

ORIGINAL ARTICLE

Genetic loss of diazepam binding inhibitor in mice impairs social interest

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Neuropsychiatric disorders in which reduced social interest is a common symptom, such as autism, depression, and anxiety, are frequently associated with genetic mutations affecting γ -aminobutyric acid (GABA)ergic transmission. Benzodiazepine treatment, acting via GABA type-A receptors, improves social interaction in male mouse models with autism-like features. The protein diazepam binding inhibitor (DBI) can act as an endogenous benzodiazepine, but a role for DBI in social behavior has not been described. Here, we investigated the role of DBI in the social interest and recognition behavior of mice. The responses of DBI wild-type and knockout male and female mice to ovariectomized female wild-type mice (a neutral social stimulus) were evaluated in a habituation/dishabituation task. Both male and female knockout mice exhibited reduced social interest, and DBI knockout mice lacked the sex difference in social interest levels observed in wild-type mice, in which males showed higher social interest levels than females. The ability to discriminate between familiar and novel stimulus mice (social recognition) was not impaired in DBI-deficient mice of either sex. DBI knockouts could learn a rotarod motor task, and could discriminate between social and nonsocial odors. Both sexes of DBI knockout mice showed increased repetitive grooming behavior, but not in a manner that would account for the decrease in social investigation time. Genetic loss of DBI did not alter seminal vesicle weight, indicating that the social interest phenotype of males lacking DBI is not due to reduced circulating testosterone. Together, these studies show a novel role of DBI in driving social interest and motivation.

KEYWORDS

diazepam binding inhibitor, GABA, knockout mice, odor discrimination, repetitive grooming, rotarod, sex differences, social interest, social recognition, testosterone

1 | INTRODUCTION

Social interaction and recognition are complex fundamental processes that enable communication, both verbal and nonverbal, with others of the same species. These interactions are facilitated by forming social relationships and are reliant upon an organism's ability to establish social memory of conspecifics.^{1,2} Importantly, mental health disorders that are commonly characterized by altered social interest and motivation, such as autism, depression and anxiety, show prominent sex differences.^{3,4} In rodents, males typically display higher levels of social interest compared with females, spotlighting sex differences that may naturally exist in social investigation and behavior.⁵⁻¹⁰

Autism, depression, and anxiety are also highly associated with genetic mutations affecting γ -aminobutyric acid (GABA) synaptic transmission and GABA type-A receptors (GABA_ARs).¹¹⁻¹³ Mice with reduced expression of glutamic acid decarboxylase 67, a key enzyme mediating GABA synthesis, display reduced sociability and impaired processing of both social and nonsocial odors.¹⁴ Furthermore, low-dose benzodiazepine treatment, acting via GABA_ARs, can improve social interaction in male mouse models with autism-like features.^{15,16} Conversely, acute administration of the benzodiazepine binding site antagonist flumazenil can decrease social interaction between male rats,¹⁷ although increased social investigation in aggressive mice treated with flumazenil has also been observed.¹⁸ Together, these findings indicate that endogenous benzodiazepine

(endozepine) actions may modulate sociability. Diazepam binding inhibitor (DBI), also known as acyl-CoA binding protein, is a 10-kDa protein initially identified through its ability to displace diazepam from GABA_AR benzodiazepine binding sites.¹⁹ High concentrations of *Dbi* mRNA expression or DBI protein immunoreactivity have been shown in several brain regions.^{20–22} Our recent work indicated that DBI acts as a positive allosteric endozepine by potentiating GABA_AR-mediated inhibitory synaptic currents in the thalamic reticular nucleus,^{23,24} although negative allosteric actions of DBI on GABA_ARs have also been described.^{25–27} DBI also plays a critical role in lipid metabolism²⁸ and the *Dbi* gene may serve ubiquitous housekeeping functions,²⁹ suggesting multiple pathways by which DBI can affect neural circuits and behavior.

Although precise roles for endogenous DBI actions in various behaviors remain unclear, both clinical and animal studies support roles for DBI in modulating behaviors in both normal and pathological states. DBI levels in cerebrospinal fluid are elevated in patients with depression³⁰ and dementia,³¹ suggesting the possibility of a role of DBI in various cognitive impairments. In addition, a DBI-overexpressing transgenic mouse displays impaired hippocampus-dependent learning and memory.³² DBI may also play roles in contextual fear conditioning and conditioned place preference behaviors.^{33,34} Central administration of DBI or a smaller cleavage product, octadecaneuropeptide, increases aggression in mice^{21,35} and anxiety in both rats and mice.^{19,36} A recent study, however, indicated that genetic loss of DBI does not alter performance in assays of anxiety-like behavior in mice such as the elevated plus maze or the open field test.³⁷ Furthermore, social isolation decreases *Dbi* mRNA expression in the mouse hypothalamus.³⁸ A role for DBI in modulating social investigation behavior, however, has not been explored.

The goal of this study was to evaluate the effects of genetic loss of DBI signaling on social interactions in mice. We hypothesized that the absence of DBI signaling by genetic knockout would lead to dysfunctional social behavior when compared with wild-type mice of the same sex. We utilized a habituation/dishabituation test to investigate both social interest and social recognition behavior, and employed other assessments of odor discrimination, motor function and repetitive grooming behavior. Our results show a role for DBI in boosting social interest levels in mice, with stronger effects in males compared with females.

