Age-differential sexual dimorphism in CHD8-S62X-mutant mouse behaviors

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Autism spectrum disorders (ASD) are ~4-times more common in males than females, and CHD8 (a chromatin remodeler)-related ASD shows a strong male bias (~4:1), although the underlying mechanism remains unclear. Chd8-mutant mice with a C-terminal protein-truncating mutation (N2373K) display male-preponderant behavioral deficits as juveniles and adults, although whether this also applies to other Chd8 mutations remains unknown. In addition, it remains unclear whether sexually dimorphic phenotypes in Chd8-mutant mice are differentially observed in males and females across different ages. We here generated new Chd8-mutant (knock-in) mice carrying a patient-derived mutation causing an N-terminal and stronger protein truncation (Chd8+/S62X mice) and characterized the mice by behavioral analyses. Juvenile Chd8+/S62X mice displayed male-preponderant autistic-like behaviors; hypoactivity and enhanced mother-seeking/attachment behavior in males but not in females. Adult male and female Chd8+/S62X mice showed largely similar deficits in repetitive and anxiety-like behavioral domains. Therefore, the CHD8-S62X mutation induces ASD-like behaviors in juvenile male mice and adult male and female mice, pointing to an age-differential sexual dimorphism and also distinct sexual dimorphisms in different Chd8 mutations (N2373K and S62X).

KEYWORDS
autism spectrum disorders, CHD8, mouse model of ASD, chromatin remodeling, sexual dimorphism, age dependence

Introduction

Autism spectrum disorders (ASD) are ~4-times more common in males than in females (Werling and Geschwind, 2013). This male–female difference depends on which ASD-risk genes are affected, with genes including CHD8, ANK2, ADNP, and TRIP12 showing a strong male bias (Stessman et al., 2017; Andreae and Basson, 2018). However, the underlying mechanisms remain unclear.

CHD8, a chromatin remodeler, is strongly associated with ASD, accounting for ~0.5% cases (Bernier et al., 2014; Barnard et al., 2015). Among ASD patients carrying CHD8
mutations, the male–female ratio is high (~85:15) (Stessman et al., 2017). Previous studies on Chd8-mutant mice have revealed various mechanisms underlying CHD8-related brain dysfunctions in ASD (Sugathana et al., 2014; Cotney et al., 2015; Wang et al., 2015; Breuss and Gleesoon, 2016; Durak et al., 2016; Katayama et al., 2016; Gompers et al., 2017; Platt et al., 2017; Andreae and Basson, 2018; Jung et al., 2018; Suetterlin et al., 2018; Wade et al., 2018; Xu et al., 2018; Zhao et al., 2018; Hulbert et al., 2020; Jimenez et al., 2020; Sood et al., 2020; Cherepanov et al., 2021; Ellingford et al., 2021; Hurley et al., 2021; Kawamura et al., 2021; Kweon et al., 2021; Weissberg and Elliott, 2021; Yu et al., 2022).

Our previous study reported that Chd8-mutant (knock-in) mice with a patient-derived, C-terminal protein-truncating mutation (N2373K) display male-preponderant behavioral deficits at pup, juvenile, and adult stages that are associated with altered neuronal, synaptic, and transcriptomic phenotypes (Andreae and Basson, 2018; Jung et al., 2018). In addition, female mice with the mutation display unique transcriptomic and synaptic/neuronal changes that are different from those in males. However, whether other CHD8 mutations induce similar sexually dimorphic phenotypes in mice remains largely unclear (Cherepanov et al., 2021; Yu et al., 2022), partly because male mice were mainly used for phenotypic analyses, or males and female mice were not separated in experimental mouse cohorts (Gompers et al., 2017; Suetterlin et al., 2018; Hulbert et al., 2020; Jimenez et al., 2020; Ellingford et al., 2021; Hurley et al., 2021; Kawamura et al., 2021).

In the present study, we generated new Chd8-mutant (knock-in) mice carrying a patient-derived mutation causing an N-terminal and stronger protein truncation (CHD8+S62X). Adult Chd8+S62X mice displayed behavioral deficits that are largely similar, but such deficits were more strongly observed in juvenile males but not females. These results suggest an age-dependent sexual dimorphism in Chd8+S62X mice.

