

Duplication of the autism-related gene Chd8 leads to behavioral hyperactivity and neurodevelopmental defects in mice

Corresponding Author: Professor Masaaki Nishiyama

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study by Kawamura and colleagues examines the neurodevelopmental and behavioral phenotypes associated with CHD8 duplication using a Chd8 knock-in (KI) mouse model.

The authors provide an extensive behavioral characterization of the model and show that treatment with risperidone can alleviate the hyperactivity observed in the KI mice. The authors also observe that Chd8 KI mice have defective cortical neurogenesis and that Chd8 overexpression alters chromatin accessibility during cortical development.

The study provides compelling evidence that Chd8 excessive dosage is critical for neurodevelopment, complementing the body of studies focusing on Chd8 haploinsufficiency. The manuscript is well written and carefully presented. However, I have several concerns on the analyses presented and the conclusions drawn, as outlined below.

- The motivation of this study is the presence of clinical reports of individuals diagnosed with neurodevelopmental disorder carrying CHD8 duplications, but this point needs to be further developed. For example, the Introduction should discuss more extensively the genetics of the CHD8 duplications (studies #13 and #24 cited). How many genes spanned in the duplicated interval? Is the breakpoint within CHD8? Also, the Discussion could be strengthened by touching on other examples of dosage-sensitive genes associated with neurodevelopmental and neurological disorders, e.g. MECP2.
- I have concerns about the analyses of the brain weight/size and the microcephaly statements made in relationship to them. How are the brain weight and brain size analyses corrected, considering that the KI embryos and pups have delayed growth? More accurate measurements could be drawn using MRI.
- I have concerns about the sex considerations in this study. The methods indicate that both female and male mice were included in the analyses. However, sex is only shown as distinct data points in Figure 6 and as separate panels in Figure 1e-f, and the effect of sex (and its interaction with genotype) never formally tested, as far as I can tell. All other experiments do not seem to consider sex as a variable.
- For the analyses in Figure 2, it would be helpful to map the timeline of the cortical deficits with greater accuracy. The authors select E14.5 and E18.5 without proposing a clear rationale for these time points. There are no data on early postnatal time points (e.g., P7) that can help clarify how those changes affect cortical lamination. Along the point made above about brain size/weight, the measurements of thickness of the layers in the adult brains in Figure 1m-n might be affected by body size, cortical region selected, and/or sex. It would be helpful to have quantification of the two populations CTIP2+ and CUX1+ binned over the ventricle-pia axis. The staining in Figure 2m is puzzling, as I would expect many BrdU+ cells still residing in the SVZ.
- For all panels in Figure 2a-i, is the number positive to the marker normalized over the number of cells (DAPI)?
- Figure 1k-n. What region of the adult cortex is being examined?
- Figure 2. Please specify is the medial or lateral cortex is being examined.

- What is the latency to enter the center in the open field analyses in Figure 5a-c?
- The data for the recognition of social novelty (Extended Figure 6h) are puzzling, as WT mice are expected to have increased preference for the novel mouse compared to the familiar mouse. Please explain. The ratios for sociability and preference for social novelty (shown in panels 5l and 5m, respectively) do not seem consistent with those showing the time spent in Extended Figure 6h and 6g.
- I believe the data for the training phase of the novel object location are missing. What about novel object recognition?
- In the fear conditioning paradigm, the Chd8 KI mice seem to have altered training to begin with, as they freeze less than their respective controls after the stimulus (Extended Figure 7g). Is this related to the reduced acoustic startle response seen in these mice (Figure 5n)? Also, for the context-dependent memory recall, while the effect of genotype is not significant, I wonder if the interaction between genotype and time is, because the trajectory seems to differ (reduced extinction in the KI mice).
- The relationship between the data presented in Extended Figure 8 and the rest of the manuscript is not clear and these results are not organically integrated in the story.
- While genetic rescue approaches are typically important in clarifying the possibility of ameliorating phenotypes through the re-introduction of the missing gene (often postnatally or in adulthood), the one presented here does not fall within this group. In fact, the authors have simply balanced out the extra copy introduced with the KI with the missing copy in an haploinsufficient line (Chd8^{+/-};+/KI). This is an elegant control and provides data on the Chd8^{+/-} line coherent with previous literature, and should be framed as such.
- There are several concerns about the analyses presented in Figure 3. First, overall, the RNAseq data show modest effect size for Chd8 KI on the transcriptome of E14.5 forebrains and lack of impact on E18.5 brains. How do the authors reconcile these observations with the significant cellular alterations observed in Figure 2? How many of the 99 downregulated and 63 upregulated genes in E14.5 forebrains survive a more stringent threshold of log2FC >|0.58|? I believe the surprisingly low impact of Chd8 mutations on the transcriptome has been reported before in other Chd8 genetic models (e.g. ref #15) and this should be clearly discussed here. The volcano plots shown in Extended Figure 3 a-b should be presented in the main figure for transparency. Second, the authors report the opposite trends of expression for transcripts in the Chd8 KI model presented here and previous studies (refs #15 and #17), but I am not clear if the cutoff used to identify DEGs in #15 and #17 were those published in those studies or those applied here by the authors. Third, for the GSEA enrichment analyses related to the brain disorders risk genes, I am unclear about the source of diseases genes used as input.
- What is the reference for the risperidone treatment regimen employed here (0.02 mg/kg for 7 days)?
- In panels 6j-n, it would be important to test for the effects of risperidone on the WT animals as well (data provided, but P-value not indicated).
- Line 102 – “Chd8^{-/-};Chd8 KI mice were fertile, appeared to grow normally, and were macroscopically indistinguishable from control littermates”. I could not find data supporting this statement, e.g. growth curves as in panels 1e and 1f.
- How many breedings are represented in Fig. 1D? It would be helpful to evaluate the statistics
- Missing references for sentences at lines 45-46.

Reviewer #2

(Remarks to the Author)

This study by Kawamura and colleagues presents a CHD8 over-expression mouse model demonstrating significant behavioral and neurodevelopmental deficits. CHD8, Chromodomain Helicase DNA-binding protein 8, is a well-studied gene genetically linked to autism. Duplications of 14q11.2 that include CHD8 have recently been identified in patients with neurodevelopmental disorders overlapping with autism. This study generated a CHD8 knockin mouse into the Rosa26 locus that results in about 2-fold over-expression of CHD8 compared to WT. Notably, the heterozygous KI mice (KI/+) exhibit growth retardation and microcephaly, contrasting with the macrocephaly observed in CHD8 haploinsufficiency models. The authors then carried out a series of profiling experiments using RNA-seq, ChIP-seq, and ATAC-seq at E14.5, finding robust evidence that CHD8 over-expression alters gene expression and chromatin accessibility, correlating with decreased NPC differentiation, and ADHD and ASD-like behavioral phenotypes observed in the KI mice. The rescue of behavioral abnormalities by crossing CHD8 KI with heterozygous KO mice is particularly compelling and adds significant value to the study.

The study is well-designed, with appropriate biological replicates and rigorous statistical analysis. The extensive experimental approach supports the central finding regarding the dosage effect of CHD8. Based on the rigor of the study, novelty and potential impact of their findings, it would be appropriate to publish in the journal of Nature Communications. However, I have several suggestions and questions for further strengthening the manuscript.

1. Given the key point of CHD8 expression dosage, it would be informative to quantify CHD8 protein levels in the genetic

rescue mice (-/+;KI/+) and include these data as well. Additionally, full-length Western blots should be provided in the supplemental figures to ensure transparency and reproducibility.

2. The authors mention that the KI allele of CHD8 is driven by the endogenous Rosa26 promoter. It is crucial to clarify whether any other regulatory elements, such as a CAGG promoter, were included in the targeting construct. This clarification is necessary to understand the precise regulation of CHD8 expression in this KI model.

3. The identification of only 9 DEGs at E18.5 compared to 162 DEGs at E14.5 is interesting. The authors should discuss potential reasons for this observation, such as in developmental role of CHD5 or compensatory mechanisms that may reduce the number of DEGs at later stages. Are there any gene expression changes in adult mice when behavioral tests are conducted?

4. The comparison of DEGs in this study with those reported in previous CHD8 haploinsufficiency (-/+) studies is insightful. However, it is essential to confirm whether the ages of the mice in the previous studies are comparable to those in this study. This distinction is important for accurately interpreting the data.

5. While the identification of 16,725 CHD8 binding peaks via ChIP-seq is noteworthy, the relatively low number of DEGs suggests that not all binding events are functionally significant. The authors might consider reanalyzing the ChIP-seq data with different peak-calling criteria to refine the analysis. Additionally, generating heatmaps centered around transcription start sites (TSS), H3K4me3, or H3K4me1 peaks (in addition to those showed in figure 4d centering around CHD8 binding peaks) in the supplemental data could provide further insights into the effects of CHD8 over-expression on enhancers.

6. The conclusion that pharmacological intervention rescued the behavioral phenotypes of CHD8 KI mice is based on a single behavioral test. This assertion may be overstated. The authors should either temper this conclusion or provide additional behavioral data to substantiate the claim. Moreover, the therapeutic relevance of risperidone in this context seems limited, and this should be acknowledged in the discussion.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done an excellent job at addressing many of the questions and concerns raised during the revision process.

I appreciate that the effects of risperidone on WT are showed and discussed, and the interpretation of findings revisited accordingly. I also appreciate adding the behavioral outcomes of risperidone at 0.2 mg/kg, which is currently included as a figure for the Reviewer only. I think these data should make into into the Supplement because the results raise a caveat that might useful for other studies that might be dosing risperidone.

My concern about the interpretation of the preference for social novelty in the 3-chamber test remains unaddressed. As the Supplementary Fig. 7H show, the authors fail to detect a preference in time spent with stranger vs. time spent with familiar mouse (irrespective of genotype). Hence the conclusion that "both Chd8 KI and WT mice showed a similar preference for a novel mouse (stranger 2) and a familiar mouse (stranger 1) in the social novelty preference test" is not supported.

Further, the conclusions drawn from the BrdU and EdU experiments in Fig. 3 are not the very clear, as currently phrased at lines 177-180. How do the authors integrate the interpretations of reduced cell cycle exit with premature exit from the S phase?

Reviewer #2

(Remarks to the Author)

In this revision, the authors have conducted additional experiments as suggested and fully address the questions and concerns I had with the manuscript.

