bearing) were used. Mice were genotyped by PCR for the YMT2 / B-related Ssty subfamily present on the Y long arm, which detects the Y chromosome.

The third line of mice carries the Y* chromosome and, interestingly, contains a variable number of X chromosomes. This line is used to test for the contributions of one versus two X chromosomes. The Y* chromosome is assumed to be a product of an aberrant recombination event between the X and Y chromosomes. In essence, Y* is considered to contain all Y-chromosome unique material in addition to a functional X centromere and a pseudoautosomal region (PAR) that is inverted in its majority. Recombination between the X and Y* chromosomes generates two new chromosomes, Y*X and XY*. Y*X is a small chromosome that contains the centromeric portion of Y* attached to G-band negative material from the X, whereas XY* is a large chromosome containing the X chromosome attached at its distal end to all of the Y* except the centromeric region (13). XY* males and XX females were originally purchased from the Jackson Laboratories (stock # 2021) and bred to produce four genotypes of mice: XX (2XF) and XY*X (1XF) females, and XY* (1XM) and XXY* (2XM) males. We genotyped all the offspring with PCR for *Xist* mRNA. In this study, only 1XM and 2XM mice (testis-bearing) were used.

All mice used in these studies were born, reared and housed at the University of Virginia School of Medicine Animal Facility in Jordan Hall under a 12 : 12 h light / dark cycle (lights off 12.00 h EDT). The day of birth was designated as post-natal day 0 (PN0). Upon weaning, at PN21, all mice were group-housed according to sex and litter until they were gonadectomised and individually housed afterwards for the remainder of the study. Food (Harlan Teklad Mouse / Rat Sterilizable Diet #7012) (Madison, WI, USA) and water were provided *ad lib*.

Five cohorts of mice were used for the present study. The first cohort (WT females = 13, WT males = 12, X^{Tfm}Y males = 13) was gonadectomised and received E₂ implants; after at least 10 days of recovery, they were tested for social investigation of an ovariectomised (OVX) female stimulus. The second (WT females = 10, WT males = 10, X^{Tfm}Y males = 12), third (XXM = 9, XYM = 8) and fourth (1XM = 6, 2XM = 9) cohorts were gonadectomised, received E₂ implants and were tested as above with habituation / dishabituation tests using a male stimulus. The fifth cohort (WT females = 12, WT males = 13, X^{Tfm}Y males = 11) was gonadectomised, received testosterone implants, recovered from surgery for at least 10 days, and was subsequently tested for social investigation of female and male stimulus mice.

Surgery and hormone treatment

Mice were gonadectomised between 70 and 120 days of age, under general isoflurane inhalant anaesthesia. Animals from cohorts 1, 2, 3 and 4 received an E₂ implant (5 mm Silastic brand silicon tube; inner diameter 1.02, outer diameter 2.16; Dow Corning, Midland, MI, USA) filled with crystalline 17 β -oestradiol and cholesterol (1 : 1; Sigma, St Louis, MO, USA). This concentration produces a high but physiological, plasma concentration of E₂ equal to that noted in the pro-oestrus phase of the mouse cycle (80–90 pg / ml) (14). We used E₂ because it enhances the display of social investigation, but does not activate the AR; thus, all animals receive equivalent hormone stimulation. Animals from cohort 5 received testosterone implants (inner diameter 1.02 mm, outer diameter 2.16 mm) filled to 10 mm in length with crystalline testosterone. These implants yield plasma testosterone levels within the physiological range for C57BL / 6J males (1–2 ng / ml) (15). Using this hormone treatment, any behavioural differences between E₂ and testosterone-treated animals can be attributed to actions of non-aromatisable androgens. In this experiment, all animals were tested with both types of stimuli (male and female). Half of the animals in each group

(randomly selected) were tested first with a female and, 1 week later, with a male stimulus; the order was reversed for the other animals.

Stimulus animals

Two types of stimulus animals were used in these studies. We used a relatively neutral stimulus, OVX females, because they have been shown to elicit high levels of social investigation (16). The other stimulus we used were gonad-intact males. Because gonad-intact males can elicit aggression and other behaviours that can potentially interfere with social investigation, all stimulus animals (males and females) were confined under a holding cell with vertical bars that allowed nose-to-nose contact during the test. Stimulus females were adult OVX C57BL / 6J mice that had not received any hormone treatment for at least 1 week before testing. Stimulus males were gonad-intact C57BL / 6J adults. Stimulus animals were individually housed and exclusively used for social recognition tests.