**Materials and methods**

**Animals**

C57BL/6J mice with the S62X knock-in mutation in the Chd8 gene, flanked by loxP sites and a neomycin cassette, were designed and generated by Cyagen. The neomycin cassette was removed by crossing heterozygous (HT) mice with protamine-Flp mice. WT and HT mice were genotyped by PCR using the following primers: 5’-GTC AGC TAG CTC AGG CTG CT-3’ (forward), 5’-GTT ACA GCC GAG CAT GTG AT-3’ (reverse). Mice were maintained at the mouse facility of the Korea Advanced Institute of Science and Technology (KAIST) and were housed under a 12-h light–dark cycle with unlimited access to food and water.

**Brain weight and size**

Brains were dissected, placed in mouse brain matrices, and coronally cut below the cortex. After measuring the weight, brains were imaged by Gel Doc. A-P (anterior–posterior) length, cortical length, and cortical area were analyzed by ImageJ.

**Immunohistochemistry**

Mice were cardiac perfused with 4% paraformaldehyde (PFA), and brains were dissected. After storing the dissected brains in 4% PFA for more than 1 day, coronal sections (50 μm) were prepared using a vibratome (Leica). Brain slices were blocked with 5% normal donkey serum and 0.2% Triton X-100 for 1 h and incubated with primary antibodies (1:500 NeuN) overnight. After washing, slices were incubated with fluorophore-conjugated secondary antibodies (1,1,000) in phosphate-buffered saline with 0.2% Triton X-100. After washing, sections were mounted with Vectorshield (Vector Laboratory), and images were acquired using a slide scanner (Axio Scan Z1, Zeiss).

**Western blot**

Protein lysates from the whole brain were prepared and loaded into gels that were manually made. Proteins were transferred to the PVDF membrane, blocked with 5% skim milk, and stained overnight using CHD8 (Bethyl, A301-224A) and β-actin (Sigma A5316) antibodies. Membranes were washed and stained with HRP-conjugated secondary antibodies. Signals were quantified using Image Studio Lite.

**Behavioral assays**

Mice at P19–28 were used for juvenile behavioral experiments. For adult experiments, mice at the age of 9–16 weeks were used. Before the testing day, the mice were handled for 10 min for 3 days to minimize stress from human handling. Mice were placed in a dark test room for 30 min for habituation just before the behavioral experiments. All behavioral assays were performed during the light-off period when mice are active unless otherwise specified. All data were analyzed using EthoVision XT (Noldus) by an experimenter blind to the genotype unless otherwise specified. Behavioral assays were carried out in the order of increasing stress to minimize the effects of stress. Details of these assays are explained below in the order they were carried out.

**Open-field test**

Mice were placed in an open field box (40 × 40 × 40 cm), and the behaviors were recorded for 60 min for adult mice or 20 min for juvenile mice. The intensity of light in the box was set at 100 lux for adult mice and 15 lux for juvenile mice. The distance moved and time spent in the center region of the open-field box (20 × 20 cm) were measured as a measure of anxiety-like behavior.
Repetitive behaviors

To measure the duration of self-grooming and digging, representing repetitive behaviors, mice were placed in a new home cage with fresh bedding, and their behaviors were recorded for 30 min. Mice spent much more time digging than self-grooming. To measure the duration of self-grooming without being confounded by digging behavior, mice were placed in a specially designed chamber (15 × 15 × 40 cm), and their behaviors were recorded for 10 min. Light intensity in the cage was set to 50 lux to minimize light-induced anxiety. The first 10 min was considered as a habituation period, and self-grooming and digging durations during the last 20 min of the recordings were quantified manually.

Light/dark test

Mice were placed in a box that contains a white open box conjoined to a closed black (dark) box. There was a small entrance to allow the mice to move freely between these two boxes. The light intensity in the light box was set to 300 lux to examine light-induced anxiety. Behaviors were recorded for 20 min, and the time spent in each box was measured.

Elevated plus-maze test

Mice were placed in the elevated plus-maze with two open arms and two closed arms (5 × 30 cm each, with 30 cm-tall walls in the closed arm). The maze was placed 50 cm above the ground. The intensity of light in the center region connected to all four arms was set to 200 lux. Times spent in closed and open arms and the total distance moved were measured.