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Response to Reviewer #1

This study by Kawamura and colleagues examines the neurodevelopmental and behavioral phenotypes associated with CHD8 duplication using a Chd8 knock-in (KI) mouse model. The authors provide an extensive behavioral characterization of the model and show that treatment with risperidone can alleviate the hyperactivity observed in the KI mice. The authors also observe that Chd8 KI mice have defective cortical neurogenesis and that Chd8 overexpression alters chromatin accessibility during cortical development.

The study provides compelling evidence that Chd8 excessive dosage is critical for neurodevelopment, complementing the body of studies focusing on Chd8 haploinsufficiency. The manuscript is well written and carefully presented. However, I have several concerns on the analyses presented and the conclusions drawn, as outlined below.

[Response] We thank the reviewer for the careful evaluation of our manuscript and for the statements that “The study provides compelling evidence that Chd8 excessive dosage is critical for neurodevelopment, complementing the body of studies focusing on Chd8 haploinsufficiency,” and that “The manuscript is well written and carefully presented.” We also thank the reviewer for suggestions that we feel have helped us to greatly improve our manuscript.

• The motivation of this study is the presence of clinical reports of individuals diagnosed with neurodevelopmental disorder carrying CHD8 duplications, but this point needs to be further developed. For example, the Introduction should discuss more extensively the genetics of the CHD8 duplications (studies #13 and #24 cited). How many genes spanned in the duplicated interval? Is the breakpoint within CHD8? Also, the Discussion could be strengthened by touching on other examples of dosage-sensitive genes associated with neurodevelopmental and neurological disorders, e.g. MECP2.

[Response] In response to this comment, we have expanded the Introduction to provide a more comprehensive overview of the genetics of duplications at the chromosome 14q11.2 locus, which includes *CHD8*. These duplications span 2 to 27 coding genes, predominantly overlapping the *CHD8* and *SUPT16H* loci. Breakpoints within the *CHD8* coding region have been identified in three cases, two of which were not associated with impaired intelligence. These observations suggest that increased expression of *CHD8* may contribute to the clinical phenotypes observed in affected individuals.

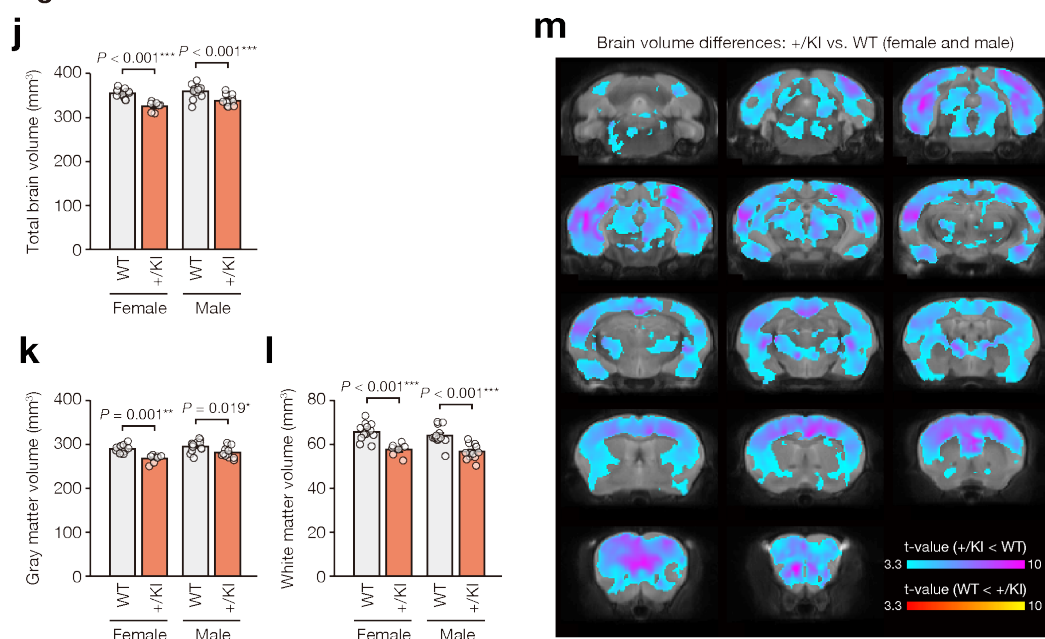
Also as suggested by the reviewer, we have incorporated examples of other dosage-sensitive genes associated with neurodevelopmental disorders in order to strengthen the Discussion. For instance, *MECP2* deletion causes Rett syndrome, characterized by developmental delay and stereotypical movements, whereas *MECP2* duplication results in *MECP2* duplication syndrome, characterized by intellectual disability and motor impairments (Chahrouh *et al.*, *Neuron* 56:422–437, 2007). Studies with mouse models of these conditions have revealed both shared and opposing effects on gene expression, synaptic function, and behavior, illustrating the complex consequences of *MECP2* dosage alterations (Ben-Shachar *et al.*, *Hum. Mol. Genet.* 18:2431–2442, 2009; Chao *et al.*, *Neuron* 56:58–65, 2007; Lu *et al.*, *Neuron* 91:739–747, 2016; Shao *et al.*, *Genes Dev.* 35:489–494, 2021). Similarly, *SHANK3* deletion is associated with Phelan-McDermid syndrome, whereas duplication of this gene is associated with neuropsychiatric disorders including ADHD and schizophrenia (Durand *et*

al., Nat. Genet. 39:25–27, 2006; Peça *et al.*, *Nature* 472:437–442, 2011; Han *et al.*, *Nature* 503:72–77, 2013). These examples highlight the impact of changes in gene dosage on neurodevelopment. In our study, we have now shown that CHD8 overexpression influences neurodevelopment. These various findings emphasize the importance of investigating both loss and gain of function for dosage-sensitive genes in order to provide a better understanding of their roles in neurodevelopmental disorders. We have now clarified these points in the Introduction and Discussion sections of the revised manuscript (page 5, lines 74-77; page 17, line 411-page 18, line 424).

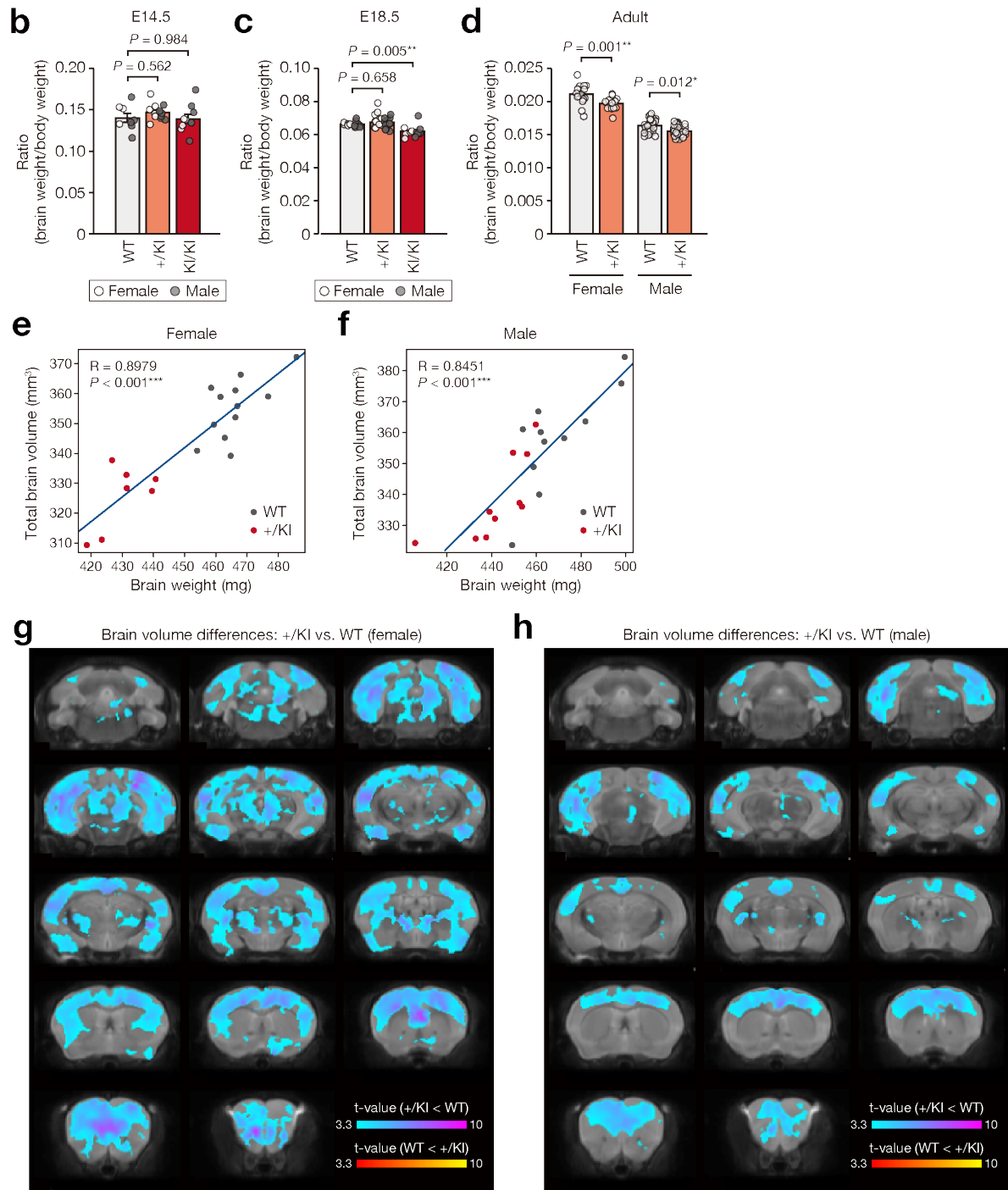
• I have concerns about the analyses of the brain weight/size and the microcephaly statements made in relationship to them. How are the brain weight and brain size analyses corrected, considering that the KI embryos and pups have delayed growth? More accurate measurements could be drawn using MRI.

[Response] We normalized brain weight by body weight and found no significant differences between WT and *Chd8* KI mice at E14.5 and E18.5 (**new Supplementary Fig. 2b,c**). In adult mice, however, *Chd8* KI mice showed a significant reduction in normalized brain weight compared with WT mice (**new Supplementary Fig. 2d**). These results suggest that brain development in *Chd8* KI mice is delayed in association with growth retardation during embryonic stages but does not fully recover in adulthood, leading to microcephaly. To further investigate brain size, we performed MRI-based measurements of brain volume in WT and *Chd8* KI mice at 8 weeks of age. The MRI results revealed a significant reduction in total brain volume as well as in the volumes of gray and white matter in *Chd8* KI mice compared with WT mice, consistent with the observed reduction in brain weight (**new Fig. 1j–l and Supplementary Fig. 2e,f**). We also observed a global reduction in volume across several brain regions of *Chd8* KI mice, with pronounced differences detected in cortical regions (**new Fig. 1m**). Female *Chd8* KI mice showed more pronounced changes than did males (**new Supplementary Fig. 2g,h**). These MRI data provide further support for the phenotype of microcephaly in *Chd8* KI mice. We have now addressed these points in the revised manuscript (page 6, line 125-page 7, line 137).