2 | MATERIALS AND METHODS

2.1 | Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. DBI heterozygous (DBI^{+/-}) and homozygous knockout (DBI^{-/-}) founder mice on the C57BL/6BomTac background, with targeted deletion of exon 2 of the *Dbi* gene on chromosome 1, were obtained from Dr. Susanne Mandrup (University of Southern Denmark). The production of these mice by the Mandrup laboratory was described previously.³⁹ At the University of Illinois, embryos generated from crossing DBI^{+/-} and DBI^{-/-} males with C57BL/6J females (Jackson

Laboratory, Bar Harbor, Maine) were surgically transferred to pathogen-free Swiss Webster females (Envigo, East Millstone, New Jersey) and carried to term to re-derive the colony. Re-derivation was performed by the Transgenic Mouse Facility of the Roy J. Carver Biotechnology Center (Fuming Pan, Director). Re-derived DBI^{+/-} male mice were backcrossed with C57BL/6J females for 3 more generations in our colony. The number of backcrossing generations was determined in consultation with Jackson Laboratories based on the strong genetic similarity between the C57BL/6BomTac and C57BL/6J strains. Breeding pairs to generate experimental mice consisted of DBI^{+/-} females crossed with DBI^{+/-} males, yielding DBI^{+/+}, DBI^{+/-} and DBI^{-/-} pups as previously described.³⁹ Mice used in the present experiments were produced in the first to fourth filial generations of this colony after backcrossing was completed. Mice were bred and housed on a 14:10 light:dark cycle with food and water available ad libitum. At weaning, mice were group housed (up to 5 mice per cage) with littermates of the same sex. A total of 39 mice were used in these studies, consisting of 19 males (DBI^{+/+} *n* = 11; DBI^{-/-} *n* = 8) and 20 females (DBI^{+/+} *n* = 11; DBI^{-/-} *n* = 9). Mice were 50 to 250 days old at the time of testing. No significant correlation was seen between age and performance on the conducted tests. In addition, mice were weighed at the time of social testing (described in further detail below); body weights of DBI^{+/+} and DBI^{-/-} mice of the same sex were not different (*P* > .4).

Stimulus animals for the social habituation/dishabituation test were 2 ovariectomized (OVX) female wild-type mice. OVX surgery was performed under isoflurane (Clipper Distributing Company, St. Joseph, Missouri) inhalation anesthesia. Bupivacaine (0.25%, 7 μ L; Hospira, Lake Forest, Illinois) was infiltrated into each surgical site to provide long-acting postoperative analgesia. The same 2 OVX females were used as stimulus mice in all social habituation/dishabituation tests in this study and housed together in the same cage in a different room than the DBI^{+/+} and DBI^{-/-} test mice.

Behavioral experiments were performed between 10 AM and 2 PM, relative to 7 PM lights-off, in accordance with previous findings that testing mouse social behaviors in a novel environment during the light phase does not significantly compromise results.⁴⁰ All mice were tested on all behavioral tests described, performed in the following order: (1) rotarod; (2) social habituation/dishabituation and (3) odor discrimination, with 1 to 70 days in between tests. Mean intertest intervals were not different between groups. Although estrous cycle stage was not assessed in female mice on days of testing, the variance in each test was comparable between males and females. Therefore, it is unlikely that estrous cycle stage influenced performance in the assessed tasks.

2.2 | Rotarod test

Rotarod testing was performed using published procedures.⁴¹ Mice were placed on the rotarod apparatus starting at 0 rotations per minute (rpm). Up to 4 mice were tested simultaneously. Once all mice were placed on dowel, the start switch was turned on to rotate the dowel at a constant acceleration rate (30 rpm). The latency of the mice to fall off was recorded by photobeam counters. As a backup, the time of falling was also noted by an experimenter using a

stopwatch. For analysis, the stopwatch values were only used in the small number of cases in which the photobeam failed to register a fall time. Each mouse underwent 4 trials per day for 4 consecutive days. Between each trial, the dowel was wiped with Clidox (Pharmaceutical Research Laboratories, Waterbury, Connecticut) and dried with a paper towel. Additionally, bedding in each chamber was replaced between testing of different mice to eliminate any residual olfactory cues and mouse droppings.

2.3 | Social habituation/dishabituation test

The following procedure was adapted from previous reports.^{10,42} On the day of testing, mice were brought to the testing room, weighed, and placed in a testing cage with fresh bedding to acclimate for 30 minutes. An inverted wire holding cell, cleaned with 70% ethanol prior to each testing period, was placed in the cage for 10 minutes, during which the test mouse could explore and habituate to the holding cell. A stimulus OVX female mouse was then placed in the holding cell for 1 minute (trial), and removed for the following 9 minutes (intertrial interval) before it was reintroduced. During the 1-minute trial, the test mouse could sniff the stimulus mouse through the wires, but physical contact was minimized to nose-to-nose touching. This procedure was repeated until the test mouse had experienced 8 trials with the same stimulus mouse. For the ninth trial, a novel stimulus OVX mouse was placed in the holding cell for 1 minute and then removed.

Trials 1 to 8 represent the habituation portion of the experiment, evaluating interest in a familiar stimulus over time. Trial 9 represents the dishabituation portion of the experiment, as the recognition of a novel mouse highlights the ability of the test mouse to discriminate between old and new social stimuli.¹ Each 90-minute testing period was recorded in 10-minute video clips, with each clip representing a trial and its subsequent intertrial period. The amount of time spent sniffing the stimulus mouse was scored from the video clips. Sniffing was defined as direct contact of the subject's nose with the wire holding cage or stimulus animal. For analysis, all clip identities were coded and randomized, and scorers were blind to trial number, genotype and sex.

2.4 | Odor discrimination test

The following procedure was adapted from previous reports.^{43,44} All odors were freshly prepared on the day of testing. Two nonsocial odors were prepared using a 1:100 dilution of almond or banana extract (McCormick and Co., Sparks, Maryland) in distilled water, and were stored in 15 mL conical tubes. A third nonsocial odor contained only water. Social odors were prepared using 2 cages containing the same sex and number of mice as the test mouse's home cage. Cages from which social odor would be obtained were not cleaned for 3 days prior to the experiment to allow for sufficient odor accumulation. Three cotton swabs were wiped across the bottom of each designated cage in a zigzag fashion and stored in 2 large glass jars, one for each set of social odors. Between each test, the jars containing social odors were washed with laboratory detergent to eliminate any residual odors from previous trials.