Habituation / dishabituation test

A habituation / dishabituation paradigm was used to assess two aspects of social behaviour: social interest in a familiar conspecific (habituation) and social recognition (dishabituation). All tests were performed during the light phase of the light:dark cycle, 3–4 h before lights off. Briefly, on the day of testing, the subject was brought into the testing room at least 30 min before the test began to habituate to the room. A holding cell was placed in the home cage of the subject for 10 min for habituation. Next, the stimulus animal was placed inside the holding cell for 1 min, then removed for 9 min (inter-trial interval) before re-introduction. This procedure was repeated until the subject had experienced eight 1-min interactions (trials) with the same stimulus animal (familiar conspecific). Nine minutes after the eighth exposure, a new stimulus animal (novel conspecific) was introduced to the subject mouse. Trials 1 to 8 represent the habituation portion of the test, and provide information on social investigation over time with a familiar conspecific. The comparison between Trials 8 and 9 represents the dishabituation portion of the paradigm and reveals the ability of the animal to discriminate between a familiar (Trial 8) and a novel (Trial 9) conspecific. A change in investigatory time between Trials 8 and 9 represents an index of social recognition and suggests that the subject has formed a social memory of the ‘familiar’ mouse during the first eight trials (16). During each 1-min interaction, the amount of time the resident spent sniffing the stimulus mouse was recorded live using a stopwatch. Sniffing was defined as direct contact with the subject’s nose touching the holding cell and / or the stimulus animal. Tests were videotaped for backup, and chemo-investigation was scored by observers blind to genotypes, sexes and hormonal treatment.

Statistical analysis

One-way ANOVA was used to analyse the effect of genotype in the total time spent investigating the stimulus mouse during the eight 1-min interactions of the habituation phase (Trials 1–8). Repeated-measures ANOVA using genotype and trial as factors was used to analyse the social recognition habituation (Trials 1–8) and dishabituation phases (Trials 8 and 9) data. Planned comparisons were performed using Fisher’s least significant difference multiple-comparison tests.

Results

Sex differences in social investigation of a female conspecific

In mice treated with E₂, WT males showed higher levels of investigation (i.e. time spent investigating) of a female than did WT females and X^{Tfm}Y males (Fig. 1A–C). Repeated-measures ANOVA on data from the habituation, Trials 1–8, revealed a significant effect of

genotype ($F_{7,303} = 6.76$; $P < 0.003$; Fig. 1A). WT males spent more time than WT females and $X^{Tfm}Y$ males investigating the stimulus male ($P < 0.05$). Similarly, an effect of trial was found ($F_{7,303} = 6.76$, $P < 0.003$; Fig. 1A), as demonstrated by a decreased chemo-investigation in each subsequent trial in comparison to Trial 1 (first exposure; $P < 0.05$). No interaction between trial and genotype was noted. Analysis of Trials 8 and 9 (last exposure to the familiar stimulus and first exposure to the novel stimulus, respectively) revealed a significant effect of trial ($F_{1,75} = 15.50$; $P < 0.0004$; Fig. 1A) that resulted from an increase in investigatory time when the novel mouse was introduced in Trial 9 (dishabituation; $P < 0.05$). Thus, all mice discriminated between familiar and novel conspecifics. Interestingly, a significant effect of genotype was noted ($F_{2,75} = 3.48$; $P < 0.04$). During this phase, WT males spent more time investigating the stimulus than WT females, and $X^{Tfm}Y$ males showed indistinguishable levels of investigation from WT males and females ($P < 0.05$). No interaction between genotype and trial was found. One-way ANOVA of the total time spent sniffing the same female revealed a significant effect of genotype ($F_{2,37} = 6.61$; $P < 0.004$; Fig. 1B). WT males spent more time than WT females and $X^{Tfm}Y$ males investigating the stimulus female ($P < 0.05$). Taken together, these data suggest that the presence and activation of a functional AR during development is essential for sex differences in investigatory time of a familiar female conspecific.