Figure 1



Supplementary Figure 2



• I have concerns about the sex considerations in this study. The methods indicate that both female and male mice were included in the analyses. However, sex is only shown as distinct data points in Figure 6 and as separate panels in Figure 1e-f, and the effect of sex (and its interaction with genotype) never formally tested, as far as I can tell. All other experiments do not seem to consider sex as a variable.

[Response] We apologize for the insufficient description and statistical analysis regarding sex of the mice in our study. In the original manuscript, the statistical results for behavioral

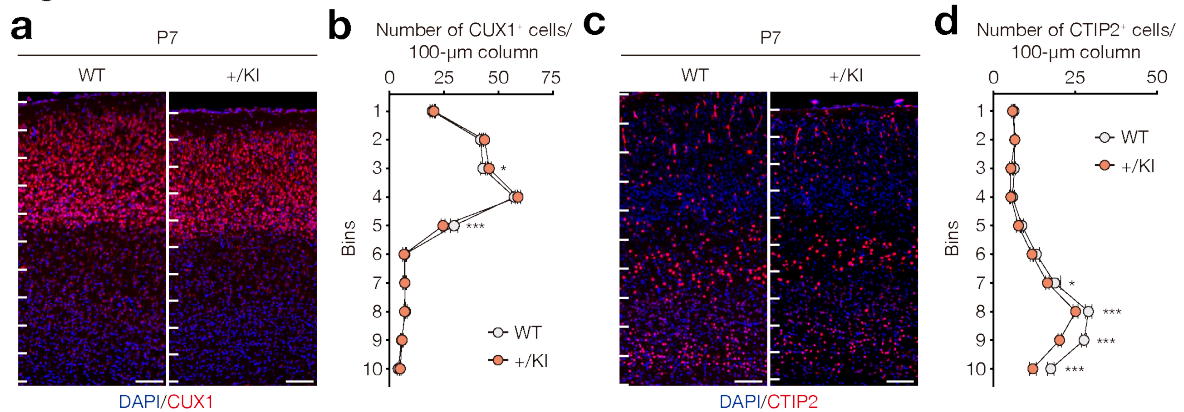
analysis were included in the original Supplementary Table 3, but the effects of sex were not explicitly shown for other analyses. In response to the reviewer's comment, we have now performed two-way ANOVA with sex and genotype to examine sex differences in brain size and behavioral phenotypes. We have included the statistical values for the effects of sex, genotype, and their interaction in the figures or figure legends, as well as more detailed statistical results in the **new Supplementary Table 3**.

The data for immunofluorescence staining of the brain were not distinguished by sex of the mice, given that both male and female *Chd8* KI mice showed a reduction in total brain volume and similar behavioral changes. However, we have clarified the number of animals of each sex used in such analysis in the figure legends. For RNA-seq, ChIP-seq, and ATAC-seq analyses, we used samples from male mice, given that previous data were mostly obtained with male mice (Katayama *et al.*, *Nature* 537:675–679, 2016) and we wanted to compare our results with these previous data. Similarly, the battery of behavioral tests was conducted with male mice. However, hyperactive behavior, which was reproducibly observed in male *Chd8* KI mice, was also observed in additional tests with female mice (Fig. 6), indicating that the phenotype is consistent across sexes. We have now clarified the statistical results in the figures and specified the handling of sex in the Methods section and figure legends of the revised manuscript (page 23, lines 529-531; page 26, lines 622-623; page 38, lines 930-936).

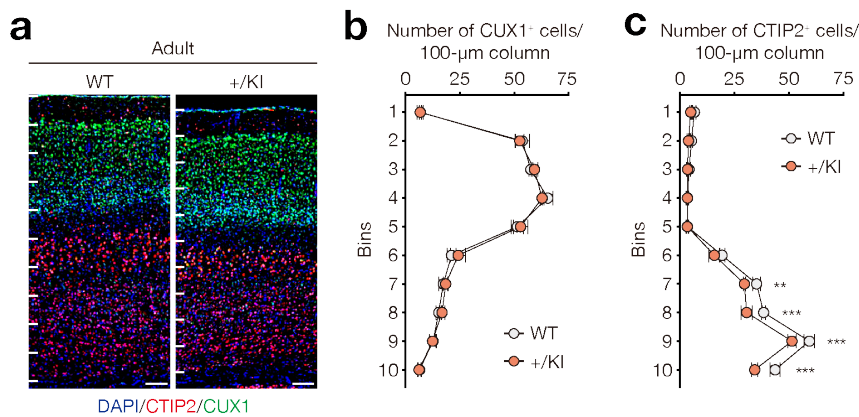
• For the analyses in Figure 2, it would be helpful to map the timeline of the cortical deficits with greater accuracy. The authors select E14.5 and E18.5 without proposing a clear rationale for these time points. There are no data on early postnatal time points (e.g., P7) that can help clarify how those changes affect cortical lamination. Along the point made above about brain size/weight, the measurements of thickness of the layers in the adult brains in Figure 1m-n might be affected by body size, cortical region selected, and/or sex. It would be helpful to have quantification of the two populations CTIP2⁺ and CUX1⁺ binned over the ventricle-pia axis. The staining in Figure 2m is puzzling, as I would expect many BrdU⁺ cells still residing in the SVZ.

[Response] We selected the embryonic days E14.5 and E18.5 on the basis that *Chd8* heterozygous mutant mice manifest delayed cortical neurogenesis at E14.5 (Katayama *et al.*, *Nature* 537:675–679, 2016; Gompers *et al.*, *Nat. Neurosci.* 20:1062–1073, 2017). These time points allowed us to capture the initial and progressive phenotypic alterations in *Chd8* KI mice. As suggested by the reviewer, we have now added data for the cortex at P7 and in adulthood to provide further insight into effects on cortical lamination. We divided the cortex into 10 equal bins along the ventricle-pia axis and quantified CTIP2⁺ and CUX1⁺ neuronal populations in each bin. The number of CUX1⁺ upper-layer neurons was altered in the cortex of *Chd8* KI mice at P7 (**new Fig. 2a,b**). In addition, we observed a significant reduction in the number of CTIP2⁺ deep-layer neurons at P7 and in adulthood (**new Fig. 2c,d and Supplementary Fig. 3a–c**). These results suggested that *Chd8* overexpression may affect the generation of upper-layer neurons in addition to deep-layer neurons during brain development. We have now addressed these points in the revised manuscript (page 7, line 146–page 8, line 153).

Figure 2



Supplementary Figure 3



The distribution of BrdU⁺ cells can be explained by the dynamics of cell cycle progression during embryonic neurogenesis. At E12.5, neural precursor cells (NPCs) manifest spatial positioning corresponding to different phases of the cell cycle, with nuclei migrating basally during G1, undergoing S phase in the basal region, and reversing their migration in G2 to reach the apical surface for mitosis (Salomoni *et al.*, *Trends Cell Biol.* 20:233–243, 2010). Cells labeled by BrdU, which were in S phase within 30 min after injection, would be expected to localize predominantly to the basal side of the ventricular zone, whereas NPCs that had progressed through the cell cycle within 2 h (EdU⁺BrdU⁻ cells) would be expected to have migrated toward the apical surface for mitosis. Our findings are consistent with previous data for which EdU was used to label cells in S phase at similar developmental stages (Ben-Reuven *et al.*, *Mol. Psychiatry* 26:1535–1550, 2021). We have now described the spatial dynamics of BrdU⁺ cells at E12.5 in detail in the Results section of the revised manuscript (page 8, lines 169-175).

• For all panels in Figure 2a-i, is the number positive to the marker normalized over the number of cells (DAPI)?

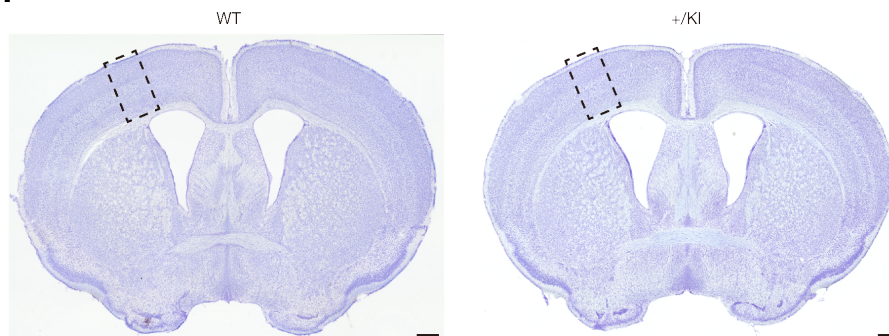
[Response] We normalized the number of cells positive for the specified markers by the area of the cortex within a 100-μm-width column, spanning from the pial surface to the ventricular surface. We have now clarified this point in the figures and Methods section of the revised manuscript (page 23, lines 539-544).

- *Figure 1k-n. What region of the adult cortex is being examined?*

[Response] We examined cortical thickness for the somatosensory cortex. To clarify the examined area, we have enclosed it within dotted lines in the Nissl staining of coronal sections (**new Supplementary Fig. 2i**). We have also now described the examined regions in the figure legends of the revised manuscript (page 51, lines 1211-1214).