Mice were brought to the testing room, weighed and placed into a testing cage with fresh bedding to acclimate for 45 minutes. For testing, cotton swabs containing either nonsocial (50 μ L of water or diluted almond/banana odorant) or social odors were inserted through the water bottle opening of the wire cage lid until ~2.5 cm of the cotton end extended into the cage. Mice were exposed to the odor for 2-minute trials with a 1-minute intertrial interval between each presentation of the subsequent odor. This procedure continued until each odor was presented 3 times, in the following order: water, almond, banana, social 1 and social 2. All tests were video recorded, and sniffing behavior was quantified post hoc. Sniffing behavior was defined as orientation of the test animal toward the cotton swab with its nose ~2 cm or closer to the tip of the swab. Video scorers were blinded to sex and genotype of the test mice.

2.5 | Evaluation of repetitive self-grooming

Repetitive self-grooming was quantified using the video clips from the social habituation/dishabituation test, and time spent grooming repetitively was measured using the 9-minute intertrial intervals, when test mice were undisturbed. Repetitive grooming was identified as a period ≥ 10 second spent grooming with no more than a 5-second interval between grooming spurts. These criteria were chosen based on average grooming times previously exhibited in C57BL/6J mice.⁴⁵ The intertrial periods following social trials 1, 4 and 8 were quantified to represent early, middle and late time-points in the habituation/dishabituation test. Video scorers were blinded to sex and genotype of test mice and to the identity of the intertrial interval.

2.6 | Seminal vesicle dissection and measurement

To assess endocrine status of male mice, body weight was measured and seminal vesicles dissected and immediately weighed at the time of euthanasia. For euthanasia, mice were sedated with pentobarbital (50 mg/kg i.p.) and decapitated. Seminal vesicle weight values were normalized to body weight for each mouse.

2.7 | Statistical analysis

Statistical analysis was performed using SAS version 9.4. Latency to fall off the rotarod (averaged across the 4 trials per day) over 4 consecutive days was analyzed using repeated measures analysis of variance (ANOVA) with day entered as a within-subjects factor, and sex and genotype as between-subjects factors. Duration of social interest was analyzed the same way over the 9 trials (familiar and novel mouse), and separately for only the first 8 trials (familiar mouse). Social recognition was analyzed similarly except including only the eighth and ninth trials, to measure the increase in interest from familiar to novel mouse. Total duration of grooming behavior was analyzed a similar way over the 3 intertrial periods examined. To determine if the animals can discriminate social from nonsocial odors, duration spent sniffing in the first trial of social odor 1 was compared with the first trial of the final nonsocial odor (banana) by entering the 2 trials as within-subject factors, and including sex and genotype as factors as in the previous analyses. The animals' responses to each of the

social odors were analyzed separately entering trial (1-3) as the within-subject factor, and sex and genotype as factors following the other analyses. In cases where sex or the interaction between sex and the other variables were significant, these ANOVAs were followed by 2-way repeated measures ANOVAs (with trial or day and genotype as factors) separated for each sex. Tukey post hoc tests were used for pair-wise comparisons of means. A Student's *t* test was used to compare seminal vesicle weight values normalized to body weight in males. In all parametric analyses, if skewness was outside the range of -1 to 1, the data were power or log transformed depending on which method produced skewness nearest zero, to improve the normality assumption. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Genetic loss of DBI does not impair rotarod learning

DBI^{+/+} and DBI^{-/-} mice were first tested using the rotarod test to assess gross motor ability and memory retention (Figure 1). Overall analysis of the rotarod data showed no significant effect of sex or an interaction between sex and genotype, indicating that both sexes performed equally well on the task. Collapsed across sex, both DBI^{+/+} and DBI^{-/-} mice displayed significantly improved performance across days as indicated by a significant main effect of day ($F_{3,105} = 34.84$, $P < .0001$), suggesting that both genotypes learned the task. No main effect of genotype was detected. However, a significant interaction between genotype and day was detected ($F_{3,105} = 5.70$, $P = .0012$), indicating that performance between DBI^{+/+} and DBI^{-/-} mice was different across days. Tukey post hoc tests showed that the 2 genotypes displayed similar performances on days 1 to 3, but DBI^{-/-} mice displayed inferior performance on day 4 compared with DBI^{+/+} mice ($P = .0028$). This result suggests that DBI^{-/-} mice do not reach as high a performance level as DBI^{+/+} mice, but do retain memory of the task and do not revert to initial performance levels. Overall, these data indicate that the genetic loss of DBI does not impair motor learning, and show comparable initial motor performance by DBI^{+/+} and DBI^{-/-} mice.

3.2 | Genetic loss of DBI reduces social interest in both males and females

To investigate the impact of a global genetic deletion of DBI on social investigation behavior, we tested DBI^{+/+} and DBI^{-/-} mice on a social habituation/dishabituation assay. The ability of the mice to habituate to a familiar conspecific and dishabituate to a novel conspecific reflects levels of social interest and social recognition, respectively. OVX females were used to provide neutral social stimuli and minimize sexual or aggressive responses. Overall analysis showed a significant main effect of sex ($F_{1,36} = 10.8$, $P = .002$) and an interaction between sex and genotype ($F_{1,36} = 5.1$, $P = .03$), indicating a difference in social interest levels between males and females (Figure 2). Therefore, males and females were analyzed separately. DBI^{-/-} male