Treatment with testosterone eliminated sex and genotype differences

WT males, WT females and $X^{Tfm}Y$ mice received testosterone to assess the effect of activational androgens on sex differences in social investigatory behaviour. Mice treated with testosterone did not display sex differences in investigatory behaviour of a female mouse (Fig. 1C, D). Analysis of trials with re-introduction of the same stimulus showed a significant effect of trial ($F_{7,279} = 10.81$; $P < 0.0001$; Fig. 1B) was caused by decreased chemo-investigation in Trials 5 to 8 compared to Trial 1 ($P < 0.05$). A trend for a genotype effect was observed, with $X^{Tfm}Y$ males showing higher levels of investigatory behaviour than WT males during the first eight trials ($F_{7,279} = 3.02$; $P = 0.06$). No interaction between genotype and trial was found. Analysis of Trials 8 and 9 showed a significant effect of trial ($F_{1,69} = 24.77$; $P < 0.0001$; Fig. 1C), revealed by increased levels of investigation on Trial 9 compared to Trial 8 ($P < 0.05$). We did not find an effect of genotype or an interaction between genotype and trial. One-way ANOVA of the total time spent investigating the familiar mouse showed no significant effect of genotype; however, a trend was noted, with $X^{Tfm}Y$ males displaying more investigatory behaviour than WT males ($F_{2,34} = 3.02$; $P = 0.06$; Fig. 1D). Taken together, sex differences between WT males and females were eliminated by testosterone treatment, with WT females showing 'male-like' levels of social investigation. Most strikingly, $X^{Tfm}Y$ males treated with testosterone also behaved like their WT littermates.

Sex differences in social investigation of a male conspecific

As in the first study, when treated with E_2 , WT males displayed more investigatory behaviour than WT females (Fig. 2A, B). However, $X^{Tfm}Y$ males behaved like WT males and also investigated a stimulus male for a longer time than did WT females. Repeated-measures ANOVA of data from the habituation trials revealed a significant effect of genotype ($F_{2,255} = 5.33$; $P < 0.011$; Fig. 2A) and a significant effect of trial ($F_{7,255} = 33.64$; $P < 0.0001$). Investigatory behaviour declined over trials. No interaction between genotype and trial was noted. All animals were able to discriminate between a novel and a familiar stimulus ($F_{1,63} = 14.86$; $P < 0.0006$; Fig. 2A) as illustrated by a significant increase in investigation during Trial 9 compared to trial 8 ($P < 0.05$). A genotype effect was found ($F_{2,63} = 3.22$; $P = 0.054$); in which WT males engaged in more investigation than WT females, whereas $X^{Tfm}Y$ males had values indistinguishable from their WT littermates. No interaction between trial and genotype was found. One-way ANOVA of trials during the

habituation period noted a significant effect of genotype ($F_{2,31} = 5.33$; $P < 0.011$; Fig. 2B) in which WT and $X^{Tfm}Y$ males engaged in more investigatory behaviour than WT females. These findings suggest that sex differences in investigation of a male conspecific do not require activation of a functional AR during development.

Testosterone treatment enhances investigatory behaviour in females

Sex differences were not present in mice treated with testosterone (Fig. 2C, D). Repeated-measures ANOVA on habituation trials revealed a significant interaction between genotype and trial ($F_{14,287} = 2.84$; $P < 0.0006$; Fig. 2C). The interaction was caused by decreased chemo-investigation in WT females and $X^{Tfm}Y$ mice for all trials compared to Trial 1. WT males maintained the same level of investigation throughout the eight trials ($P < 0.05$). Analysis of Trials 8 and 9 showed a trial effect ($F_{1,69} = 29.50$; $P < 0.0001$; Fig. 2C), all animals engaged in more investigation during Trial 9 compared to Trial 8 ($P < 0.05$). No effect of genotype or an interaction was found. Analysis of total time spent investigating the familiar male did not reveal any effect of genotype (Fig. 2D). Taken together, testosterone treatment eliminated sex differences in social investigation of a male conspecific, with WT females exhibiting enhanced levels of investigation.

No effects of sex chromosome complement

As described above, our results indicate that activation of AR during development is not required for mice to display high levels of social investigation when the stimulus animal is a male. Thus, we tested the hypothesis that WT and $X^{Tfm}Y$ males behaved in a similar manner as a result of having XY sex chromosomes. Because E_2 and testosterone treatment produced similar results in males, all animals in these experiments were treated with E_2 . First, to assess the contribution of the Y chromosome, we compared XY and XX FCG males. Repeated-measures ANOVA on habituation trials showed a significant effect of trial ($F_{7,135} = 9.94$; $P < 0.0001$; Fig. 3A), caused by decreased chemo-investigation starting on Trial 3 compared to Trial 1. No effect of genotype or an interaction was found. Analysis of Trials 8 and 9 showed a significant effect of trial ($F_{1,33} = 11.86$; $P < 0.004$; Fig. 3A), as illustrated by significantly higher levels of investigation in Trial 9 compared to Trial 8 ($P < 0.05$). There were no effects of genotype, nor was an interaction present. Analysis of total time spent investigating the familiar male showed no effect of genotype (Fig. 3B). Our findings showed that the presence or absence of the Y chromosome did not alter 'male-like' levels of social investigation exhibited toward a male mouse.

