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Inheritance of steroid-independent male sexual behavior in male offspring of B6D2F1 mice

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Abstract

The importance of gonadal steroids in modulating male sexual behavior is well established. Individual differences in male sexual behavior, independent of gonadal steroids, are prevalent across a wide range of species, including man. However, the genetic mechanisms underlying steroid-independent male sexual behavior are poorly understood. A high proportion of B6D2F1 hybrid male mice demonstrate steroid-independent male sexual behavior (identified as “maters”), providing a mouse model that opens up avenues of investigation into the mechanisms regulating male sexual behavior in the absence of gonadal hormones. Recent studies have revealed several proteins that play a significant factor in regulating steroid-independent male sexual behavior in B6D2F1 male mice, including amyloid precursor protein (APP), tau, and synaptophysin. The specific goals of our study were to determine whether steroid-independent male sexual behavior was a heritable trait by determining if it was dependent upon the behavioral phenotype of the B6D2F1 sire, and whether the differential expression of APP, tau, and synaptophysin in the medial preoptic area found in the B6D2F1 sires that did and did not mate after gonadectomy was similar to those found in their male offspring. After adult B6D2F1 male mice were bred with C57BL/6J female mice, they and their male offspring (BxB₁) were orchidectomized and identified as either maters or “non-maters.” A significant proportion of the BxB₁ maters were sired only from B6D2F1 maters, indicating that the steroid-independent male sexual behavior behavioral phenotype of the B6D2F1 hybrid males, when crossed with C57BL/6J female mice, is inherited by their male offspring. Additionally, APP, tau, and synaptophysin were elevated in the medial preoptic area in both the B6D2F1 and BxB₁ maters relative to the B6D2F1 and BxB₁ non-maters,

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Conflict of Interest

All authors declare that there are no conflicts of interest.

respectively, suggesting a potential genetic mechanism for the inheritance of steroid-independent male sexual behavior.

Keywords

steroid-independent male sexual behavior; B6D2F1 mouse; behavioral genetics; APP; tau; synaptophysin

Introduction

An individual's fitness and contribution of genes to the next generation depends on sexual behavior, which itself has been subject to strong selection throughout the course of evolution. In most rodents, male sexual behavior is strictly dependent upon the presence of gonadal steroids [reviewed in (Hart, 1974; Hull, 2006)]. In contrast, other vertebrates, particularly larger mammals including humans, do not have an absolute requirement for gonadal steroids. Individual variation in response to castration is one of the defining characteristics of several hormone-dependent social behaviors. It is not unusual for men to demonstrate male sexual behavior years after surgical or chemical castration (Heim and Hirsch, 1979).

Unlike most laboratory rodents, a significant proportion (~30%) of B6D2F1 hybrid male mice (offspring of C57BL/6 dam X DBA/2 sire F0 cross) retain copulatory behavior for as many as 25 weeks after orchidectomy (herein after referred to as maters; (Clemens et al., 1988; McGill and Manning, 1976; Thompson et al., 1976). Several studies have utilized the hybrid B6D2F1 mouse to investigate potential neuroendocrine mechanisms which may govern gonadal steroid-independent male sexual behavior. A comparison of the hormonal profile of maters vs. non-maters revealed that there were no differences in plasma T, estradiol (E2, which is converted from T by aromatase in the brain), and dihydrotestosterone (DHT; non-aromatizable metabolite of T) concentrations – all of which were at low or undetectable measures in circulation (Clemens et al., 1988; Sinchak et al., 1996). mRNA levels of estrogen receptor-alpha (ER α), androgen receptor (AR), and aromatase enzyme in the medial preoptic area (MPOA), an area critical for male sexual behavior, were also equivalent between maters and non-maters (Park et al., 2009). Finally, maters administered flutamide (androgen receptor antagonist), letrozole (aromatase inhibitor), or ICI 182,780 (an ER antagonist), continued to copulate, demonstrating that any remaining levels of non-gonadal steroids were not responsible for the persistent copulation in the maters (Park et al., 2009). The difficulty in determining whether neuroendocrine differences regulate persistent copulation in these males has led to studies investigating alternative mechanisms.