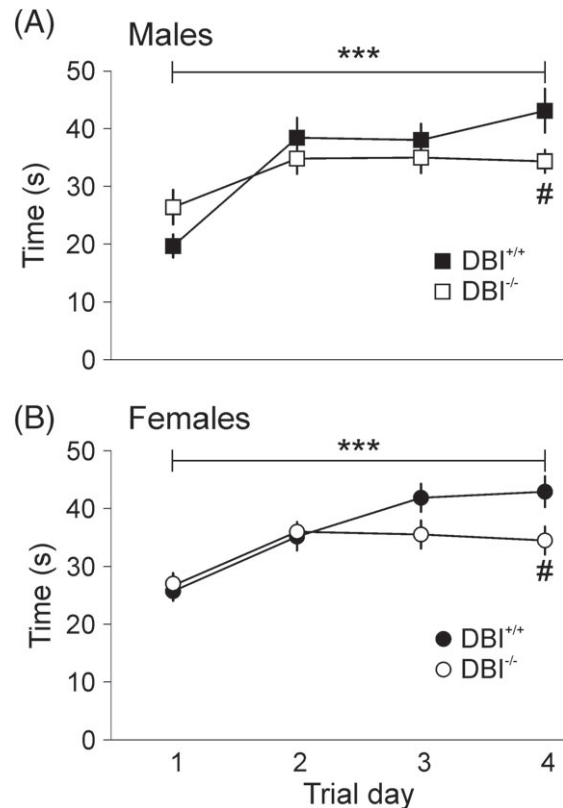


FIGURE 1 DBI^{+/+} and DBI^{-/-} mice display similar gross motor coordination and learning of the rotarod task. Latency to fall(s) off the accelerating rotarod over the course of 4 days (each day the average of 4 trials). A, Mean \pm SEM of latency to fall in DBI^{+/+} (filled squares) and DBI^{-/-} (open squares) males. B, Mean \pm SEM of latency to fall in DBI^{+/+} (filled circles) and DBI^{-/-} (open circles) females. *** Main effect of day indicating significantly improved performance in both groups ($P < .001$); # Difference between DBI^{+/+} and DBI^{-/-} mice of the same sex on day 4 ($P < .05$)

mice spent significantly less time investigating both familiar and novel OVX mice compared with DBI^{+/+} males (Figure 2A,C). We found a significant effect of trial ($F_{8,136} = 17.9$, $P < .0001$) and genotype ($F_{1,18} = 16.5$, $P = .0007$), but no interaction between them. Thus, the social interest of male DBI^{-/-} mice was significantly and equally impaired across all trials compared with DBI^{+/+} mice. DBI^{-/-} female mice also exhibited a reduction in social interest compared with DBI^{+/+} females (Figure 2B). Because the female data were positively skewed, we used a square root transformation to normalize the data. We found a significant effect of trial ($F_{8,143} = 28.1$, $P < .00001$), genotype ($F_{1,18} = 4.80$, $P = .04$), and trial-by-genotype interaction ($F_{8,143} = 3.9$, $P = .0004$), indicating that female DBI^{-/-} mice were impaired on specific trials compared with DBI^{+/+} females. Tukey post hoc analysis showed a significant difference between genotypes on trials 4, 6 and 7 (all $P < .05$), but not on other trials. Taken together, these results indicate a role for DBI signaling in modulating the motivation for social investigation in both males and females.

Analysis of total time spent investigating the familiar mouse in trials 1 to 8 showed that social interest levels in DBI^{-/-} males and females of both genotypes were reduced compared with DBI^{+/+} males (Figure 2C). Specifically, there was a significant effect of genotype ($F_{1,36} = 16.1$, $P < .001$) and sex ($F_{1,36} = 14.6$, $P < .001$) in the 2-

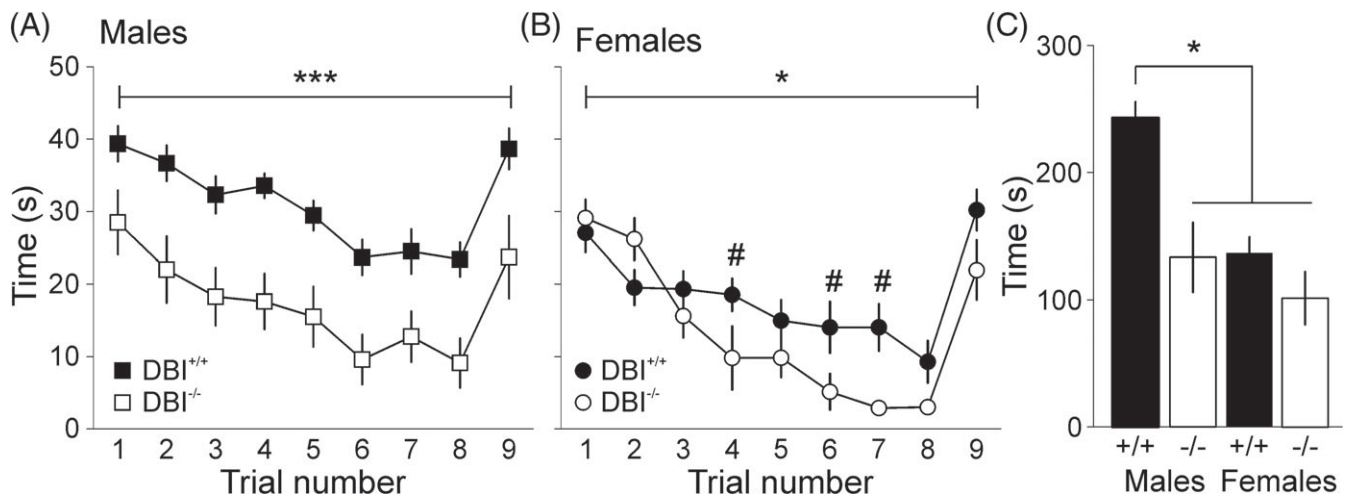


FIGURE 2 $DBI^{-/-}$ mice of both sexes display impaired performance in the social habituation/dishabituation test. A, Mean \pm SEM for time (s) spent investigating an OVX female by adult $DBI^{+/+}$ (filled squares) and $DBI^{-/-}$ (open squares) males across all trials. Trials 1 to 8 represent repeated presentation of the same stimulus mouse; trial 9 represents introduction of a novel stimulus mouse. B, Mean \pm SEM for time(s) spent investigating an OVX female by adult $DBI^{+/+}$ (filled circles) and $DBI^{-/-}$ (open circles) females across all trials. C, Total time spent by $DBI^{+/+}$ (filled bars) and $DBI^{-/-}$ (open bars) mice investigating the same stimulus mouse through the first 8 trials. ***A, B: Significant difference between $DBI^{+/+}$ and $DBI^{-/-}$ mice across the task ($P < .001$ males, $P < .05$ females). Note that for males the difference is seen in all trials. * C: Significant difference between $DBI^{+/+}$ males and other 3 groups in overall time spent investigating across trials 1 to 8 ($P < .05$). # Significant difference between $DBI^{+/+}$ and $DBI^{-/-}$ females on specific trials