Next, to assess the contribution of the numbers of X chromosomes we compared E_2 -treated Y^* male mice carrying one or two X chromosomes. Both males (1X and 2X) behaved similarly (Fig. 3C, D). Repeated-measures ANOVA of the habituation period showed a significant effect of trial ($F_{7,119} = 5.64$; $P < 0.0001$; Fig. 3C), caused by decreased chemo-investigation in Trial 8 compared to Trial 1 ($P < 0.05$). Analysis of Trials 8 and 9 revealed a significant effect of trial ($F_{1,29} = 49.75$; $P < 0.0001$; Fig. 3C) with more investigation in Trial 9 compared to Trial 8 ($P < 0.05$). An interaction between genotype and trial ($F_{1,29} = 8.54$; $P < 0.0001$) was found in which 1X males engaged in more investigation of the novel male (trial 9) than 2X males. Thus, although both males were able to discriminate between a familiar and novel male conspecific, the presence of 2X chromosomes generated lower levels of investigation of the novel male stimulus. No effect of genotype was found in total time spent investigating the male stimulus during the habituation phase (Fig. 3D). In sum, our data indicated that the number of X chromosomes does not affect 'male-like' levels of investigation of another male.

Discussion

Sex differences in behaviour normally result from a combination of hormones, genes and environmental factors. Our data show that the hormonal milieu and type of stimulus animal modify sex differences in social investigatory behaviour. Moreover, in males, the activation of a functional AR during development determines the sex-specific response to a female mouse. Treatment with testosterone increases investigation behaviour in all mice compared to the same measures in mice treated with E₂; this suggests a role for AR in activation of this behaviour.

Testosterone effects behaviour in X^{Tfm}Y males: evidence for a 'novel' AR?

Our findings show that X^{Tfm}Y males respond to testosterone, and this hormone produces different patterns of social investigation compared to E₂ treatment. Given that X^{Tfm}Y mice lack a functional AR, it is unlikely these behavioural responses are caused by ligand binding to the 'classic' nuclear AR. Thus, our data support the hypothesis that the actions of testosterone in X^{Tfm}Y males are mediated by pathways alternate to the ones involving the activation of the classical nuclear AR (i.e. activation of a novel AR encoded by a different gene than nuclear AR).

Indeed, several *in vitro* studies have provided evidence for the presence of a membrane AR. In these studies, the administration of cell-impermeable androgen-conjugates such as dihydrotestosterone (DHT)-bovine serum albumin (BSA) (17) or testosterone-BSA (18) supports the existence of membrane ARs in different cell lines, including the rat pituitary (18). Recently, an *in vivo* study assessing the role of nuclear AR in anabolic-androgenic steroid self-administration provided further evidence for the existence of membrane ARs (17). Male hamsters were allowed to self-administer DHT, BSA or DHT-BSA. Males developed a significant preference for DHT or DHT-BSA, but not BSA. In this same study, X^{Tfm}Y and WT male rats showed equivalent levels of DHT self-administration. Taken together, these data suggest that androgen-self administration may be mediated by membrane ARs. Unfortunately, the Tfm mutation in rats is not complete because the mutation in the ligand region of the receptor leaves a small number of partially functional nuclear ARs (19). Thus, it is possible that the observations in rats were a result of the activation of such receptors by the supra-physiological levels of androgens used. Conversely, the half-life of DHT-BSA conjugates *in vivo* is currently unknown, and there is a possibility that free DHT can activate nuclear AR. As predicted by the location and type of the mutation, X^{Tfm}Y mice do not produce any functional nuclear ARs (20), providing an additional model to assess the existence of novel ARs. Although previous studies have shown that E₂-treatment produced the same amount of aggression in WT males and X^{Tfm}Y males (21) and, conversely, testosterone-treatment did not eliminate differences in anxiety between WT and X^{Tfm}Y males (22), no-one has designed an experiment to ask about untraditional ARs in the Tfm mouse.