A gene expression analyses of the MPOA between B6D2F1 hybrid maters and non-maters revealed over 500 differentially expressed genes (Park et al., 2010). We further investigated two genes in particular, amyloid beta (A4) precursor protein (*A β* , located on chromosome 16 in the mouse) and microtubule associated protein tau (*Mapt*, located on chromosome 11 in the mouse), which are normally associated with Alzheimer's Disease. Expression of these genes and proteins were significantly higher in maters relative to the non-maters (Park et al., 2010). Furthermore, transgenic male mice that overexpressed either APP or tau, the two

proteins products of *App* and *Mapt*, respectively, displayed enhanced sexual behavior after orchidectomy compared to control wildtype littermates, demonstrating that the relationship of APP and tau with male sexual behavior were both beyond correlational (Bharadwaj et al., 2013; Park et al., 2010). Because the normal function of both genes have been associated with synaptic plasticity, this led to an investigation that revealed that relative to B6D2F1 non-maters, the maters exhibited higher levels of synaptophysin, a protein generally enriched in synapses and associated with synaptic connectivity (*synaptophysin* gene located on the X chromosome) (Bharadwaj et al., 2013).

One assumption underlying any genetic mechanisms of steroid-independent male sexual behavior is that behavioral qualities of sires should pass to male offspring, and thus we designed a study to test the hypothesis that the behavioral phenotype of male offspring would be more closely related to that of the sire's behavioral phenotype. In addition we assessed levels of APP, tau and synaptophysin in the MPOA of the both the sires and sons to determine the relationship between the behavioral trait of steroid-independent male sexual behavior and the protein levels of these candidate genes.

Methods

Animals

Male B6D2F1 hybrid mice ($n=36$; *Mus musculus*) were bred by crossing C57BL/6J (B6) females with DBA/2J males. Adult hybrid male mice were crossed with another cohort of B6 females to produce male BXB₁ offspring. All male mice were produced at the University of Virginia, Charlottesville, weaned at 20–21 days and housed singly until the onset of the experiment (between 50 and 80 d of age). All of the mice were maintained on a 12:12 light:dark cycle (light off at 1200h EST) and received food (Harlan Diet 8604; Harlan Teklad, Madison, WI) and water *ad libitum*. All procedures were performed according to the AALAC guidelines and approved by the University of Virginia Animal Use and Care Committee. All surgeries were performed while the animals were anesthetized with isoflurane.

Sexual experience prior to orchidectomy and mating with B6 females

Although prior mating experience is not necessary for the expression of steroid-independent male sexual behavior in B6D2F1 hybrid male mice, it does increase the proportion of males that demonstrate male sexual behavior after long-term orchidectomy (Manning and Thompson, 1976). Thus, B6D2F1 hybrid males were provided sexual experience with hormonally primed stimulus female B6 mice on at least three separate occasions prior to being paired overnight with an ovary-intact B6 female mouse. The next day, all B6D2F1 hybrid males underwent orchidectomy. Of the 36 B6 dams paired with B6D2F1 males, 29 produced litters. When the male BXB₁ offspring reached adulthood, they were also provided sexual experience with hormonally primed stimulus female B6 mice on at least three separate occasions prior to being orchidectomized.

Behavioral Testing after orchidectomy

B6D2F1 hybrid males were tested for male sexual behavior every two weeks for 8 weeks after orchidectomy. Males were considered to be maters if they demonstrated mounts, intromissions and the ejaculation reflex on the last behavioral test (n=8). Males were considered non-maters (n=7) if they did not display any of the components of male sexual behavior during the last test. Any males that did not demonstrate consistent behavior and did not fulfill either of the criteria were classified as behavioral 'intermediates' and were excluded from analyses in this study.