Three-chamber test

For the three-chamber (Silverman et al., 2010) test, mice were isolated for 3 days before the experiment to avoid the effect of prior interactions. A mouse was placed in an empty three-chamber apparatus (40 × 60 cm) and allowed to explore the environment and habituate for 10 min. The behavior of the subject was recorded for 10 min. Following habituation, a small cage containing a stranger mouse (129/SvJae strain; S1) was placed in one of the corners. Another cage containing an object (O) was placed in the other corner of the apparatus, followed by recordings for 10 min. After 10 min, the object was replaced with another stranger mouse (S2), and the behaviors were recorded for 10 min. Time spent sniffing objects or unfamiliar mice was measured.

Dyadic social interaction

After 3-day social isolation, mice were habituated in the open-field box for 10 min a day before testing. On the test day, two mice with identical genotypes and sex that have not met each other before were placed in the open-field box, and their behaviors were recorded for 10 min. The duration of social interactions was quantified manually. Light intensity was set to 50 lux.

Juvenile play

Mice were isolated and habituated in their home cage with fresh bedding for 10 min a day before testing. On the test day, two mice with identical genotypes and sex that have not met each other before were placed in a new home cage with fresh bedding, and their behaviors were recorded for 10 min. Light intensity was set to 15 lux. The duration of social interactions was quantified manually.

Juvenile maternal homing

The juvenile maternal homing test was performed as previously described (Zhan et al., 2014). Mice at P19 were separated from their mothers at least 30 min before the test. The test consists of two phases; a nest-homing phase followed by a maternal homing phase. In the nest-homing phase, bedding materials from the home cage where the separated mouse used to spend time with the mother and siblings (Nest) and fresh bedding (New) were placed in the opposite corners of an open field box (40 × 40 × 40 cm). Mice were placed in one of the empty corners, and behaviors were recorded for 3 min. In the maternal homing phase, an empty chamber and a chamber containing the mother of the subject mouse were placed in the two remaining empty corners of the box. Mice were placed in the corner with home-cage beddings (Nest), and their behaviors were recorded for 5 min. Light intensity in the center of the box was set to 15 lux, and time spent at each corner was measured.

LABORAS analysis

The Laboratory Animal Behavior Observation Registration and Analysis System (LABORAS, Metris) is a system that allows the quantification of mouse behaviors over a long period of time (usually days) without perturbation by researchers (Quinn et al., 2003). The system consists of a cage placed above a vibration-sensitive platform and a connected program (LABORAS 2.6). Mice were individually placed in the apparatus, and their behaviors were recorded for 96h. The first 24h were considered as the time for habituation and were not analyzed. Data analysis was performed using LABORAS 2.6 program (Metris).

Ultrasonic vocalization

Male mice were placed in a home cage with an age-matched unfamiliar C57BL/6J female mouse. For pup
USV, pups were separated from their mothers and placed in a plastic container. Ultrasonic vocalizations (USVs) were recorded for 5 min using an ultrasound microphone (Avisoft) and Avisoft Recorder software. Recorded USVs were analyzed as previously described (Kim et al., 2018).

**Analysis and statistics**

All experiments were performed and analyzed by researchers blind to the genotype. Outliers were identified and excluded using ROUT test (Q = 1%). To compare male and female mouse
phenotypes, two-way ANOVA with sex and genotype as main variables was used. For two-way ANOVA, normality was assumed. Statistical tests were performed using Graphpad Prism 9 and SigmaPlot 12.0. Statistical details, including the sex, age, and number of mice, are described in Supplementary Table 1.

Results

Generation and basic characterization of male and female Chd8+/S62X mice

To test if a heterozygous (HT) CHD8-S62X mutation differentially induces autistic-like behaviors in male and female mice, we generated and characterized Chd8+/S62X mice, carrying a patient-derived N-terminal protein-truncating mutation (Figures 1A,B; Supplementary Figure 1; O’Roak et al., 2012). Male and female Chd8+/S62X mice, with CHD8 protein levels ~50% of wild-type (WT) mice, were born at normal Mendelian ratios and exhibited largely normal body weight, gross brain morphology, and brain size (Figures 1C–F).