Metabolites of testosterone, such as 3 α -diol (23) or 3 β -diol (24), could be involved in behaviour via their activation of ER β or GABA_A receptors. A recent study showed that gonad-intact ER β knockout (KO) males behave similarly to WT males in terms of chemo-investigation times and their ability to discriminate between a familiar and a novel OVX female (25). Moreover, when tested for olfactory preferences, gonad-intact WT and ER β KO males behave essentially the same (9). Taken together these data suggest that activation of ER β is not responsible for a male-like behavioural response. Thus, it is unlikely that our observations of male-like behaviour in X^{Tfm}Y mice was caused by activation of ER β . Moreover, because E₂-only treatment did not cause male-like levels in X^{Tfm}Y mice, it is plausible that ER activation is not mediating this phenomenon. Finally, because the implants used in the present study provide physiological levels of testosterone (15) and data from

X^{Tfm}Y male rats show decreased aromatase activity in brain (26), we believe the observed effects of testosterone do not occur via activation of ER.

The effect of GABA_A on social behaviours has been primarily assessed in the context of its ability to reduce aggressive behaviour at the same time as promoting social investigation in mice (27) and social learning of food preference in Mongolian gerbils (28). Interestingly, a recent study suggested that GABAergic neurones in the olfactory bulb have a negative effect on olfactory learning in rats (29). Thus, it is not clear whether the anxiolytic effects of GABA_A receptor activation on social behaviour are a result of a dysfunction in olfaction. Because our data indicate that testosterone-treated X^{Tfm}Y mice are able to discriminate between familiar and novel conspecifics, we consider that their ability to reach 'male-like' levels of investigation in response to testosterone is not mediated by GABA_A activation.

In males, AR is involved in sex differences of social investigatory behaviour directed toward females but not males

In rodents, the habituation / dishabituation paradigm is primarily utilised to assess social recognition (16), although it also provides information on recurrent social interest in a familiar animal. Previous studies using similar tasks have reported sex differences in the duration of investigation times, in which males spend more time investigating the stimulus animal than females (30–34). To the best of our knowledge, the present study is the first report of a direct role of AR in the determination of this sexual dimorphism for a specific type of social stimulus animal (OVX female).

In mice, the AR plays a role in the development of sex differences in olfactory investigation of a female but not a male conspecific. When treated with E₂ and depending on the sex of the stimulus animal, X^{Tfm}Y males showed either levels of investigation similar to WT females, which were lower than WT males, or they acted like WT males. When the stimulus animal was a female, E₂-treated X^{Tfm}Y males behaved like WT females as opposed to WT males. Thus, the absence of a functional nuclear AR during developmental organisation and / or puberty, affected the ability of X^{Tfm}Y males to express E₂-mediated 'male-like' investigatory behaviour of females. By contrast, when the stimulus animal was a male mouse, gonadectomised WT and X^{Tfm}Y males showed similar levels of chemo-investigation under both types of hormonal conditions, suggesting that the activation and / or presence of a nuclear AR during neonatal development is not required to recognise another male.

Social investigatory behaviour of a male, by another male, is not regulated by sex chromosome complement

Given that WT and X^{Tfm}Y males showed similar patterns of investigatory behaviour of a male stimulus mouse, we asked whether sex chromosome complement was part of the mechanism responsible for this behavioural response. We tested two mouse models, FCG and Y*, both of which were gonadectomised and treated with E₂.

FCG males help elucidate whether 'male-like' patterns of social investigation of another male might be attributed to the presence or absence of Y chromosome genes. Gonadectomised and E₂-treated XY and XX males showed similar habituation and dishabituation responses to a male stimulus. In a previous study we used the FCG mouse, treated them with testosterone after gonadectomy, and tested for social recognition of an OVX female. Interestingly, as in the present study, no effect of sex chromosomes was found (12). Using Y* males to assess the potential role of X chromosomes, we observed that gonadectomised and E₂-treated males with 1X and 2X chromosomes behaved the same, showing that 'male-like' levels of investigation of another male is not a result of the absence of genes that typically escape X inactivation. A recent study assessed differences in

sociability between intact, castrated and testosterone- treated 1X and 2X males using three-chamber choice tasks (35). Gonad-intact 2X, males exhibited increased investigation of a male mouse compared to an inanimate object and castration followed by testosterone-treatment eliminated this difference. These data suggested that differences in circulating androgen levels (testosterone or E₂) account for differences in sociability between 2X and 1X males because gonad-intact 2X males showed a preference for same-sex conspecifics. In the present study, we showed that independent of the number of X chromosomes, gonadectomised and E₂-treated males spent equivalent amounts of time spent investigating a male conspecific. Thus, we consider that differences in sociability between intact 2X and 1X males are a result of differences in activation of ER as a result of differences in circulating testosterone and its subsequent aromatisation to E₂. In sum, we conclude that sex chromosome complement is not one of the underlying mechanisms responsible for male mouse patterns of investigatory behaviour of familiar male conspecifics.