Orchidectomized male BXB₁ offspring were paired every two weeks with a stimulus B6 female in their home cage for two hours. Eight weeks after orchidectomy, BXB₁ males were tested for gonadal steroid-independent male sexual behavior. Males were considered to be maters if they demonstrated mounts, intromissions and the ejaculation reflex (n=10) and non-maters (n=27) if they did not display any of the components of male sexual behavior. Similar to their sires, any males that did not meet either criteria were classified as behavioral intermediates and were not included in the analyses of this study.

Testing for male sexual behavior was conducted as previously described in (Park et al., 2009). All tests were conducted under dim red lights during the dark phase of the light/dark cycle. All males were placed singly into Plexiglas arenas (17.8cm w × 17.8cm h × 25.4 cm l) with their home cage bedding which had not been changed for 2 weeks. After the males habituated to the arena for one hour, tests began with the introduction of a hormone-treated stimulus B6 female mouse into the arena. Stimulus females were ovariectomized and injected subcutaneously with 5 µg estradiol benzoate (dissolved in sesame oil) 48h prior to testing. Three to five hours prior to testing, stimulus females were injected subcutaneously with 5 µg progesterone. Once the male mounted, the test continued to a criterion of a successful ejaculatory reflex or for 120 min, whichever occurred first. If the stimulus female became unreceptive during testing she was replaced with a receptive female.

All tests were videotaped and scored by an observer blind to the classification of the individuals. During each behavioral test, the behavioral components recorded were: mount latency (ML; time from the introduction of a receptive female to the first mount), intromission latency (IL; time from the introduction of a receptive female to the first intromission), and ejaculation latency (EL; interval between the first intromission and ejaculation).

Western Blot Analyses

At the completion of the sexual behavior tests, all males were sacrificed. Brains were dissected, rapidly frozen, and then stored at -80°C. Frozen brains were shipped to the University of MA, Boston, and all procedures were conducted in accordance with our animal protocol, approved by the University of MA, Boston IACUC. Brains dissected from B6D2F1 maters that ejaculated on both behavioral tests conducted on weeks 6 and 8 post-castration (n=5), along with brains from one of their BXB₁ sons that were identified as maters were chosen to analyze protein levels in the MPOA and frontal cortex. Additionally, brains from B6D2F1 non-maters along with brains from one of their BXB₁ sons that were

identified as non-maters were chosen for Western blot analyses ($n=5/\text{group}$; B6D2F1 non-maters were randomly chosen using a random number generator to match the sample size of the maters). All brains were cut into $100\mu\text{m}$ thick coronal sections with a Leica cryostat. Based on the Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 1997), the MPOA and frontal cortex were dissected and homogenized in Thermo Scientific Tissue Protein Extraction Reagent (TPER) plus HALT protease inhibitor chilled on ice. Samples were stored at -80°C .

For protein extraction, brain tissue homogenates were thawed and centrifuged, and total protein concentrations were determined by BCA (bicinchoninic acid) Protein Assays (Pierce Chemical Co., Rockford, IL). Samples were loaded into a 10% polyacrylamide gel and subjected to electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked in 10% milk in Tween TBS overnight at 4°C then warmed to room temperature and rinsed.

They were then incubated with either anti-APP antibodies (Millipore, Billerica, MA), anti-Tau monoclonal antibody, clone 46 produced in mouse (1:10,000; Sigma-Aldrich Corp., T9450) followed by polyclonal goat anti-mouse (1:10,000; BioRad, 170–5047), or monoclonal anti-synaptophysin produced in rabbit (1:10,000; Millipore, catalog MAB368) followed by polyclonal goat anti-rabbit (1:10,000; Millipore, AP307P). This was followed by detection using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co.). Later, blots were re-probed with antibody against β -actin (1:50,000; Sigma-Aldrich Corp. A1978). After rinsing, the blots were incubated for 1 h in an HRP-conjugated goat anti-mouse IgG secondary antibody (1:10,000; Jackson) followed by chemiluminescent detection.