Hypoactivity and enhanced mother-seeking/attachment behavior in male but not female Chd8+/S62X juveniles

Given that ASD is characterized by early symptomatic manifestations and Chd8+/N2373K mice (C-terminal truncation) showed autistic-like enhanced anxiety-like behaviors in pup and juvenile stages (Jung et al., 2018), we examined early behavioral deficits in Chd8+/S62X mice. Chd8+/S62X pups separated from their mothers emitted largely normal levels of USVs, except for a tendency for a moderate decrease in the latency to first call, compared with WT mice (Figures 2A–F; see Supplementary Table 1 for statistical details). Juvenile male and female Chd8+/S62X mice showed decreased levels of locomotor activity in the open-field test compared with...
WT mice, although center-zone activity was normal, as supported by a significant genotype difference (Figure 3A). However, the Mann–Whitney U test performed for the lack of the genotype-sex interaction, which does not allow multiple comparisons, revealed hypoactivity in males but not in females, although there was a tendency for hypoactivity in females. Male and female Chd8+/S62X mice also showed normal levels of anxiety-like behaviors in elevated plus-maze and light–dark tests and social interaction in the juvenile play test, compared with WT mice (Figures 3B–D).

Intriguingly, in the maternal homing test, in which a juvenile mouse is separated from its mother for 30 min and given the nest material from the home cage that it shared with its mother, male and female Chd8+/S62X mice spent similar amounts of time with the home-cage, or fresh, nest materials, compared with WT mice, as shown by time spent in targets (i.e., home-cage/nest and fresh) and the nest-fresh preference index (difference in time spent for different targets over total time spent) (Figure 3E).
In the second phase of the test, in which juvenile mice are allowed to reunite with their mother, male, but not female, Chd8<sup>+/S62X</sup> mice spent more time with the reunited mother and less time with the home-cage nest material, compared with WT mice, as shown by time spent in targets (i.e., mother and nest) and the mother-nest preference index (Figure 3E). The Mann–Whitney U test was also used here for the lack of the genotype-sex interaction.

These results suggest largely normal social communication in Chd8<sup>+/S62X</sup> pups and largely normal anxiety-like behavior in juvenile Chd8<sup>+/S62X</sup> males and females but hypoactivity and anxiety-like mother-seeking/attachment behaviors in male Chd8<sup>+/S62X</sup> juveniles but not in female Chd8<sup>+/S62X</sup> juveniles.

**Largely normal social behaviors in adult male and female Chd8<sup>+/S62X</sup> mice**

Adult male and female Chd8<sup>+/S62X</sup> mice showed largely normal social approach and social novelty recognition in the three-chamber test compared with WT mice, as supported by time spent sniffing targets and the preference index (Figure 4A). Males and females also showed normal levels of direct social interaction in the dyadic social-interaction test compared with WT mice (Figure 4B).

Upon encountering a novel female mouse, male Chd8<sup>+/S62X</sup> mice emitted normal levels of courtship ultrasonic vocalizations (USVs) compared with WT mice (Figure 4C). These results suggest largely normal social behaviors and communication in adult male and female Chd8<sup>+/S62X</sup> mice.

**Differential increases in repetitive behaviors in adult male and female Chd8<sup>+/S62X</sup> mice**

In tests of repetitive behaviors, adult male and female Chd8<sup>+/S62X</sup> mice showed similarly increased digging but normal self-grooming behavior in novel home cages, as supported by two-way ANOVA (significant genotype difference for digging) (Figure 5A). However, additional
Differential increases in repetitive behaviors in adult male and female Chd8<sup>+/-S62X</sup> mice

In LABORAS cages, a familiar environment where mouse movements are monitored for consecutive 72 h, male and female Chd8<sup>+/S62X</sup> mice showed similarly increased self-grooming in light-off phases, as supported by two-way ANOVA and the Mann–Whitney U test (Figure 5B). These results collectively that adult male and female Chd8<sup>+/S62X</sup> mice show differential increases in repetitive behaviors.

A decrease in center-region time in mutant females but not in mutant males.