way ANOVA, and Tukey post hoc tests showed that $DBI^{+/+}$ males performed better than the other 3 groups (all $P < .05$). Note that $DBI^{-/-}$ males displayed a level of social interest that was not different from $DBI^{+/+}$ or $DBI^{-/-}$ females, and that $DBI^{+/+}$ and $DBI^{-/-}$ females were not different from each other. These data suggest that the impact of a genetic loss of DBI on social interest is more severe in males than in females, and that $DBI^{-/-}$ mice lack the sex difference in social interest levels typical of $DBI^{+/+}$ mice.

3.3 | Genetic loss of DBI does not impair social recognition in either sex

To determine whether genetic loss of DBI impairs social recognition, we analyzed the ability of mice to dishabituate from a familiar stimulus mouse to a novel mouse. We compared the difference in time spent sniffing the stimulus mouse in trial 8 compared with trial 9, which corresponded to the last exposure to the familiar stimulus mouse and the first exposure to the novel stimulus mouse, respectively. All mice displayed dishabituation from the familiar stimulus mouse to the novel stimulus mouse (Figure 2A,B). Collapsed across genotypes, there was a significant effect of sex ($F_{1,36} = 5.5$, $P = .03$), trial ($F_{1,34} = 82.9$, $P < .0001$), and marginally nonsignificant interaction of sex and trial ($F_{1,34} = 2.8$, $P = .10$), indicating altered performance between males and females from trials 8 to 9. Due to this sex

difference, males and females were analyzed separately. In males, there was a significant effect of trial ($F_{1,17} = 34.3$, $P < .0001$) and genotype ($F_{1,18} = 13.1$, $P = .002$), but no interaction between them. In females, significant trial ($F_{1,17} = 64.7$, $P < .0001$) and genotype ($F_{1,18} = 4.4$, $P = .05$) effects were observed, but no interaction effect. The lack of an interaction effect in either males or females indicates that both $DBI^{+/+}$ and $DBI^{-/-}$ mice responded similarly to the introduction of a new stimulus mouse, and investigation time by $DBI^{-/-}$ mice was reduced compared with $DBI^{+/+}$ mice of the same sex. Furthermore, all groups showed similar values for the difference in investigation time in trial 9 compared with trial 8 (Table 1). Specifically, 2-way ANOVA showed no significant effect of genotype ($F_{1,36} = 0.12$, $P = .73$), sex ($F_{1,36} = 1.71$, $P = .2$) or interaction between sex and genotype ($F_{1,36} = 0.03$, $P = .87$). Taken together, these data suggest that $DBI^{-/-}$ mice of both sexes retain social recognition abilities despite a reduction in social interest.

3.4 | Increased levels of repetitive self-grooming in both male and female $DBI^{-/-}$ mice

A distinctive characteristic of $DBI^{-/-}$ mice is a fur phenotype that is identifiable by weaning.⁴⁶ The mice are oily to the touch and develop a reddish hue along with patchy alopecia. It is possible that $DBI^{-/-}$ mice may compensate for this fur condition with an increase in

TABLE 1 Difference in investigation time between trials 8 and 9 in the social habituation/dishabituation task

	Males		Females	
	$DBI^{+/+}$	$DBI^{-/-}$	$DBI^{+/+}$	$DBI^{-/-}$
Mean \pm SEM, difference in investigation time, trial 9 vs trial 8	15.25 \pm 2.33	14.59 \pm 4.84	20.92 \pm 4.09	18.94 \pm 4.00

Mean \pm SEM time spent(s) investigating novel stimulus mouse (trial 9) compared with familiar stimulus mouse (trial 8). No differences were observed between $DBI^{+/+}$ and $DBI^{-/-}$ mice of either sex.

grooming behavior, thus potentially interfering with their ability to engage in social behavior. To account for this, we evaluated the repetitive grooming behavior of the mice as they performed the social habituation/dishabituation task, focusing on the intertrial periods following trials 1, 4 and 8 to quantify grooming across early, middle, and late portions of the task (Figure 3). Because overall analysis of the data showed large differences in the variances between groups, the data were transformed by adding the value 1 to the response and then taking the log base 10 to meet the equal variance assumption. Analysis of the transformed data showed a significant interaction between sex, genotype and trial ($F_{2,70} = 4.0$, $P = .02$); therefore, the data were analyzed separately by sex. In males, there was a significant effect of trial ($F_{2,34} = 5.8$, $P = .007$) and genotype ($F_{1,17} = 32.2$, $P < .0001$) but no interaction between them, indicating that $DBI^{-/-}$ mice showed higher levels of grooming at all trial points. In females, there was a significant effect of trial ($F_{2,36} = 6.1$, $P = .005$), genotype ($F_{1,18} = 72.7$, $P < .0001$) and an interaction of trial and genotype ($F_{2,36} = 8.9$, $P < .0001$), suggesting differences in repetitive grooming between genotypes during certain trials. Tukey post hoc analysis showed that $DBI^{-/-}$ females were different from $DBI^{+/+}$ females during trials 1 and 4 (both $P < .0001$), but not trial 8 ($P > .05$). Although the loss of DBI appears to induce a more severe reduction of social interest in male mice, both male and female mice exhibit increased levels of repetitive grooming. Therefore, it is unlikely that the increase in repetitive grooming behavior alone can account for the DBI-mediated impairment in social interest.