The intensities of each of the candidate proteins and β -actin were visualized and quantified directly using the Bio-Rad Chemi Doc XRS+ Imager and Image Lab software. Levels of each of the proteins were normalized to those of β -actin in each sample. Manuals of the BioRad Image Lab software are available on their website (<http://www.bio-rad.com/>).

Statistical Analyses

One-way ANOVAs were used to analyze differences in composition of litters sired by the female B6 X male B6D2F1 hybrid cross. Differences in the proportion of B6D2F1 hybrid males and their male offspring demonstrating steroid-independent male sexual behavior were assessed with the Chi-square measure. One-way ANOVAs were used to analyze protein levels between groups. Effect sizes were further estimated by calculating eta-squared (η^2) for ANOVAs and Phi (ϕ) for Chi-square tests. Post-hoc comparisons were conducted using the Fisher Protected Least Significant Difference test where appropriate. Observed differences were considered significant if $p < 0.05$.

Results

Steroid-independent male sexual behavior of B6D2F1 hybrid males and their male offspring

Of the 36 B6D2F1 hybrid male mice that were paired with B6 dams, 29 sired a litter; of these 29, 8 were categorized as maters (28%) and 7 were categorized as non-maters (24%; Figure 1A and Table 1). The other 14 males did not meet the criterion for characterization of either a mater or non-mater. B6D2F1 maters sired a total of 25 BXB₁ male offspring, and non-maters sired a total of 12 BXB₁ male offspring (Table 1). There were no differences in litter composition between B6D2F1 maters and non-maters, in either the number of male offspring [F -value (1,13) = 1.62, p = 0.23, η^2 =0.12], the number of female offspring [F -value (1,13) = 0.12, p = 0.73, η^2 =0.01], or litter size [F -value (1,13) = 0.41, p = 0.53, η^2 =0.03].

A significantly higher proportion of B6D2F1 maters sired a litter that contained at least one BXB₁ male offspring that was a mater (75%) relative to B6D2F1 non-maters (14%; Figure 1B; χ^2 (1) = 5.53, p < 0.05, ϕ = 0.89). Of the 25 BXB₁ male offspring from the B6D2F1 maters, 7 were identified as maters. One of the litters that was sired by one of the B6D2F1 non-maters contained 3 BXB₁ maters (Table 2). Due to the relatively high number of maters in this one litter, this led to the finding that there were no statistical differences between B6D2F1 hybrid maters and non-maters in the total number of offspring sired that were maters [F -value (1,13) = 0.91, p = 0.36, η^2 =0.07].

There were no differences in ejaculation, intromission, or mount latencies on the last male sexual behavior test session between the male offspring maters that were sired from either B6D2F1 hybrid maters or non-maters (Table 3; all p -values > 0.05 for all comparisons; η^2 =0.07, 0.53, and 0.04 for ejaculation, intromission, or mount latencies, respectively).

APP, tau, and synaptophysin in the MPOA of maters differs significantly from non-maters

Because previous studies determined that increased APP, tau, and synaptophysin expression levels in the MPOA of B6D2F1 hybrid male mice were associated with steroid-independent male sexual behavior, we analyzed levels of these proteins in not only the B6D2F1 maters and non-maters, but their male offspring as well (Bharadwaj et al., 2013; Park et al., 2010). In concordance with previously reported results, Western blots of APP, tau, and synaptophysin from MPOA tissues showed that all three proteins were significantly higher in B6D2F1 maters relative to those in B6D2F1 non-maters [p <0.05 for all comparisons; Figure 2A–C; APP: F -value (1,8) = 7.034, p < 0.05, η^2 =0.88; tau: F -value (1,8) = 8.476, p < 0.05, η^2 =0.94; synaptophysin: F -value (1,8) = 5.764, p < 0.05, η^2 =0.72]. Notably, higher APP, tau, and synaptophysin levels were only observed in the MPOA, and not the frontal cortex, indicating that the increased levels of these proteins are region specific [p > 0.05 in all comparisons for the frontal cortex; data not illustrated; APP: F -value (1,8) = 2.931, p > 0.05, η^2 =0.36; tau: F -value (1,8) = 0.016, p > 0.05, η^2 =0.002; synaptophysin: F -value (1,8) = 1.445, p > 0.05, η^2 =0.19].