In addition, Chd8<sup>+/-S62X</sup> mice spent less time in open arms of the elevated plus-maze and more time in the closed arms, as supported by the genotype difference (Figure 6C), further suggesting enhanced anxiety-like behaviors in adult Chd8<sup>+/S62X</sup> mice. However, additional t-test and Mann–Whitney U test indicated that male Chd8<sup>+/S62X</sup> mice showed decreased open-arm time and increased closed-arm time while female Chd8<sup>+/S62X</sup> mice showed only increased closed-arm time.

In the light–dark test, Chd8<sup>+/S62X</sup> mice spent less time in the light chamber compared with WT mice, although additional t-test and Mann–Whitney U test indicated insignificant changes in both male and female mutant mice compared with WT mice (Figure 6D). Therefore, adult male and female Chd8<sup>+/S62X</sup> mice display differential increases in anxiety-like behaviors.

Discussion

In the present study, we investigated the impacts of a patient-derived, N-terminal protein-truncating CHD8 mutation (S62X) on male and female mice at behavioral levels across postnatal stages. Our results indicate that the CHD8-S62X mutation induces age-differential sexual dimorphism in behavioral deficits.
The current results indicate that repetitive and anxiety-like behavioral deficits are observed in adult male and female Chd8
+/-S62X
mice, whereas hypoactivity and excessive mother seeking/attachment are mainly observed in juvenile Chd8
+/-S62X
males. Statistical analyses of the results by two-way ANOVA frequently yielded significant genotype differences but the lack of genotype-sex interactions, pointing to similar directions in the behavioral changes in male and female Chd8
+/-S62X
mice at juvenile and adult stages. Additional t-tests, however, indicated an interesting difference between Chd8
+/-S62X
juveniles and adults; juvenile males but females showed behavioral deficits (hypoactivity and excessive mother seeking), while adult males and females showed largely similar deficits (repetitive behaviors and anxiety-like behaviors). Therefore, juvenile male-preponderant behavioral deficits seem to be changed to adult behavioral deficits that are similarly observed in males and females, suggestive of age-differential sexually dimorphic behavioral deficits in Chd8
+/-N2373K
mice. These results differ from the male-preponderant behavioral deficits that are persistently observed in Chd8
+/-N2373K
mice across pup, juvenile, and adult stages (Jung et al., 2018).

The current results also suggest age-differential behavioral deficits in Chd8
+/-S62X
mice. In the anxiety-like behavioral domain, Chd8
+/-S62X
pups show largely normal mother separation-induced USVs, while Chd8
+/-S62X
juveniles show...
partially increased anxiety-like behaviors (increased mother seeking but normal open-field center time [males]), which is followed by increased anxiety-like behavior in adults (decreased open-field center time [females] and increased closed-arm time [males and females]. In the locomotor activity domain, Chd8<sup>S62X</sup> juveniles (males) show open-field hypoactivity, whereas Chd8<sup>S62X</sup> adults (males and females) show normal open-field activity, further supporting the idea that deficits in a particular behavioral domain change across postnatal stages in Chd8<sup>S62X</sup> juveniles. These results collectively suggest that different CHD8 mutations could lead to different patterns of male–female differences with varying severities and time courses of development. These data will help design future experiments with different research focuses such as male–female differences, temporal phenotypic alterations, and phenotypic strengths.

ASD exhibits the largest male–female ratio (~4–5:1) among neuropsychiatric disorders (Werling and Geschwind, 2013). Previous studies have shown that the male–female ratio can differ depending on the identity of the ASD-risk genes involved (Stessman et al., 2017). Our study extends this concept by showing that, even within the same gene, two different mutations (N2372K vs. S62X) can change the extent and time course of sexual dimorphisms in autistic-like phenotypes. This new concept may transcend ASD and apply to other neuropsychiatric disorders with a strong male bias (i.e., 2–4-times), such as intellectual disability, attention-deficit hyperactivity disorder, and schizophrenia (Abel et al., 2010; May et al., 2019).

In summary, our results indicate that the CHD8-S62X mutation in mice leads to age-differential sexual dimorphism in behavioral deficits.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Committee on Animal Research at KAIST.

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Author contributions

SL and HKw performed mouse behavioral experiments. HKA and EK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2022.1022306/full#supplementary-material

SUPPLEMENTARY TABLE 1

Statistical details.
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