Supplementary Figure 2

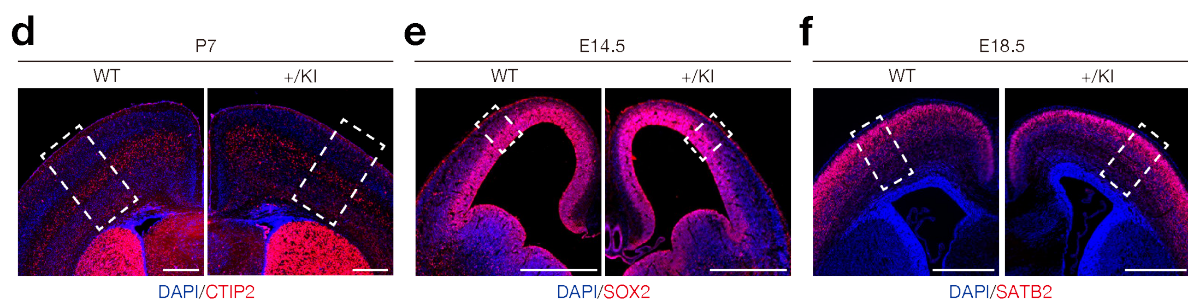
i



- *Figure 2. Please specify is the medial or lateral cortex is being examined.*

[Response] In this study, we examined and counted cells in the lateral cortex of the embryonic brain. To clarify the specific area examined, we have now included low-magnification immunofluorescence images of the embryonic brain with the examined region highlighted by dotted lines (**new Supplementary Fig. 3d-f**) and have modified the text accordingly (page 53, line 1230-page 54, line 1254).

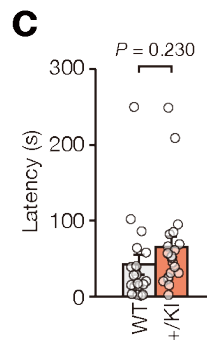
Supplementary Figure 3



- *What is the latency to enter the center in the open field analyses in Figure 5a-c?*

[Response] We examined the latency to enter the central area in the open-field test and found no difference between *Chd8* KI and WT mice (**new Fig. 5c**). We have now addressed this point in the revised manuscript (page 13, lines 290-292).

Figure 5



• The data for the recognition of social novelty (Extended Figure 6h) are puzzling, as WT mice are expected to have increased preference for the novel mouse compared to the familiar mouse. Please explain. The ratios for sociability and preference for social novelty (shown in panels 5l and 5m, respectively) do not seem consistent with those showing the time spent in Extended Figure 6h and 6g.

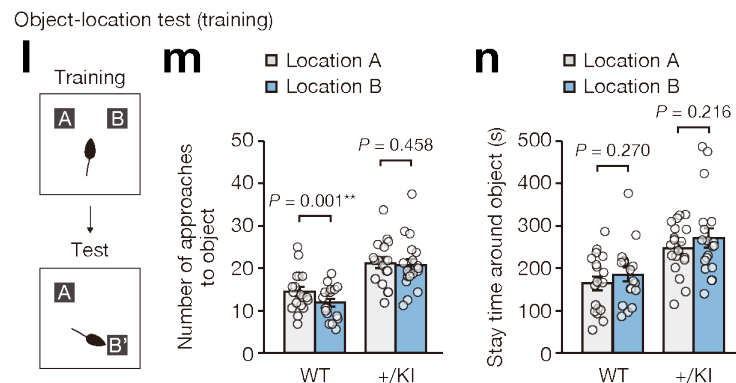
[Response] As the reviewer points out, under our experimental conditions we occasionally fail to detect a clear preference for the novel mouse in the social novelty–preference test. This might be due to certain environmental or procedural factors or genetic background, with the conditions requiring further optimization (Pearson *et al.*, *Behav. Brain Res.* 213:189–194, 2010; Jabarin *et al.*, *Mol. Autism* 13:41, 2022). However, in the present study, we performed all behavioral tests under the same standardized conditions, with careful counterbalancing to minimize bias. We have also reexamined the ratios for sociability and preference for social novelty in the three-chamber test and found that they were properly calculated as “stranger/(stranger + empty cage)” and “familiar/(stranger + familiar),” respectively. However, the original y-axis labels of “stranger vs. empty cage” and “stranger vs. familiar” may have been misleading in their representation of these calculations. To avoid misunderstanding, we have revised these labels to “Social preference index” and clarified the exact calculations in the figure legends and Methods section of the revised manuscript (page 34, line 833–page 35 line 837; page 60, lines 1324–1327).

• I believe the data for the training phase of the novel object location are missing. What about novel object recognition?

[Response] In the training phase of the novel object–location test, we detected no difference in the duration of time spent at each location between *Chd8* KI and WT animals, whereas WT mice made fewer approaches to one of the objects than to the other (**new Supplementary Fig. 7l–n**). This suggests that the observed preference of mice for the novel location during the test session likely reflects memory of the object’s location rather than a preference for the object itself. In addition, *Chd8* KI mice showed increased numbers of approaches to and an increased time spent around both objects compared with WT mice, which may be related to their increased activity levels. With regard to the novel object–recognition test, we did not perform this test because of the variable preferences of mice for certain objects and the potential for inconsistent outcomes. Given the behavioral alterations of *Chd8* KI mice in both the object–location test and Barnes maze test, which assesses spatial recognition, we believe that these mice may have mild memory deficits. We

have now included the data for the training phase of the novel object–location test and addressed these points in the revised manuscript (page 14, lines 316-320).

Supplementary Figure 7

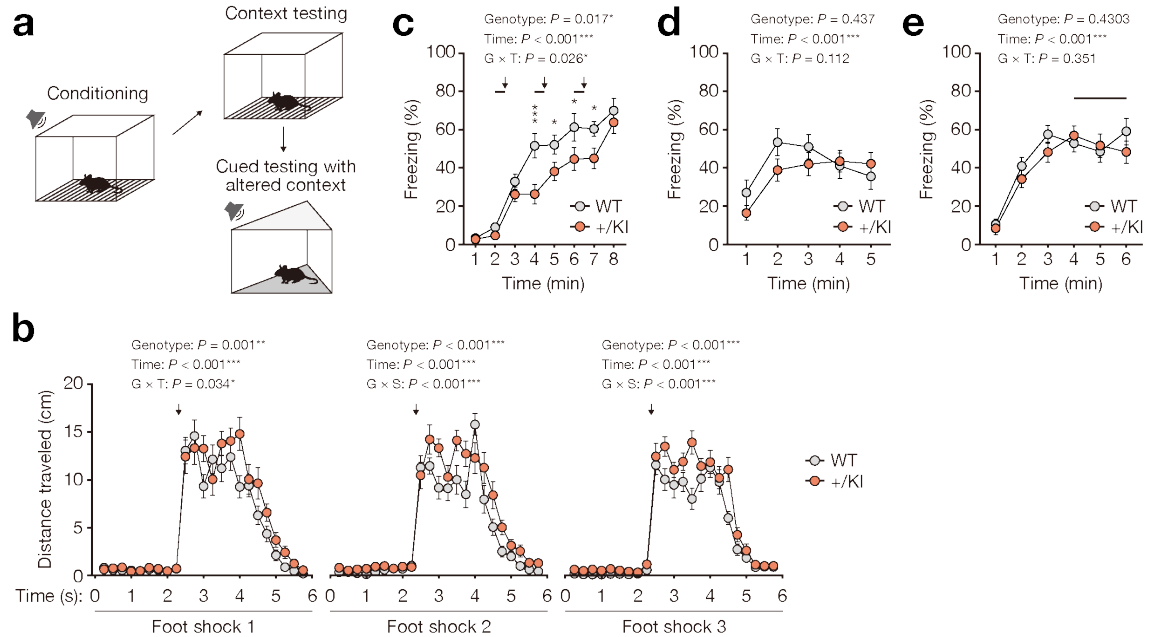


• In the fear conditioning paradigm, the *Chd8* KI mice seem to have altered training to begin with, as they freeze less than their respective controls after the stimulus (Extended Figure 7g). Is this related to the reduced acoustic startle response seen in these mice (Figure 5n)? Also, for the context-dependent memory recall, while the effect of genotype is not significant, I wonder if the interaction between genotype and time is, because the trajectory seems to differ (reduced extinction in the KI mice).

[Response] The acoustic startle response has been shown to be enhanced by fear (Davis and Astrachan, *J. Exp. Psychol. Anim. Behav. Process.* 4:95–103, 1978; Davis *et al.*, *Behav. Brain Res.* 58:175–198, 1993). As suggested by the reviewer, it is possible that an attenuated fear response may be related to the reduced acoustic startle response in *Chd8* KI mice. Another possibility is that the reduced freezing behavior of these mice might reflect increased locomotor activity in addition to an impaired fear response. We found that the distance traveled immediately after each shock was consistently higher for *Chd8* KI mice than for WT mice (**new Supplementary Fig. 8b**). We have now clarified these points in the Results section of the revised manuscript (page 14, line 329–page 15, line 335). In the case of context-dependent memory recall, we found no significant interaction between genotype and time as assessed by two-way repeated-measures ANOVA (genotype $\times$ time, $P = 0.112$) (Supplementary Fig. 8d). To clarify this point, we have now provided the individual effects of each factor and their interaction revealed by the statistical analysis in the figure, with further detailed statistical results included in **new Supplementary Table 3**.

Supplementary Figure 8

Fear-conditioning test



• *The relationship between the data presented in Extended Figure 8 and the rest of the manuscript is not clear and these results are not organically integrated in the story.*

[Response] We apologize for the insufficient description of this point. To clarify the relation of these data to and their integration into our manuscript, we have moved their description to the end of the Results section and provided a detailed explanation regarding the rationale for this analysis. Monoamine neurotransmitter systems are associated with neuropsychiatric disorders and behavioral abnormalities. In addition, we found that the hyperactivity of *Chd8* KI mice was ameliorated by risperidone (Fig. 6i-n), which acts as an antagonist at the D2 dopamine receptor and the 5-HT_{2A} serotonin receptor. This prompted us to investigate dopamine and serotonin levels in these mice. Our monoamine analysis revealed a tendency for the concentration of serotonin to be increased in the nucleus accumbens of *Chd8* KI mice (Supplementary Fig. 10b), raising the possibility that dysregulation of the serotonergic system may contribute to the hyperactive phenotype of these mice. We therefore believe that the results of our monoamine analysis provide further insight into the phenotype of *Chd8* KI mice. We have now addressed these points in the revised manuscript (page 16, lines 379-382).