Activational effects of androgens in mouse social investigation

As mentioned above, several studies using similar behavioural paradigms to that employed in the present study have reported sex differences in the duration of investigatory times (males > females). For example, although gonad-intact males spend more time investigating conspecifics than do gonad-intact females (32), castrated males and females exhibit similar low levels of investigation toward conspecifics (36) and female mice spend similar time investigating familiar conspecifics (habituation) across the oestrous cycle (25). Thus, sex differences in time spent investigating conspecifics appear to be influenced by activational effects of hormones.

Indeed, our data suggest that an oestrogenic metabolite of testosterone is sufficient for the expression of sex-specific patterns of social investigation in adult mice, independently of the sex of the stimulus. Our findings are in agreement with work conducted in aromatase knockout (ArKO) males tested for social recognition using OVX stimulus mice and a habituation / dishabituation task (37). Gonad-intact ArKO males, which are unable to convert testosterone into E₂ as a result of a targeted mutation in the aromatase gene, have deficits in social recognition as revealed by their lack of habituation / dishabituation responses to a female conspecific. Furthermore, when castrated ArKO males received a combination of E₂ and dihydrotestosterone propionate (DHTP), social recognition was restored to WT male levels. Because E₂ and DHTP were not tested individually it is not clear which (or if) both hormones are required. However, given that gonad-intact ArKO males (with impaired social recognition) have similar or even higher levels of DHT than WT males (38), it was hypothesised that, in adult male mice, social recognition of an OVX female depends predominantly on the activational actions of oestradiol. Our data confirm and expand upon this point.

Testosterone treatment increased the amount of time WT females spent investigating conspecifics but did not have major effects on WT males. In WT females, testosterone, but not E₂, induced 'male-like' levels of investigation of male and female stimuli. Interestingly, several studies have assessed the effect of activational androgens on social recognition in males (36, 39–41). For example, male rats castrated as adults showed decreased levels of investigation compared to gonad-intact males, and testosterone replacement restored behaviour to gonad-intact levels (33). It has also been suggested that social recognition in males is modulated by androgen-dependent vasopressinergic neurones, whereas, in the absence of testosterone, castrated males are able to transition into a vasopressinergic-independent modulation similar to the one suggested for females (36, 41). The present study is the first to show the ability of females to respond to activational androgens, possibly through the activation of AR, and reach 'male-like' levels of social investigation; however, whether this is via vasopressinergic regulation remains to be elucidated. Given that female

behaviour is different depending on the hormone treatment, we hypothesise that females, unlike males, require non-aromatisable testosterone to express high levels of chemo-investigation of social stimuli. As mentioned above, males did not display large differences in behaviour when E₂ versus testosterone was given, suggesting that E₂ is sufficient to ensure 'male-like' levels of investigation.

AR and the sexual differentiation of social behaviours

The ability of male odour cues to stimulate c-fos expression in olfactory pathway relay centres [i.e. medial amygdala, bed nucleus of the strial terminalis (BNST) and medial preoptic area (mPOA)] is sexually dimorphic because only females show neural activation (42). After exposure to male-soiled bedding, X^{Tfm}Y male mice showed a female-like pattern of c-fos expression in the BNST and mPOA (5). Neonatal treatment of female mice with DHT eliminated the ability of male odours to induce neural activation in the BNST and mPOA (10). These findings suggested that developmental activation of AR blocks adult c-fos responses to male odour. Interestingly, our behavioural findings indicate that the absence of a functional AR during development does not affect the ability of X^{Tfm}Y males to express WT male levels of investigation of another male. In the present study, we did not assess neural activation. However, it would be interesting to determine how a lack of AR activation during development affects c-fos expression in response to processing of social cues from an awake active individual (auditory, visual and olfactory) versus cues present solely in soiled-bedding (olfactory). Because AR does not mediate male social investigation of another male, WT males may show a pattern of c-fos similar to the response noted in X^{Tfm}Y and WT females after exposure to male-soiled bedding.