3.5 | $DBI^{-/-}$ mice can discriminate social vs nonsocial odors

The reduced degree of social interest displayed by the male $DBI^{-/-}$ mice could reflect an impaired receptivity to social odors. To test for this possibility, we evaluated the same cohort of mice on an odor discrimination task (Figure 4). To determine if the animals can discriminate social from nonsocial odors, the time spent sniffing in the first trial of social odor 1 was compared with the first trial of the final nonsocial odor (banana), such that both the social and nonsocial odors were novel to the mouse. The analysis showed a highly significant difference between the responses to the 2 odors ($F_{1,34} = 62.4$, $P < .0001$), showing that the animals were more interested in the social odors than the nonsocial odors. No significant effects of sex, genotype or interactions between sex and genotype were detected, indicating that all mice showed a preference for the social odor regardless of sex or genotype.

The animals' responses to each of the social odors were analyzed separately. Across the 3 trials for social odor 1, we found a significant effect of trial ($F_{2,68} = 14.3$, $P < .0001$), indicating that the mice habituated to the odor. Although the effect of the sex-by-genotype interaction was not significant ($F_{1,34} = 3.3$, $P = .08$), it was close enough to justify separating the analysis by sex. In males, there was a significant effect of trial ($F_{2,32} = 3.8$, $P = .03$), but not genotype, and no interaction of genotype and trial. Males thus showed habituation to social odor 1, but performance was not different between $DBI^{+/+}$ mice and $DBI^{-/-}$ mice. In females, there was a significant effect of trial ($F_{2,36} = 15.8$, $P < .0001$) and genotype ($F_{1,18} = 4.7$, $P = .04$),

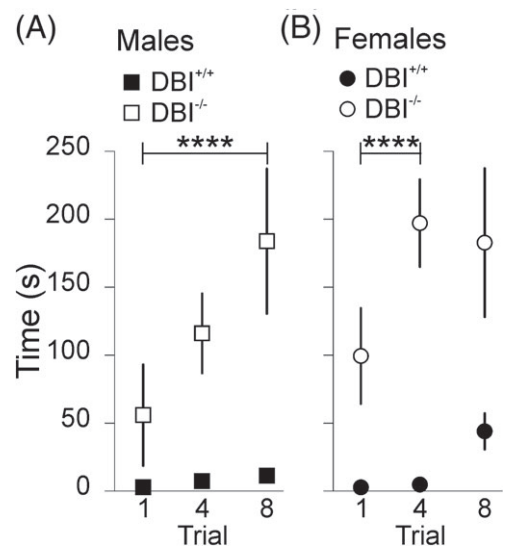


FIGURE 3 $DBI^{-/-}$ mice display increased time spent in repetitive grooming compared with $DBI^{+/+}$ mice. Mean \pm SEM for time(s) spent in repetitive grooming behavior by males (A, $DBI^{+/+}$ filled squares, $DBI^{-/-}$ open squares) and females (B, $DBI^{+/+}$ filled circles, $DBI^{-/-}$ open circles). **** Significant difference between $DBI^{+/+}$ and $DBI^{-/-}$ mice within each sex across all trials (males) and trials 1 and 4 (females) ($P < .0001$)

indicating that both genotypes habituated to social odor 1, but that $DBI^{-/-}$ females spent less time investigating the odor than $DBI^{+/+}$ females. No interaction between trial and genotype was detected in females. For social odor 2, the data were positively skewed, and therefore log transformed to improve normality. Analysis of the transformed data showed a significant effect of trial ($F_{2,68} = 9.6$, $P = .0002$) and genotype ($F_{1,34} = 6.4$, $P = .02$). The effect of sex and the interactions were not significant. This result indicates that both males and females habituated to social odor 2, and that $DBI^{-/-}$ mice of both sexes spent less time investigating the odor than $DBI^{+/+}$ mice.

As with social odor 2, responses to all 3 of the nonsocial odors were positively skewed and required a log transformation to normalize the data. For each of the nonsocial odors, there was a significant effect of trial (water: $F_{2,68} = 25.0$, $P < .0001$; almond: $F_{2,68} = 33.3$, $P < .0001$; banana: $F_{2,68} = 18.6$, $P < .0001$), indicating that all mice showed habituation to the nonsocial odors regardless of genotype. In the water trials, an effect of genotype was also observed ($F_{1,34} = 8.6$, $P = .006$), with $DBI^{-/-}$ mice displaying less interest in the cotton swab compared with $DBI^{+/+}$ mice. In the almond trials, we found an effect of genotype ($F_{1,34} = 7.5$, $P = .01$) and a genotype-by-sex interaction ($F_{1,34} = 4.7$, $P = .04$), indicating that males and females should be analyzed separately. When separated by sex, there was a significant effect of genotype in males ($F_{1,16} = 11.4$, $P = .004$) but not in females. This result indicates that male $DBI^{-/-}$ mice spent less time investigating the almond odor than $DBI^{+/+}$ males, a genotype difference that is not seen in females. In the banana trials, a genotype effect was also found ($F_{1,34} = 6.8$, $P = .01$) but no effect of sex or any interactions, indicating that $DBI^{-/-}$ mice of both sexes were less interested in the banana odor than $DBI^{+/+}$ mice.