• *While genetic rescue approaches are typically important in clarifying the possibility of ameliorating phenotypes through the re-introduction of the missing gene (often postnatally or in adulthood), the one presented here does not fall within this group. In fact, the authors have simply balanced out the extra copy introduced with the KI with the missing copy in an haploinsufficient line (*Chd8*^{+/-}; *+KI*). This is an elegant control and provides data on the *Chd8*^{+/-} line coherent with previous literature, and should be framed as such.*

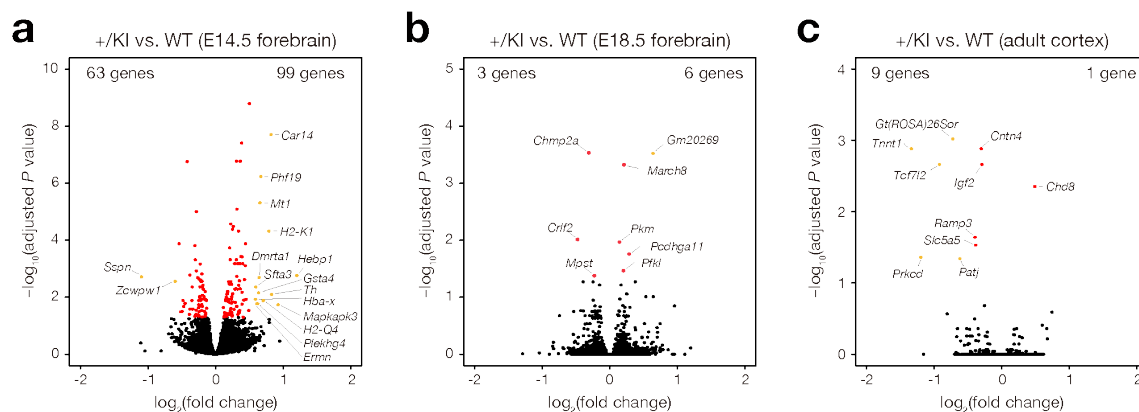
[Response] As pointed out by the reviewer, *Chd8*^{+/-}; *Chd8* KI mice served as a control to balance gene dosage rather than to rescue the phenotype. The amelioration of the abnormal social behavior and increased anxiety-like behavior of *Chd8*^{+/-} mice by the additional copy

of the gene provided by the *Chd8* KI allele emphasizes the physiological functionality and efficacy of the *Chd8* transgene in our genetically engineered model. We have now addressed this point by framing the *Chd8*^{+/-}; *Chd8* KI mice as a control for gene dosage in the revised manuscript (page 15, lines 349-353).

• There are several concerns about the analyses presented in Figure 3. First, overall, the RNAseq data show modest effect size for Chd8 KI on the transcriptome of E14.5 forebrains and lack of impact on E18.5 brains. How do the authors reconcile these observations with the significant cellular alterations observed in Figure 2? How many of the 99 downregulated and 63 upregulated genes in E14.5 forebrains survive a more stringent threshold of $\log_2FC > |0.58|$? I believe the surprisingly low impact of Chd8 mutations on the transcriptome has been reported before in other Chd8 genetic models (e.g. ref #15) and this should be clearly discussed here. The volcano plots shown in Extended Figure 3 a-b should be presented in the main figure for transparency. Second, the authors report the opposite trends of expression for transcripts in the Chd8 KI model presented here and previous studies (refs #15 and #17), but I am not clear if the cutoff used to identify DEGs in #15 and #17 were those published in those studies or those applied here by the authors. Third, for the GSEA enrichment analyses related to the brain disorders risk genes, I am unclear about the source of diseases genes used as input.

[Response] As the reviewer points out, our RNA-seq analysis revealed few DEGs in the forebrain at E18.5, whereas many more were detected in the E14.5 forebrain. It is possible that these results reflect in part the limitations of bulk RNA-seq analysis, which can obscure changes in specific cell types and brain regions by averaging gene expression across heterogeneous cell populations. By E18.5, compared with E14.5, the forebrain has undergone substantial changes, including differentiation of glial cells and an increase in the proportion of basal ganglia, leading to greater cellular diversity. This increased diversity might dilute gene expression changes associated with the reduction in the number of deep-layer neurons in mice with *Chd8* overexpression. Further studies are warranted to delineate changes in gene expression within specific cell types and brain regions of *Chd8* KI mice from early development through to adulthood. Application of a more stringent threshold of $|\log_2FC| > 0.58$ to our RNA-seq data for the E14.5 forebrain revealed that 15 out of the original 63 upregulated genes (*Hebp1*, *Mapkapk3*, *Th*, *Car14*, *H2-K1*, *H2-Q4*, *Phf19*, *Mt1*, *Dmrta1*, *Chd8*, *Gsta4*, *Plekhg4*, *Ermn*, *Sfta3*, and *Hba-x*) and 2 out of the original 99 downregulated genes (*Sspn* and *Zcwpw1*) meet this threshold. We have now moved the volcano plots to the main figure and highlighted these genes that show changes with this threshold (**new Fig. 3a–c**). Furthermore, as mentioned by the reviewer, *Chd8* heterozygous mutations have previously been shown to result in only minor but global changes in gene expression (Katayama *et al.*, *Nature*, 537:675–679, 2016). Despite these modest transcriptomic alterations, however, *Chd8* mutant mice manifest marked neurodevelopmental and behavioral phenotypes, including macrocephaly and behavioral abnormalities. These findings suggest that the cumulative or cooperative effects of subtle changes in the expression of numerous genes due to *Chd8* mutations may underlie these phenotypes. Similarly, *Chd8* overexpression in our study resulted in neurodevelopmental and behavioral abnormalities, despite only minor changes in gene expression. This consistency across different CHD8 dosage models further supports the notion that even slight perturbations in CHD8 levels can have substantial functional consequences, highlighting the key role of CHD8 in neurodevelopment. We have now added these points to the Results and Discussion sections of the revised manuscript (page 9, lines 188-194;

Figure 3



We apologize for the insufficient description of the gene sets used for GSEA in this study. We reanalyzed the data set from Katayama *et al.* (*Nature* 537:675–679, 2016) and independently defined the gene set with a cutoff of $P < 0.05$ for our analyses. In the case of the data set from Gompers *et al.* (*Nat. Neurosci.* 20:1062–1073, 2017), we utilized the gene set defined by Chen *et al.* (*Neuron* 109:3775–3792, 2021), which also applied a cutoff of $P < 0.05$. Of note, we observed an opposite trend in the expression changes between *Chd8*^{+/-} and *Chd8* KI mice not only in GSEA with these gene sets but also in correlation analysis for DEGs identified in the forebrain of *Chd8* KI mice at E14.5 (Fig. 3i,j). This consistency supports the dosage-sensitive effects of CHD8 on gene regulation. Regarding the source of disease-associated gene sets used for GSEA, we adopted the gene sets curated in the original reference 46. These gene sets were derived from the following databases: ASD genes from SFARI (human module, gene scores 1 and 2), ADHD genes from ADHDgene (<http://adhd.psych.ac.cn>), intellectual disability genes from IDGenetics (<http://www.ccgenomics.cn/IDGenetics>), schizophrenia genes from SZDB2.0 SNP data sets (<http://www.szdb.org>), and microcephaly genes from DisGeNET (<https://www.disgenet.org/home>). We have now provided detailed descriptions of the gene sets for DEGs in *Chd8*^{+/-} mice and for the disease gene sets, including their sources, in the Methods section of the revised manuscript (page 27, lines 633–642).

• *What is the reference for the risperidone treatment regimen employed here (0.02 mg/kg for 7 days)?*

[Response] Our treatment regimen was based on a previously described protocol (Peñagarikano *et al.*, *Cell* 147:235–246, 2011). In this previous study, risperidone (0.2 mg/kg) was administered by daily intraperitoneal injection for 7 consecutive days, followed by behavioral testing on days 8, 9, and 10. Drug administration was continued during the testing days, with the drug being delivered ~1 h before testing. However, we observed a strong sedative effect when WT mice were treated with risperidone at 0.2 mg/kg (**Fig. R1 for reviewing purposes only**). We therefore reduced the dosage to mitigate this sedative effect and finally chose a dose of 0.02 mg/kg, which has also been used in several other studies of mice with minimal effects on locomotor activity (Su *et al.*, *Eur. J. Pharmacol.* 564:123–130, 2007) as well as in clinical trials for individuals with ASD (McCracken *et al.*, *N. Engl. J. Med.* 347:314–321, 2002). We have now clarified these points in the Methods

section of the revised manuscript (page 30, lines 722-728).

Figure R1 (for reviewing purposes only)

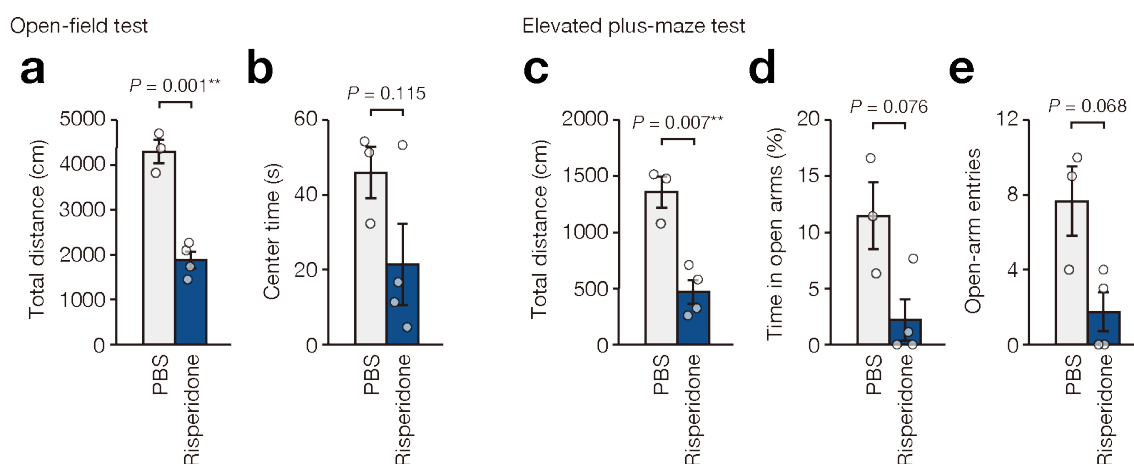
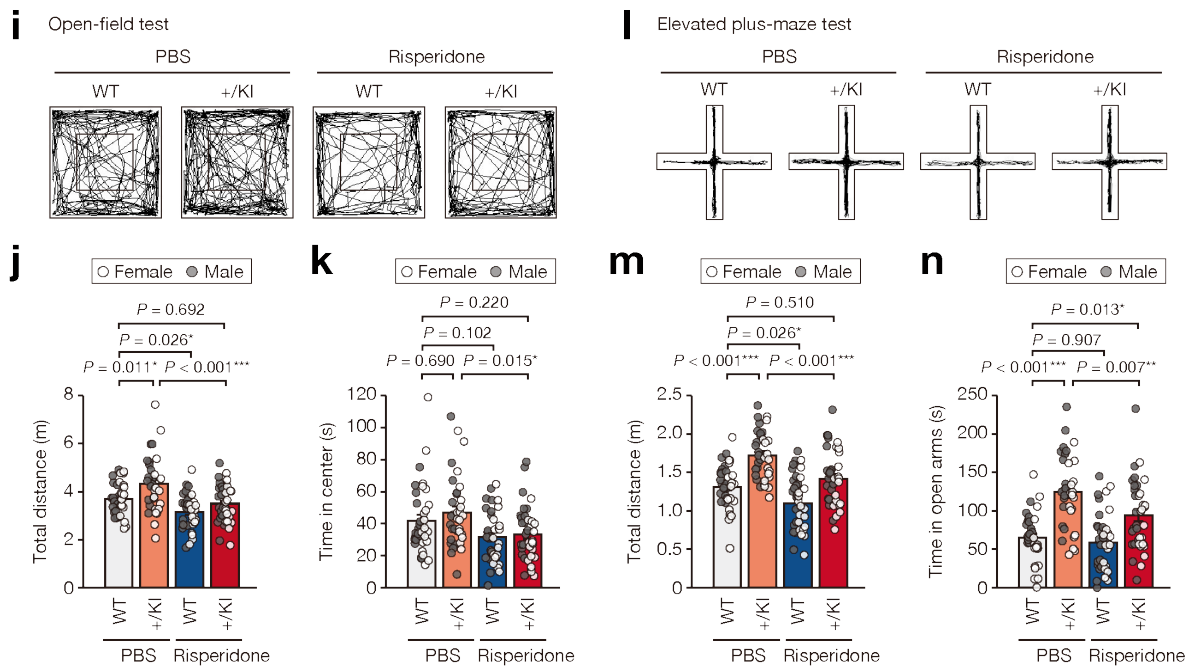