Similar to their hybrid B6D2F1 sires, APP, tau, and synaptophysin from MPOA tissues were elevated in BXB₁ male offspring that were maters relative those that were non-maters [p <0.05 for all comparisons; Figure 2D–F; APP: F -value (1,8) = 5.418, p < 0.05, η^2 =0.5;

tau: $F\text{-value}_{(1,8)} = 6.329$, $p < 0.05$, $\eta^2 = 0.86$; synaptophysin: $F\text{-value}_{(1,8)} = 9.279$, $p < 0.05$, $\eta^2 = 0.79$]. Additionally, no differences in levels of APP, tau, or synaptophysin in the frontal cortex were observed [$(p > 0.05$ in all comparisons; data not illustrated; APP: $F\text{-value}_{(1,8)} = 0.332$, $p > 0.05$, $\eta^2 = 0.04$; tau: $F\text{-value}_{(1,8)} = 0.214$, $p < 0.05$, $\eta^2 = 0.03$; synaptophysin: $F\text{-value}_{(1,8)} = 2.743$, $p > 0.05$, $\eta^2 = 0.33$].

Discussion

Our findings demonstrate for the first time that steroid-independent male sexual behavior is a heritable behavioral trait that is predominantly passed down from B6D2F1 hybrid male mice that exhibit steroid-independent male sexual behavior to their male offspring when crossed with B6 female mice. 75% of the litters from this cross contained a BXB₁ male offspring mater, while only one of the 7 litters from the cross between B6D2F1 non-maters and B6 female mice contained BXB₁ maters (14%; Figure 1B). Our findings are in accordance with results of a prior study in which steroid-independent male sexual behavior was observed in some of the male offspring from certain recombinant inbred mouse lines generated from the B6D2F1 hybrid mice (Coquelin 1991); however, the behavioral phenotypes of the sires were not tracked in the prior study and it remained unknown whether those male offspring identified as maters were originally sired from B6D2F1 maters or non-maters.

It has been previously suggested that one potential genetic mechanism underlying the inheritance of steroid-independent male sexual behavior may be based upon allelic heterozygosity between multiple loci, and that a specific chromosomal loci allelic distribution pattern with B6 alleles at some loci and DBA/2 alleles at other loci regulates inheritance of steroid-independent male sexual behavior (Coquelin 1991). While B6, DBA/2J, and B6D2F1 mice share common alleles at several loci, many loci in the B6D2F1 genome contain two different alleles. The different alleles are designated here as *B* or *D* to reflect their origins from the respective B6 or DBA/2J parental strains (Fig. 3). B6D2F1 hybrid male mice inherit *B* alleles on the maternal homologue and *D* alleles on the paternal homologue, and thus are heterozygous at all loci. When crossed with B6 female mice, the resulting BXB₁ offspring will be homozygous (*B/B*) for approximately 1/2 of loci, and approximately 1/2 heterozygous (*B/D*) at the other loci (Figure 3). Thus, BXB₁ offspring sired from B6D2F1 maters have the potential to inherit the same *B* and *D* allelic distribution pattern at chromosomal loci for steroid-independent male sexual behavior and could inherit the behavioral phenotype. Although further experiments are needed to test these hypotheses, it is interesting to note that if these hypotheses are validated, it may be possible for a male offspring from the cross of a B6D2F1 non-mater with a B6D2F1 female to demonstrate steroid-independent male sexual behavior, but only if the hybrid B6D2F1 female possessed the specific chromosomal loci allelic distribution pattern necessary for steroid-independent male sexual behavior.