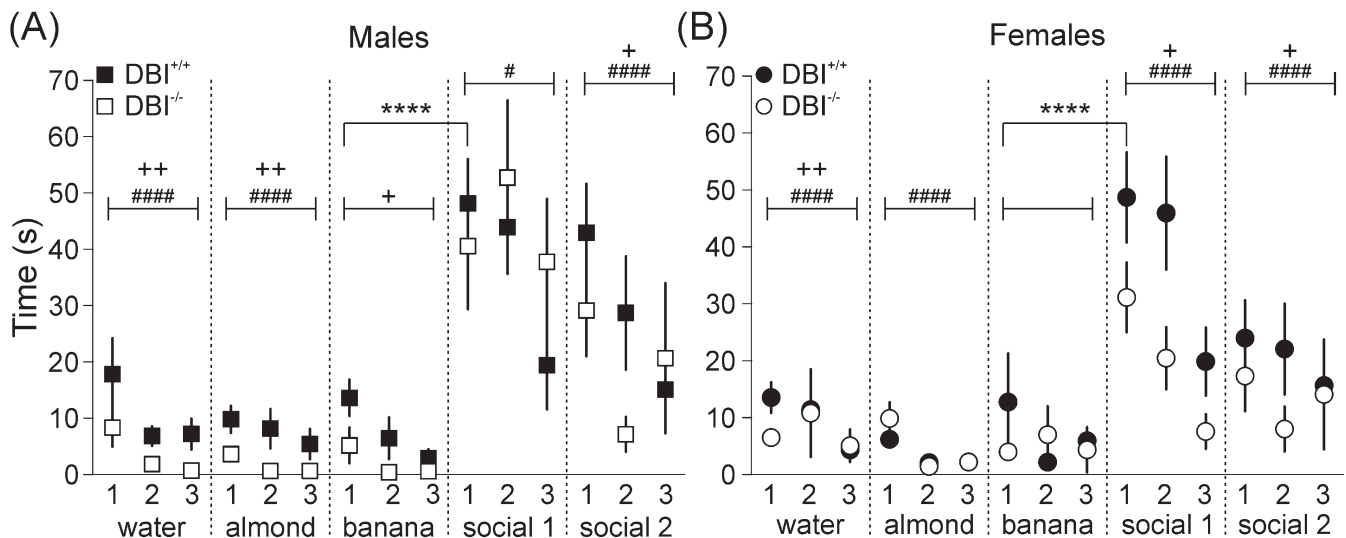


FIGURE 4 DBI^{+/+} and DBI^{-/-} mice can distinguish social vs nonsocial odors. Mean \pm SEM of time spent by males (A, DBI^{+/+} filled squares, DBI^{-/-} open squares) and females (B, DBI^{+/+} filled circles, DBI^{-/-} open circles) sniffing 3 nonsocial odors (water, almond, banana) and 2 social odors (swabs of used cages housing unfamiliar mice) during 3 consecutive presentations of each odor. ****Significant difference in comparison of last non-social odor (banana) to social odor 1 ($P < .0001$). # Significant effect of trial within the same odor ($\#P < .05$, #### $P < .0001$). + Significant effect of genotype within the same odor ($+P < .05$, ++ $P < .01$)

3.6 | Seminal vesicle weights are not changed in DBI^{-/-} males

Social behavior in male mice is modulated by testosterone feedback,^{10,47} and the social interest phenotype of DBI^{-/-} males is similar to that seen in castrated male wild-types.^{9,48} We thus postulated that the reduced social interest of DBI^{-/-} male mice may reflect lower circulating testosterone levels. To evaluate the endocrine status of DBI^{+/+} and DBI^{-/-} male mice, we measured the seminal vesicle weight normalized to overall body weight, a biomarker of peripheral androgen actions.^{49,50} Seminal vesicle weight was not different between the 2 genotypes (expressed as percentage of total body weight: DBI^{+/+} $0.89\% \pm 0.02\%$; DBI^{-/-} $0.83\% \pm 0.05\%$; $t(14) = 1.05$, $P = .31$). Therefore, the behavioral differences seen between these groups are unlikely to reflect lower circulating testosterone.

4 | DISCUSSION

In these studies, we sought to determine whether genetic loss of DBI affects social behavior in mice. Our data show an effect of genetic loss of DBI on social interest levels in mice, with a greater effect in males than in females. Moreover, we determined that DBI^{-/-} mice of either sex can competently discriminate social vs nonsocial odors, and that motor learning does not appear to be impaired by the genetic loss of DBI. These results suggest that the reduced social interest phenotype of DBI^{-/-} males is not caused by impaired olfactory or pheromonal receptivity of social cues, or altered motor function that would reduce the propensity of mice to move around the testing cage. These data also show that DBI^{-/-} mice of both sexes display increased repetitive grooming. The grooming phenotype, however, does not appear to be correlated to the degree of social interest; both male and female DBI^{-/-} mice displayed increased

baseline levels of grooming compared with respective DBI^{+/+} of the same sex when measured during the 9-minute period following the first presentation of a social stimulus mouse, a time point at which only DBI^{-/-} males displayed reduced social interest compared with wild-types. Furthermore, a lack of change in seminal vesicle weight between DBI^{+/+} and DBI^{-/-} males indicates that the differences in social interest between these genotypes are not reflective of lower circulating testosterone. Together, our results provide evidence of a role for DBI in modulating social behavior, and indicate a stronger phenotype in males compared with females.

DBI^{-/-} mice lack the typical sex difference in levels of social interest, suggesting that DBI may be involved in the development and/or maintenance of sexually dimorphic neural circuits mediating social behavior. In this regard, 2 neuropeptide systems are strong candidates as potential targets of DBI actions. The vasopressin system, which is integral in the regulation of social behavior,⁵¹ exhibits strong sexual dimorphism, with elevated vasopressin-immunoreactive fiber density in the lateral septum in male rats⁵² and mice⁵³ compared with female conspecifics. Furthermore, vasopressin V1b receptor knockout male mice show reduced interest in bedding soiled by either males or females.⁵⁴ The vasopressin system may thus be a target of DBI-dependent modulation of social behavior. Oxytocin is another neuropeptide that may be critically involved in these behaviors. Recent studies indicate sex-specific relationships of oxytocin receptor expression in the amygdala to social interest, with social interest levels of male rats and mice being positively correlated to expression in the medial amygdala, and social interest in female rats being negatively correlated to expression in the central amygdala.^{55,56} Therefore, it would be interesting to investigate the structural and functional impacts of genetic loss of DBI on circuitry of these vasopressin and oxytocin systems implicated in social behavior.