Fig. R1 (for reviewing purposes only) A strong sedative effect of treatment with risperidone at 0.2 mg/kg in WT mice. **a,b**, Total distance traveled (**a**) and time spent in the central area (**b**) for the open-field test performed with WT mice treated with PBS vehicle or risperidone. **c–e**, Total distance traveled (**c**), time spent in the open arms (**d**), and number of entries into the open arms (**e**) for the elevated plus-maze test performed with WT mice treated with vehicle or risperidone. Risperidone (0.2 mg/kg) was administered by daily intraperitoneal injection for 7 consecutive days, with the final injection being administered 1 h before behavioral analysis. All data are means $\pm$ s.e.m. P values were determined with the unpaired Student's t test. $^{**}P < 0.01$.

• *In panels 6j–n, it would be important to test for the effects of risperidone on the WT animals as well (data provided, but P -value not indicated).*

[Response] In response to this comment, we have now indicated the P values for the effects of risperidone on WT mice in the figure. Detailed statistical results have also been included in the figure legend and Supplementary Table 3. A slight but significant reduction in total distance traveled was apparent for the WT animals after risperidone treatment in both behavioral tests (Fig. 6j,m). However, no difference was detected in the time spent in the central area for the open-field test or in the open arms for the elevated plus-maze test between vehicle- and risperidone-treated WT mice (Fig. 6k,n). These findings suggest that the behavioral results may reflect a mild sedative effect. We have clarified the statistical results of the behavioral analysis and the potential effects of risperidone on WT mice in the revised manuscript (page 16, lines 374-376).

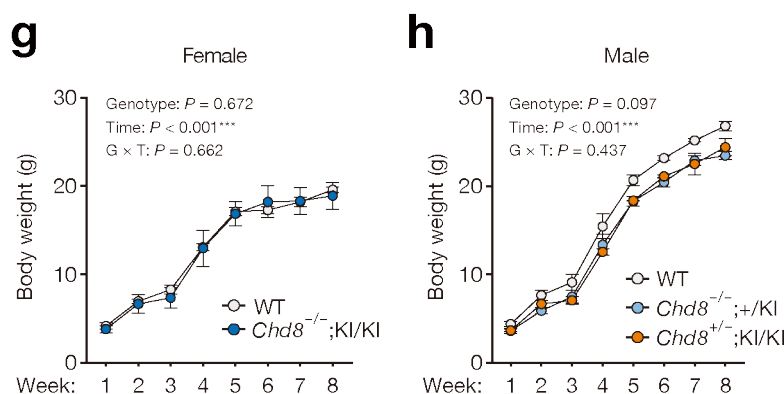
Figure 6



• Line 102 – “*Chd8*^{-/-};*Chd8* KI mice were fertile, appeared to grow normally, and were macroscopically indistinguishable from control littermates”. I could not find data supporting this statement, e.g. growth curves as in panels 1e and 1f.

[Response] We have now provided growth curves for *Chd8*^{-/-};/+KI, *Chd8*^{-/-};/KI/KI, and *Chd8*^{+/-};/KI/KI mice (new Supplementary Fig. 1g,h). Despite the low expected frequency of these genotypes, we obtained such mice among 119 offspring generated during the revision period. Body weight for mice of these genotypes was similar to that for control littermates, supporting the statement that they grow normally. We have now addressed this point in the Results section of the revised manuscript (page 6, lines 111-113).

Supplementary Figure 1



• How many breedings are represented in Fig. 1D? It would be helpful to evaluate the statistics

[Response] During the revision process, we expanded the data set from 6 litters in the original manuscript to 13 litters so as to increase the reliability of our analysis. The chi-square test confirmed that both *Chd8*^{-/-} and KI/KI mice were significantly less frequent than the expected Mendelian ratio (**new Fig. 1d**). We have now addressed this point in the revised manuscript (page 6, lines 108-111).

Figure 1

d

Genotyping of mice at 3 weeks of age

Number of animals (from 13 litters)

(*Chd8*^{+/-};+/KI × *Chd8*^{+/-};+/KI):

Genotype	<i>Chd8</i> ^{+/+}	<i>Chd8</i> ^{+/-}	<i>Chd8</i> ^{-/-}	P value
+/+	4 (3.9%)	12 (11.8%)	0 (0%)	0.049*
+/KI	20 (19.8%)	28 (27.7%)	12 (11.8%)	0.301
KI/KI	1 (0.9%)	18 (17.8%)	6 (5.9%)	0.032*
P value	0.007**	0.522	0.049*	

• *Missing references for sentences at lines 45-46.*

[Response] We have now cited the appropriate references (Grayton *et al.*, *Prog. Neurobiol.* 99:81–91, 2012; Takumi *et al.*, *Curr. Opin. Neurobiol.* 48:183–192, 2018) in the revised manuscript (page 4, lines 52-54).

Response to Reviewer #2

This study by Kawamura and colleagues presents a CHD8 over-expression mouse model demonstrating significant behavioral and neurodevelopmental deficits. CHD8, Chromodomain Helicase DNA-binding protein 8, is a well-studied gene genetically linked to autism. Duplications of 14q11.2 that include CHD8 have recently been identified in patients with neurodevelopmental disorders overlapping with autism. This study generated a CHD8 knockin mouse into the Rosa26 locus that results in about 2-fold over-expression of CHD8 compared to WT. Notably, the heterozygous KI mice (KI/+) exhibit growth retardation and microcephaly, contrasting with the macrocephaly observed in CHD8 haploinsufficiency models. The authors then carried out a series of profiling experiments using RNA-seq, ChIP-seq, and ATAC-seq at E14.5, finding robust evidence that CHD8 over-expression alters gene expression and chromatin accessibility, correlating with decreased NPC differentiation, and ADHD and ASD-like behavioral phenotypes observed in the KI mice. The rescue of behavioral abnormalities by crossing CHD8 KI with heterozygous KO mice is particularly compelling and adds significant value to the study.

The study is well-designed, with appropriate biological replicates and rigorous statistical analysis. The extensive experimental approach supports the central finding regarding the dosage effect of CHD8. Based on the rigor of the study, novelty and potential impact of their findings, it would be appropriate to publish in the journal of Nature Communications. However, I have several suggestions and questions for further strengthening the manuscript.

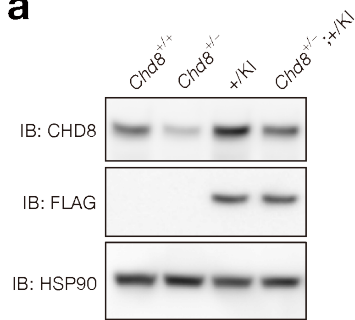
[Response] We thank the reviewer for the careful evaluation of our manuscript and for the statements that “The rescue of behavioral abnormalities by crossing CHD8 KI with heterozygous KO mice is particularly compelling and adds significant value to the study,” and that “The study is well-designed, with appropriate biological replicates and rigorous statistical analysis.” We also thank the reviewer for suggestions that we feel have helped us to greatly improve our manuscript.

1. Given the key point of CHD8 expression dosage, it would be informative to quantify CHD8 protein levels in the genetic rescue mice (-/+;KI/+) and include these data as well. Additionally, full-length Western blots should be provided in the supplemental figures to ensure transparency and reproducibility.

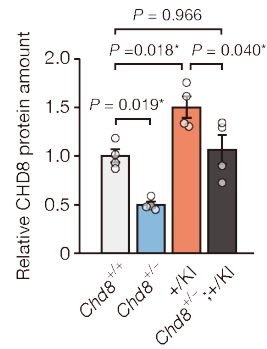
[Response] As suggested by the reviewer, we examined CHD8 expression at the protein level by immunoblot analysis. The abundance of CHD8 protein was restored to WT levels in the brain of *Chd8*^{+/-}; *Chd8* KI mice at E14.5 (**new Supplementary Fig. 9**). We have addressed this issue in the Results section of the revised manuscript (page 15, lines 353-354). We have also now included full-length immunoblots in **new Supplementary Figure 11**.