We now have a list of candidate genes that are differentially expressed in the MPOA that correlate with steroid-independent male sexual behavior, and this list is essential towards identifying the chromosomal loci allelic distribution pattern for steroid-independent male sexual behavior (Bharadwaj et al., 2013; Park et al., 2010). It would be of interest to

determine in future studies whether the BXB₁ maters also demonstrate a similar pattern of gene expression in the MPOA that correlates with steroid-independent male sexual behavior, as it would provide further evidence for the heritability of this behavioral phenotype. It would not be surprising to observe considerable overlap in differential gene expression based on our results of the Westerns in which the expression of APP, tau, and synaptophysin are significantly higher in the MPOA of both B6D2F1 and BXB₁ maters relative to B6D2F1 and BXB₁ non-maters, respectively (Figure 2).

Although the exact mechanism by which these proteins regulate steroid-independent male sexual behavior has yet to be determined, recent findings demonstrating a strong association between increased complexity in dendritic architecture in the MPOA with steroid-independent male sexual behavior may provide some insight (Bharadwaj et al., 2013). Synaptophysin may play a significant role in the regulation of synaptic plasticity [(Kwon and Chapman, 2011); reviewed in (Evans and Cousin, 2005)], and recent studies investigating the function of APP and tau have revealed an association with promoting spine formation and proper spine development: (1) in rodents, APP expression peaks around the second postnatal week, during robust synaptogenesis (Loffler and Huber, 1992); (2) increased expression of APP promotes synapse differentiation, synaptogenesis, and increased spine number (Ashley et al., 2005; Lee et al., 2010; Loffler and Huber, 1992; Neve et al., 2000; Qiu et al., 1995; Torroja et al., 1999; Wang et al., 2005; Wang et al., 2009); and (3) small interfering RNA (siRNA) against APP caused impaired synaptic activity *in vivo* (Herard et al., 2006). Furthermore, studies have also implicated a strong relationship between tau and spine density, as tau has been found to be integral in the formation and maintenance of synaptic connections (Dickstein et al., 2010; Thies and Mandelkow, 2007). Recently, tau has been found to play an integral role in the dynamic rearrangement of cytoskeletal fibers vital for synaptic plasticity: utilizing tau-siRNA to knockdown expression of tau led to a significant decrease in spine density (Chen et al., 2012). The results of our Western blots indicate that elevated production of APP and tau, two proteins often associated with neural pathology, along with synaptophysin, could help neurons maintain synaptic connections after orchidectomy and support a behavior that would otherwise be lost.

The neural network that regulates sexual behavior in males has been mapped in several species (Hull et al., 2006). The medial amygdala receives projections from the main and accessory olfactory bulbs (Davis et al., 1978; Lehman and Winans, 1982), and sends its projections to the mPOA and bed nucleus of the stria terminalis (Gomez and Newman, 1992; Kevetter and Winans, 1981; Maragos et al., 1989). B6D2F1 hybrid maters retain the ability to discriminate between female and male mice while non-maters have lost this ability (Park et al., 2009), and this finding further supports the hypothesis that the neural circuitry connecting the MPOA to the olfactory system that governs male sexual behavior may remain intact in the maters but not in the non-maters.

It is also noteworthy that all three proteins were not elevated in another brain area, the frontal cortex, in either the B6D2F1 sires or the BXB₁ offspring, indicating that the differential expression of APP, tau and synaptophysin are site specific. However, because the results of our study did not demonstrate a direct functional relationship between increased levels of these proteins and increased steroid-independent male sex behavior, we cannot rule

out the possibility that increased levels of these proteins are a result of increased steroid-independent male sexual behavior or that the two are both downstream of another factor regulating steroid independent male sexual behavior. However, transgenic mice that overexpress either APP or tau have demonstrated facilitated steroid-independent male sexual behavior, suggesting a causal relationship (Bharadwaj et al., 2013; Park et al., 2010). Further experiments investigating steroid-independent male sexual behavior in conditional overexpressors or knockouts of candidate genes would directly probe whether the relationship is more than correlational.