The recent findings that low-dose benzodiazepine treatment can improve social interest in male mice harboring genetic mutations associated with either Dravet syndrome or autism^{15,16} indicate that

positive allosteric modulation of GABA_ARs can promote social interest and motivation. The present results, showing reduced social interest with loss of DBI, are thus in agreement with a model of DBI acting (at least on balance) via positive allosteric GABA_AR modulation to boost social interest, and suggest a role for DBI endoepine actions in modulating social behavior. To date, the thalamic reticular nucleus remains the only brain area identified in which DBI is confirmed to act as a positive endoepine,^{23,24} but it is highly likely that there are other discrete sites in the brain in which DBI exerts similar actions, in addition to sites of negative GABA_AR modulation by DBI. Therefore, it will be interesting in the future to investigate region-specific and cell-type-specific actions of DBI, particularly in circuits involved in social motivation, to further elucidate the potential contributions of DBI acting as an endoepine in the modulation of these behaviors.

Social interest levels in wild-type male mice are reduced upon castration, and restored with testosterone replacement.^{9,10,48} Testosterone is converted in the brain to estradiol via aromatization or to nonaromatizable androgens via 5- α reduction,^{57,58} mediating effects by activation of estradiol and androgen receptors, respectively. Although the precise roles of estradiol and androgen receptors in mediating testosterone effects on social interest in mice are still a matter of some debate,^{10,47} the similar levels of social interest observed in DBI^{-/-} males and both DBI^{+/+} and DBI^{-/-} females suggested that the male-specific reduction in social interest may reflect lower circulating testosterone levels and/or altered neural response to testosterone feedback with the loss of DBI in males. The biosynthesis of steroid hormones may be upregulated by DBI acting as an endogenous ligand of the mitochondrial benzodiazepine receptor, now referred to as 18 kDa translocator protein (TSPO),^{59,60} although the role of TSPO activation in steroidogenesis has recently been called into question.⁶¹ Seminal vesicle weights, however, were not different in DBI^{-/-} males compared with DBI^{+/+}, suggesting that the reduction in social interest is not a proxy for altered testis production of testosterone. It is possible, however, that genetic loss of DBI alters neural expression of steroid conversion enzymes such as aromatase or 5- α reductase, and/or estrogen or androgen receptors, but this has not been tested. On the other hand, murine *Dbi* mRNA expression in the ependyma surrounding the third ventricle is reduced upon castration, and this effect is reversed by androgen treatment,⁶² indicating that neural *Dbi* gene expression is androgen-sensitive in mouse, as also shown in human and rat tissues.^{63,64} Our findings of reduced social interest but unchanged seminal vesicle weight in males with genetic loss of DBI suggest that DBI may act as an intermediary in the male brain to at least partially effect the actions of testosterone on social behavior.

The results of the odor discrimination task indicate that the ability to distinguish between social and nonsocial odors is largely intact in the genetic absence of DBI, although reduced investigation by DBI^{-/-} mice of swabs containing either social or nonsocial odors was also observed. DBI promotes early postnatal neurogenesis in the mouse olfactory bulb,²⁵ and neurogenesis in the adult olfactory bulb appears to be important for murine odor discrimination.^{65,66} These contributions of DBI to olfactory neurogenesis are thus likely related to the olfactory phenotype observed in the present studies. The

DBI^{-/-} mice, however, also displayed reduced interest in cotton swabs containing only water; this effect was more prominent in males than in females. Therefore, the reduced degree of investigation of the odorants may also simply reflect a lower level of motivation for investigating the cotton swabs as objects. In relation to the social interest phenotype, however, it is important to note that all mice tested showed a prominent increase in the amount of time investigating the first social odor compared with the last nonsocial odor (banana). Therefore, the ability to discriminate between social and nonsocial odors is intact in DBI^{-/-} mice of either sex, and the reduced degree of social interest observed in DBI^{-/-} mice does not reflect an inability to correctly perceive and investigate social odorants.

A secondary finding of this study is the presence of high levels of repetitive self-grooming behavior in the DBI^{-/-} mice. An increase in the amount of repetitive self-grooming is commonly used as a parameter of autistic-like behavior in mice.^{67,68} Presently, it is unclear whether the increase in repetitive grooming seen in DBI^{-/-} mice reflects autistic-like stereotyped behavior or if it is largely a byproduct of the skin and fur phenotype of these mice. Topical treatment with Vaseline or latex restores hepatic fat levels of DBI^{-/-} mice to DBI^{+/+} values,⁶⁹ indicating that amelioration of the effects of DBI deficiency in the skin can have robust effects in other tissues. It is probable, however, that the application of a topical ointment would itself induce increased grooming behavior. Alternatively, generation of mouse models with DBI deleted only in the nervous system would help to resolve whether DBI acts in the brain to drive repetitive grooming and/or other stereotyped behaviors.

In summary, our results indicate a novel role for DBI in boosting social interest. The effects of genetic loss of DBI on social interest do not appear to be a result of impaired olfactory discrimination, gross motor learning or movement ability, or a side effect of increased repetitive self-grooming. In addition, the apparent absence of a difference in circulating testosterone in males lacking DBI, coupled with previous research indicating androgen-sensitivity of DBI gene expression, suggests that DBI may act to mediate at least some effects of testosterone in the brain. This work thus provides a basis for further investigation of the mechanisms by which DBI acts to shape social behavior.

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