Supplementary Figure 9

a

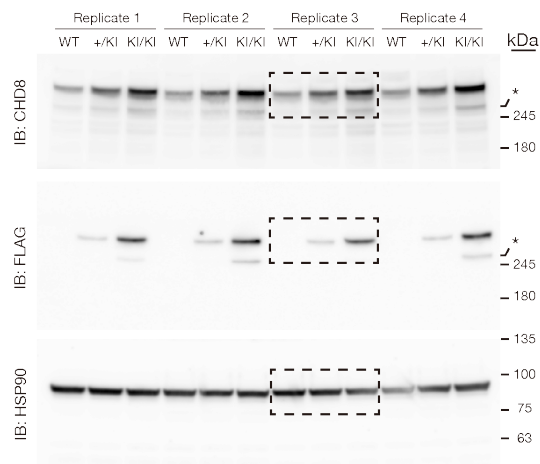


b

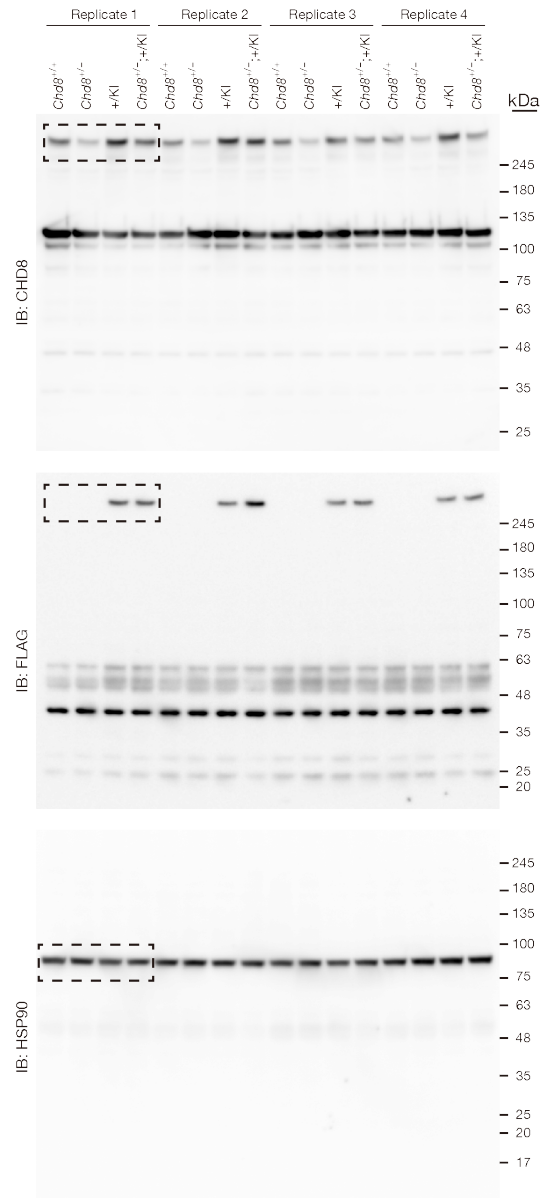


Supplementary Figure 11

Figure 1b



Supplementary Figure 9a

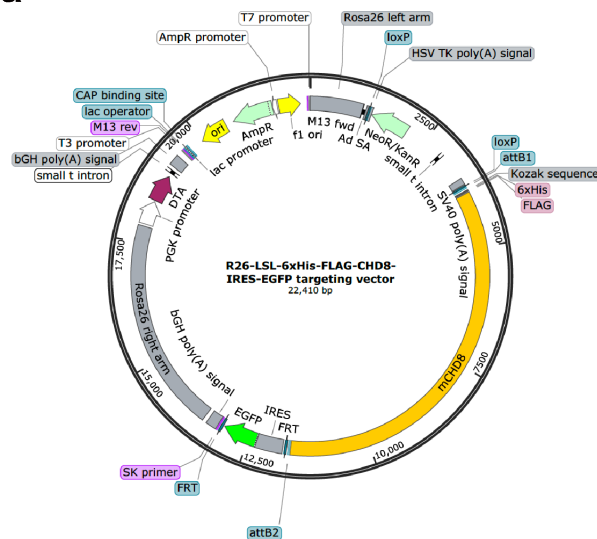


2. The authors mention that the KI allele of *CHD8* is driven by the endogenous *Rosa26* promoter. It is crucial to clarify whether any other regulatory elements, such as a CAGG promoter, were included in the targeting construct. This clarification is necessary to understand the precise regulation of *CHD8* expression in this KI model.

[Response] We have now included a map of the targeting vector in the revised manuscript (new Supplementary Fig. 1a). This map shows that the *CHD8* coding sequence is preceded by a splice acceptor (SA) site, a loxP site, an attB1 site, a Kozak sequence, and His and FLAG tag sequences in *Chd8* KI mice, but there are no additional regulatory elements, such as a CAGG promoter, included in the construct.

Supplementary Figure 1

a



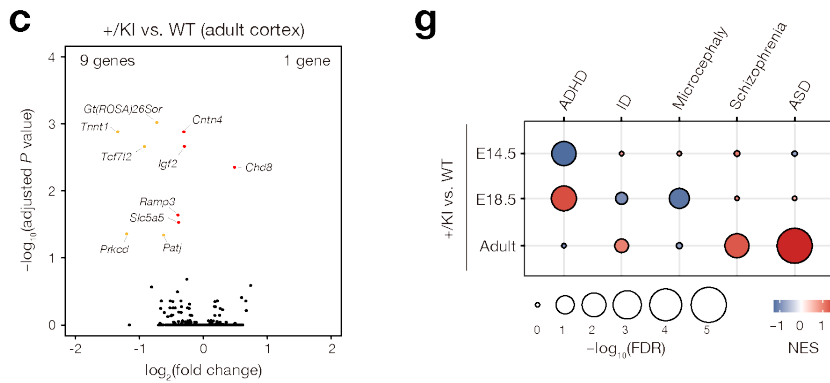
3. The identification of only 9 DEGs at E18.5 compared to 162 DEGs at E14.5 is interesting. The authors should discuss potential reasons for this observation, such as in developmental role of *CHD5* or compensatory mechanisms that may reduce the number of DEGs at later stages. Are there any gene expression changes in adult mice when behavioral tests are conducted?

[Response] As suggested by the reviewer, we have now added a more detailed discussion of the difference in the number of DEGs between E14.5 and E18.5. *CHD8* is highly expressed in the ventricular zone and subventricular zone at E14.5, and its expression gradually decreases thereafter (Kasah *et al.*, *J. Anat.* 233:755–769, 2018). Deletion of *Chd8* at this embryonic stage leads to significant changes in gene expression and cortical developmental defects that become apparent before E18.5 (Kweon *et al.*, *Cell Rep.* 34:108780, 2021; Dong *et al.*, *J. Neurosci.* 42:8373–8392, 2022). These findings suggest that early transcriptional programs are highly sensitive to *CHD8* dosage alterations, resulting in more pronounced gene expression changes at E14.5 compared with E18.5. We also consider the possibility of compensatory biological mechanisms—such as feedback loops, activation of alternative pathways, and homologous gene expression—that might mitigate the effects of *Chd8* deletion at later developmental stages (Marie *et al.*, *PNAS* 115:E8246–E8255, 2018). Furthermore, we acknowledge the limitations of the bulk RNA-seq analysis performed in our study, which may obscure changes in specific cell types and brain regions as a result of

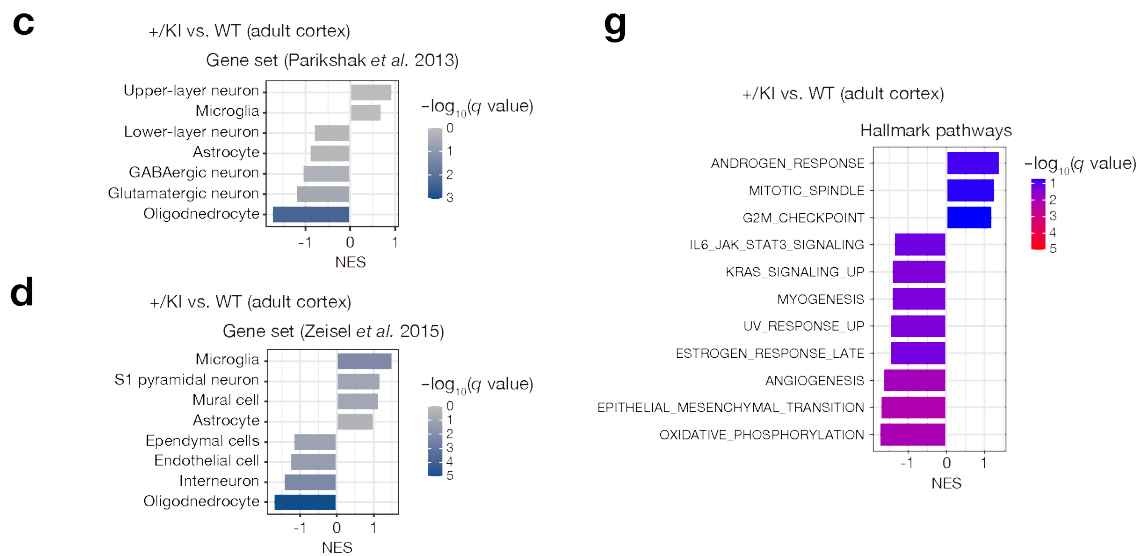
the averaging of gene expression across heterogeneous cell populations. By E18.5, compared with E14.5, the forebrain has undergone substantial changes, including differentiation of glial cells and an increase in the proportion of basal ganglia, leading to greater cellular diversity. Further studies are warranted to delineate alterations in gene expression within specific cell types and brain regions in *Chd8* KI mice from early development through to adulthood. We have now addressed these points in the revised manuscript (page 19, lines 439-455).

As suggested, we also performed RNA-seq analysis for the cortex of adult *Chd8* KI mice. We identified 10 DEGs including *Cntn4* and *Tcf7l2* (**new Fig. 3c**), both of which are associated with neurodevelopmental disorders (Fernandez *et al.*, *Am. J. Hum. Genet.* 74:1286–1293, 2004; Iossifov *et al.*, *Nature* 515:216–221, 2014). GSEA revealed upregulation of genes associated with ASD and schizophrenia in the adult cortex of *Chd8* KI mice, with such upregulation not being observed in the forebrain at embryonic stages (E14.5 and E18.5) (**new Fig. 3g**). Furthermore, GSEA for cell type-specific markers revealed downregulation of oligodendrocyte markers in the adult cortex of these mice (**new Supplementary Fig. 4c,d,g**). We believe that our new results provide further insight into the molecular and cellular changes associated with *Chd8* overexpression in adulthood. We have now addressed these points in the revised manuscript (page 9, lines 192-194; page 9, line 203-page 10, line 209).