Considering B6D2F1 maters and non-maters are genetically identical and are generally heterozygous at all loci (variant alleles may be present but are presumably rare), it is significant to note that not all of the male offspring sired from B6D2F1 hybrid maters were maters. These results are congruent with a prior finding in which only ~30% of BXD mice from two different recombinant lines that demonstrated steroid-independent male sexual behavior, indicating that this behavioral phenomenon is fixed by a factor that may not be entirely genetic (Coquelin, 1991). This variability in steroid-independent male sexual behavior may be attributed to several environmental factors that have yet to be determined along with their interactions with the genetic factors that have been discussed. These include novel vs. home environment (see (Wee and Clemens, 1989), differential maternal care, and perinatal exposure to circulating gonadal steroids that have organizational effects on sexual differentiation, potentially due to intrauterine position. This last factor is especially intriguing given that several steroid-mediated cellular mechanisms that establish sex differences in synaptic patterning in the MPOA of the rat have been well characterized and correlated with male sexual behavior (Amateau and McCarthy, 2004; Schwarz et al., 2008). Specifically, estradiol is a crucial regulator of dendritic spines in the MPOA not only during development, but adulthood as well (Amateau and McCarthy, 2004; Calizo and Flanagan-Cato, 2000; Schwarz et al., 2008; Todd et al., 2007; Woolley and McEwen, 1992); whether differential exposure to steroids during development impact dendritic morphology and in turn, influence steroid-independent male sexual behavior, remains to be elucidated.

Many of the mice in our study could not be classified as either maters or non-maters, and were identified as behavioral ‘intermediates.’ Although not included in the analyses of this study, these ‘intermediates’ warrant further investigation into whether the degree of environment and/or genetic factors play a role on the level of expression of steroid-independent male sexual behavior.

One further limitation of our study was that our B6D2F1 generation was rather limited in size and the non-mater group within the paternal generation was only n=3. As this study required testing a number of offspring per sire, practical considerations limited the number of animals that could be tested. Additionally, it was difficult to predict at the outset of the study exactly how many non-maters would be produced, as mater or non-mater status can only be determined many weeks following castration. Nevertheless, a larger sample size may have expanded the inferences that could be drawn from this group.

Interestingly, one of the B6D2F1 males that was identified as a non-mater sired a litter that contained 3 BXB₁ males that were identified as maters (Table 2). One explanation for this

may be that the ability to demonstrate steroid-independent male sexual behavior may not have manifested in this B6D2F1 male; it has been documented that in some B6D2F1 hybrid males, there is a “difficult period” after castration in which steroid-independent male sexual behavior may not be evident until months after orchidectomy (McGill and Manning 1976). Alternatively, some important environmental factors may have suppressed the expression of steroid-independent male sexual behavior in this B6D2F1 male; however, the exact role of the environment in steroid-independent male sexual behavior has yet to be elucidated.

In summary, we provide evidence in support of steroid-independent male sexual behavior as a heritable behavioral phenotype, and that the behavioral status of the B6D2F1 sire is an important factor when crossing a B6D2F1 hybrid male with a B6 female. The mechanism by which inheritance of this behavioral trait appears to incorporate both genetic and non-genetic factors, involving APP, tau, and synaptophysin; however, the exact mechanisms by which this inheritance occurs will need to be further investigated in order to provide a better understanding of individual differences in male sexual behavior.