Sex differences in olfactory discrimination have been reported. Indeed, even though females are able to detect lower thresholds of urinary odorants (43), males are superior in olfactory discrimination. Using a food-motivated paradigm, Wesson *et al.* (44) assessed the role of aromatised androgens in olfactory discrimination. In their study, ArKO and WT male and female mice were gonadectomised and E₂-treated. WT males, ArKO males and ArKO females discriminated urinary odours significantly faster than WT females. Moreover, ArKO and WT males did not differ from each other on any test. Thus, it was suggested that sex differences in olfactory discrimination are a result of the organisational actions of perinatal androgens (possibly via AR activation) in males. The present study complements these findings, showing that males require a functional AR during neonatal development for the expression of E₂-mediated 'male-like' social investigation of a female conspecific.

Our findings are consistent with those showing that the AR plays a role in the sexual differentiation of social behaviours. In bedding and partner preference tests, X^{Tfm}Y males and WT females behaved similarly as opposed to WT males (5). Interestingly, in the previous study, all animals were treated with E₂, which may have bypassed the activation of 'novel' ARs and / or nonclassical androgenic actions in X^{Tfm}Y animals. Thus, it is possible that testosterone- or DHT-treatment would have rescued a 'male-like' response in X^{Tfm}Y mice in those behavioural tests. We predict that, in the absence of a functional nuclear AR, activation of a 'novel' AR compensates during both organisation and adulthood. Regardless of the differences in experimental design and behavioural paradigms used, the findings of this previous study are consistent with our findings that AR plays an important role in the organisation of sex differences in the olfactory investigation of conspecifics.

Finally, X^{Tfm}Y mice are analogous to human patients with complete androgen insensitivity syndrome (CAIS). CAIS individuals have a 46, XY karyotype, undescended testes that remain in the abdominal cavity and normal-to-high levels of androgens (45). Typically, CAIS patients are raised as girls and report a female gender identity (46). However, a few studies have reported that some CAIS individuals show male typical behaviours, including a

male gender identity as adults (47–49). In the present study, we found that, under specific social and hormonal conditions, X^{Tfm}Y males have the capacity to behave like WT males. Thus, we introduce the possibility that the presence and activation of a ‘novel’ AR may be involved in the expression of ‘male-like’ behaviours in individuals lacking the classic nuclear receptor.

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Fig. 1.

Social investigation of a female conspecific. Mean $\pm$ SEM time spent (s) investigating an ovariectomised (OVX) female by adult gonadectomised and oestradiol- (A, B) or testosterone-treated (C, D) wild-type (WT) males, X^{Tfm}Y males and WT females. The subjects were exposed to the same stimulus animal during the first eight trials. On the ninth trial, a novel OVX female stimulus was introduced. (A, C) Data from all trials. (B, D) Total time spent by the subjects investigating the same stimulus female during the first eight trials. The number of subjects in each group is shown in parenthesis below the bars. *Significantly different from Trial 1 for all genotypes ($P < 0.05$). **Significantly different from Trial 8 for all genotypes ($P < 0.05$). Δ Significantly different from both other groups ($P < 0.05$).

Fig. 2. Social investigation of a male conspecific. Mean $\pm$ SEM time spent investigating (s) a gonad-intact male by adult gonadectomised and oestradiol- (A, B) or testosterone-treated (C, D) wild-type (WT) males, X^{Tfm}Y males and WT females. The number of subjects in each group is shown in parenthesis below the bars. *Significantly different from Trial 1 for all genotypes ($P < 0.05$). +Significantly different from Trial 8 for WT females and X^{Tfm}Y males ($P < 0.05$). ΔSignificantly different from both other groups ($P < 0.05$).

Fig. 3.

Sex chromosome effect on male social investigation of a male conspecific. Mean $\pm$ SEM time spent (s) investigating a male stimulus mouse by gonadectomised and oestradiol (E_2)-treated XY (XYM) and XX (XXM) males (A, B), or 1X (1XM) and 2X (2XM) males (C, D). The number of subjects in each group is shown in parenthesis below the bars.

*Significantly different from Trial 1 for both genotypes ($P < 0.05$). **Significantly different from Trial 8 for both genotypes ($P < 0.05$).