Figure 3



Supplementary Figure 4



*4. The comparison of DEGs in this study with those reported in previous *Chd8* haploinsufficiency (-/+) studies is insightful. However, it is essential to confirm whether the ages of the mice in the previous studies are comparable to those in this study. This distinction is important for accurately interpreting the data.*

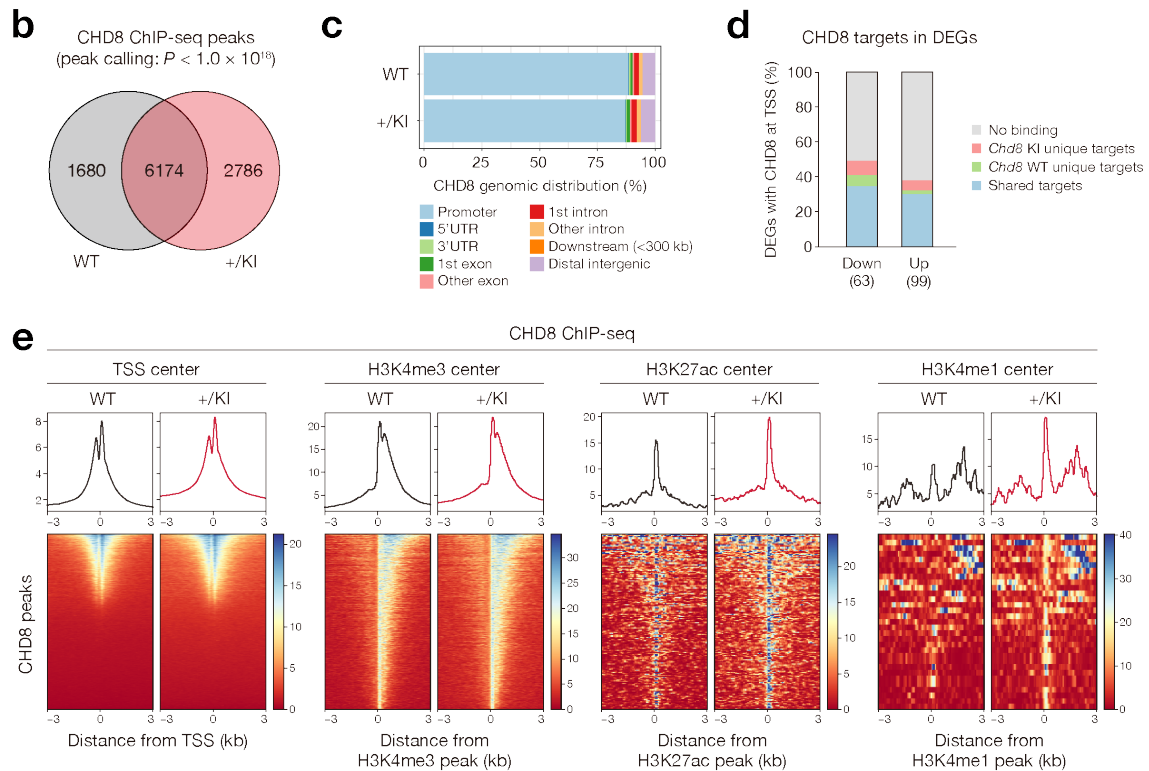
[Response] We performed RNA-seq analysis for the forebrain of *Chd8* KI mice at E14.5 and E18.5. We compared these data with previously identified up- and downregulated genes (P value of <0.05) in the whole brain of *Chd8*^{+/-} mice at the same developmental stages, E14.5 and E18.5 (Katayama *et al.*, *Nature* 537:675–679, 2016), as well as in the forebrain of *Chd8*^{+/-} mice at E14.5 (Gompers *et al.*, *Nat. Neurosci.* 20:1062–1073, 2017). Despite the differences in sample specificity between whole brain and forebrain, we observed a similar negative correlation in gene expression changes across both data sets from independent studies (Fig. 3i,j). This consistency underscores the dosage-sensitive effects of *CHD8* on gene regulation. We have now clarified these points in the Results section and provided detailed descriptions of the gene sets for DEGs in *Chd8*^{+/-} mice in the Methods section of the revised manuscript (page 10, lines 221-225; page 27, lines 639-642).

5. While the identification of 16,725 CHD8 binding peaks via ChIP-seq is noteworthy, the relatively low number of DEGs suggests that not all binding events are functionally significant. The authors might consider reanalyzing the ChIP-seq data with different peak-calling criteria to refine the analysis. Additionally, generating heatmaps centered around transcription start sites (TSS), H3K4me3, or H3K4me1 peaks (in addition to those showed in figure 4d centering around CHD8 binding peaks) in the supplemental data could provide further insights into the effects of CHD8 over-expression on enhancers.

[Response] As suggested by the reviewer, we adopted a stricter criterion for peak calling in ChIP-seq analysis in order to select only prominent signals for CHD8 binding. Application of a more stringent peak-calling threshold ($P < 1 \times 10^{-18}$) resulted in a reduction in the number of CHD8 binding peaks from the original 16,725 ($P < 1 \times 10^{-12}$) to 8960 (**new Supplementary Fig. 5b,c**), with CHD8 being bound at the TSS of <50% of DEGs in the forebrain of *Chd8* KI mice at E14.5 (**new Supplementary Fig. 5d**). These results suggest that many transcriptional changes may be mediated through enhancer activity or other indirect effects resulting from CHD8 overexpression. Whereas this stringent threshold enhances the specificity of binding site identification, the substantial decrease in peak number might potentially overlook relevant binding events. We have therefore retained the original peak data set as the primary basis for our main analyses and have also included the additional data from the more stringent peak-calling criterion to provide further insight and validation (page 11, lines 241-246).

We have also expanded our analysis to include heat maps centered not only around CHD8 binding peaks but also around key histone marks, specifically H3K4me3, H3K27ac, and H3K4me1. Our results reveal enhanced binding of CHD8 at regions marked by H3K27ac and H3K4me1 in *Chd8* KI mice, whereas no difference was observed at the TSS or H3K4me3 regions (**new Supplementary Fig. 5e**). These results support increased binding of CHD8 to enhancer regions as a result of *Chd8* overexpression. We have now addressed these points in the revised manuscript (page 11, lines 252-255).

Supplementary Figure 5



6. The conclusion that pharmacological intervention rescued the behavioral phenotypes of CHD8 KI mice is based on a single behavioral test. This assertion may be overstated. The authors should either temper this conclusion or provide additional behavioral data to substantiate the claim. Moreover, the therapeutic relevance of risperidone in this context seems limited, and this should be acknowledged in the discussion.

[Response] We agree with the reviewer that our conclusions regarding the effects of risperidone on the behavioral phenotypes of *Chd8* KI mice should be cautious. Risperidone treatment ameliorated hyperactive behavior, as evidenced by a reduction in the total distance traveled in both the open-field test and the elevated plus-maze test (Fig. 6i-n). However, other behavioral abnormalities observed in *Chd8* KI mice were not assessed for their response to risperidone, and we acknowledge this as a limitation in the revised manuscript. Furthermore, a slight but significant reduction in the total distance traveled in response to risperidone treatment was observed for WT mice in both behavioral tests (Fig. 6j,m), suggesting that the behavioral results may reflect a mild sedative effect of the drug. We believe that risperidone shows potential for amelioration of certain behavioral phenotypes in *Chd8* KI mice, but its broader relevance and translational value for CHD8-associated phenotypes require further investigation. We have now moderated our conclusions and acknowledged the limited therapeutic relevance of risperidone in the revised manuscript (page 16, lines 374-376; page 20, line 488-page 21, line 493).

Response to Reviewer #1

The authors have done an excellent job at addressing many of the questions and concerns raised during the revision process.

[Response] We thank the reviewer again for the constructive suggestions that we feel have helped us to greatly improve our manuscript.

I appreciate that the effects of risperidone on WT are showed and discussed, and the interpretation of findings revisited accordingly. I also appreciate adding the behavioral outcomes of risperidone at 0.2 mg/kg, which is currently included as a figure for the Reviewer only. I think these data should make into into the Supplement because the results raise a caveat that might useful for other studies that might be dosing risperidone.

[Response] As suggested, we agree that these findings provide an important caveat that may be informative for other studies investigating risperidone dosing. We have now included the behavioral data for risperidone at 0.2 mg/kg as a **new Supplementary Figure 9c–g** in the revised manuscript (page 16, lines 367-371).

My concern about the interpretation of the preference for social novelty in the 3-chamber test remains unaddressed. As the Supplementary Fig. 7H show, the authors fail to detect a preference in time spent with stranger vs. time spent with familiar mouse (irrespective of genotype). Hence the conclusion that "both Chd8 KI and WT mice showed a similar preference for a novel mouse (stranger 2) and a familiar mouse (stranger 1) in the social novelty preference test" is not supported.

[Response] We apologize for the previous misinterpretation of the results shown in Supplementary Fig. 7h. As the reviewer points out, both *Chd8* KI and WT mice did not show a significant preference for the novel mouse (stranger 2) over the familiar mouse (stranger 1) in the social-novelty preference test, suggesting a lack of clear social novelty preference under our experimental conditions. We have now revised the corresponding sentence accordingly and have clarified this point in the Results section of the revised manuscript (page 13, lines 301-305).

Further, the conclusions drawn from the BrdU and EdU experiments in Fig. 3 are not the very clear, as currently phrased at lines 177-180. How do the authors integrate the interpretations of reduced cell cycle exit with premature exit from the S phase?

[Response] Our BrdU and EdU experiments suggested that NPCs in *Chd8* KI mice prematurely progress through S phase while remaining in the cell cycle, rather than exiting the cell cycle and differentiating into neurons. This shift toward sustained cell cycle progression likely limits neuronal differentiation, leading to the reduction in deep-layer neuron generation in mice with *Chd8* overexpression. Supporting this interpretation, previous studies have shown that cell-cycle regulation can influence the timing and outcome of cellular differentiation, including the transition from proliferation to neurogenesis (Salomoni et al., Trends Cell Biol. 20:233–243, 2010). Furthermore, our RNA-seq analysis in the forebrain of *Chd8* KI mice revealed a downregulation of neuronal differentiation

markers and an upregulation of genes associated with the G2-M checkpoint and E2F targets, both indicative of increased proliferative activity (Fig. 3d,f and Supplementary Fig. 4e). Taken together, our findings suggest that an increase in cell cycle progression may disturb the balance between NPC maintenance and differentiation, thereby contributing to the reduction in deep-layer neuron generation in mice with *Chd8* overexpression. We have now clarified this point in the Results section of the revised manuscript (page 8, lines 161-164; page 8, line 173-page 9, line 179).

Response to Reviewer #2

In this revision, the authors have conducted additional experiments as suggested and fully address the questions and concerns I had with the manuscript.

[Response] We thank the reviewer for the positive evaluation of our manuscript. We also appreciate the time and effort taken in reviewing our paper. This reviewer has no more suggestions.