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Highlights

- ~30% B6D2F1 male mice demonstrate steroid-independent male sex behavior (SI-MSB).
- Elevated APP, tau, and synaptophysin impact SI-MSB.
- SI-MSB was found to be a heritable trait.
- This trait is passed down mainly from B6D2F1 mice that demonstrate SI-MSB.
- APP, tau, and synaptophysin were higher in B6D2F1 males and sires that show SI-MSB.

Figure 1.

(A) Percentage of B6D2F1 male mice (n=36) that were either maters or non-maters. (B) Percentage of B6D2F1 males that sired a litter that contained at least one BXB₁ mater.

*significantly higher than B6D2F1 non-maters ($p < 0.05$).

B6D2F1: MPOA**A. APP** **B. Tau** **C. Synaptophysin** **BXB₁: MPOA****D. APP** **E. Tau** **F. Synaptophysin**

Figure 2. Higher levels of APP, tau, and synaptophysin in B6D2F1 and BXB₁ maters relative to non-maters

Mean (+SEM) of (A) APP, (B) tau, and (C) synaptophysin in the MPOA in B6D2F1 maters and non-maters. Mean (+SEM) of (D) APP, (E) tau, and (F) synaptophysin in the MPOA in BXB₁ males that were maters or non-maters. *, significant difference ($p < 0.05$); levels adjusted for loading controls (β -actin) in the MPOA.

Figure 3. Hypothetical pairs of homologous chromosomes in inbred and hybrid mice and their offspring

A) All loci on homologous chromosomes are homozygous in B6 females and heterozygous at all loci in B6D2F1 hybrid males as the B6D2F1 hybrid males inherited only B alleles on the maternal homologue and only D alleles on the paternal homologue. B) Depending on the paternal homologue the BXB₁ male offspring inherited (either B or D alleles), the males are either homozygous or heterozygous at all loci, respectively. Whether each locus will be homozygous (~50%) or heterozygous (~50%) will be random. Depicted are only three potential examples of hypothetical pairs of chromosomes for three different BXB₁ male offspring as each locus will randomly be either homozygous or heterozygous. We propose that the chromosomal loci pattern important for steroid-independent male sexual behavior (i.e., those that are heterozygous for the candidate genes that play a role in steroid-independent male sexual behavior) is passed down from the B6D2F1 hybrid sire to the BXB₁ male offspring

Table 1

Litter composition (mean $\pm$ SEM) of the cross between female B6 and male B6D2F1 mice. There were no differences in litter size, average number of maters, or the number of male or female offspring between maters and non-maters.

Female B6 X Male B6D2F1	Maters (n=8)	SEM	Non-maters (n=7)	SEM
Average Litter Size	6.4	0.5	5.7	1.0
Average # of males	3.0	0.6	2.0	0.5
Average # of females	3.4	0.6	3.7	0.7
Average # of Maters	0.9	0.2	0.4	0.4

Table 2

Proportions of BXB_1 male offspring that were maters and non-maters. There were no significant differences in the number of maters sired between the male B6D2F1 hybrids that were maters or non-maters.

BXB_1 Male offspring	<i>n</i>	%	BXB_1 Maters	BXB_1 Non-maters
Sired from Mater	25	68%	7 (19% of total)	18 (49% of total)
Sired from Non-mater	12	32%	3 (8% of total)	9 (24% of total)

Table 3

Mean $\pm$ SEM of ejaculation (EL), intromission (IL), and mount (ML) latencies of B6D2F1 hybrid male maters and their offspring on the last session testing for male sexual behavior. There were no statistical differences in ejaculation, intromission, or mount latencies between the male offspring maters that were sired from either B6D2F1 hybrid maters or non-maters.

	EL	SEM	IL	SEM	ML	SEM
B6D2F1 hybrid males ($n=8$)	1317.1	268.3	1328.5	384.2	646.6	389.3
BXB ₁ male offspring from maters ($n=8$)	2654.8	847.4	546.1	298.4	1392	782.7
BXB ₁ male offspring from non-mater ($n=3$)	1755.3	743.7	1818	536.6	428	115.1