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Title
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Permalink
https://escholarship.org/uc/item/0ck9n78k

Journal
Nature neuroscience, 20(8)

ISSN
1097-6256

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Publication Date
2017-08-01

DOI
10.1038/nn.4592

Peer reviewed
Germline Chd8 haploinsufficiency alters brain development in mouse

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The chromatin remodeling gene CHD8 represents a central node in neurodevelopmental gene networks implicated in autism. We examined the impact of germline heterozygous frameshift Chd8 mutation on neurodevelopment in mice. Chd8+/del5 mice displayed normal social interactions with no repetitive behaviors but exhibited cognitive impairment correlated with increased regional brain volume, validating that phenotypes of Chd8+/del5 mice overlap pathology reported in humans with CHD8 mutations. We applied network analysis to characterize neurodevelopmental gene expression, revealing widespread transcriptional changes in Chd8+/del5 mice across pathways disrupted in neurodevelopmental disorders, including neurogenesis, synaptic processes and neuroimmune signaling. We identified a co-expression module with peak expression in early brain development featuring dysregulation of RNA processing, chromatin remodeling and cell-cycle genes enriched for promoter binding by Chd8, and we observed increased neurological proliferation and developmental splicing perturbation in Chd8+/del5 mice. This integrative analysis offers an initial picture of the consequences of Chd8 haploinsufficiency for brain development.

DNA packaging determines the transcriptional potential of a cell and is central to the development and function of metazoan cell types. Chromatin remodeling complexes control the local chromatin state, yielding either transcriptional activation or repression. Pluripotency, proliferation and differentiation are dependent on genomic regulation at the chromatin level, and proteins that control chromatin packaging are critical in development and cancer. Although many chromatin remodeling factors function across organ systems, case-solving efforts have linked mutations of chromatin genes with specific, causal roles in neurodevelopmental disorders (NDDs). This finding is particularly strong for rare and de novo mutations in autism spectrum disorder (ASD). Understanding how mutations to chromatin remodeling genes affect transcriptional regulation during brain development may reveal developmental and cellular mechanisms driving NDDs.

A key gene that has emerged from studies profiling rare and de novo coding variation in ASD is the gene CHD8, which encodes the chromatin remodeler CHD8 (chromodomain helicase DNA-binding protein). In addition to ASD, human individuals harboring CHD8 mutations exhibit macrocephaly, distinct craniofacial morphology, mild-to-severe intellectual disability (ID) and gastrointestinal problems. Homozygous deletion of Chd8 in mice is lethal at early embryonic stages. Chd8 knockdown in zebrafish recapitulates macrocephaly and gastrointestinal phenotypes, suggesting a high degree of evolutionary conservation of CHD8 function in development. It has been proposed that CHD8 achieves this regulatory function in brain development by binding to relevant gene promoters and enhancers. Although recent mouse studies indicate that Chd8 is required in neurogenesis and that mutations to Chd8 cause behavioral phenotypes, important questions remain regarding the role of Chd8 in regulating neurodevelopment, brain structure and behavior via direct and indirect transcriptional regulation. Characterizing the functional impact of germline heterozygous CHD8 mutation on brain development could reveal specific and generalizable mechanisms linking chromatin biology to NDD pathology. Toward this goal, we generated a germline 5-base-pair (bp) deletion in Chd8 using CRISPR/Cas9 genome engineering and assayed neuroanatomical, behavioral and transcriptional phenotypes associated with Chd8 haploinsufficiency in the developing mouse brain.

RESULTS
Mice harboring heterozygous germline Chd8 mutation exhibit megalencephaly
We used CRISPR/Cas9 targeting of C57BL/6N oocytes to generate mice harboring 5-bp or 14-bp deletions in Chd8 exon 5, upstream of

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Received 7 September 2016; accepted 23 May 2017; published online 26 June 2017; doi:10.1038/nn.4592
the majority of identified human mutations\(^8\) (Fig. 1a–c). F0 mutation carrier lines were expanded via breeding to wild-type C57BL/6N (WT) mice. Heterozygous male mice were bred for at least four generations before further experiments, and multiple litters were used for all experiments to eliminate potential off-target mutations. Consistent with an earlier study\(^10\), the presumed Chd8 frameshift alleles resulted in embryonic lethality in homozygous mutants, but heterozygous (Chd8\(^{+/del5}\)) mice were viable, reached a normal lifespan and were fertile irrespective of sex.

We performed quantitative reverse-transcription PCR (qRT-PCR) and western blot analysis on brain lysates, using an N-terminus Chd8 antibody that has been used previously used for Chd8 chromatin immunoprecipitation followed by deep sequencing (ChIP-seq)\(^12\). Heterozygous mutation resulted in decreased Chd8 transcript and protein at embryonic day (E)14.5, postnatal day (P)0 and in adults (Fig. 1d,e and Supplementary Fig. 1a–c). We identified a band representing full-length Chd8 (\(\sim 280\) kDa), which was consistently significantly reduced in Chd8\(^{+/del5}\) mice at all stages (Supplementary Fig. 1a–c). We also observed a smaller \(\sim 110\) kDa band, similar in size to what has been reported as a short Chd8 isoform\(^13\), which displayed inconsistent trends of lower expression in Chd8\(^{+/del5}\) mice (Supplementary Fig. 1a–c). We performed RNA sequencing on E12.5 WT mice and

![Figure 1](https://example.com/figure1.png)

**Figure 1** Chd8\(^{+/del5}\) mouse model. (a) Location of case mutations in human CHD8\(^+\) (top) and corresponding guideRNA sequence homology for Cas9-targeting of mouse Chd8 (bottom). (b) Schematic of mouse line generation. Het, heterozygous; del, deletion. (c) Sequence trace showing 5-bp deletion in exon 5. (d) qRT-PCR showing reduction of RNA in Chd8\(^{+/del5}\) (HT) forebrain at P0 (**\(P = 0.0076\); \(n = 9\) WT, 8 Chd8\(^{+/del5}\)). (e) Western blot of Chd8\(^{+/del5}\) mice, showing reduction of Chd8 protein in Chd8\(^{+/del5}\) forebrain at E14.5 (**\(P = 0.02\); \(n = 6\) WT, 6 Chd8\(^{+/del5}\) and P0 (**\(P = 0.0089\); \(n = 9\) WT, 9 Chd8\(^{+/del5}\)). (f) Whole-mount brain of Chd8\(^{+/del5}\) mouse at P0 reveals increased cortical length (denoted by orange arrow and line), indicative of malformational OB, olfactory bulb; Cx, cortex; MB, midbrain;Cb, cerebellum. (****\(P < 0.0001\); WT \(n = 4\) male, 6 female; Chd8\(^{+/del5}\) \(n = 10\) male, 10 female). (g) Representative coronal sections of WT and Chd8\(^{+/del5}\) brains at P7 visualized with Nissl stain. Scale bar, 1 mm. Pink shading, measured neocortical area; NCx, neocortex; St, striatum; CC, corpus callosum; HC, hippocampus; Th, thalamus; HTh, hypothalamus. (h) Plots of cortical area, thickness at 30% and 70% distance from the dorsal midline and cortical hemispheric circumference (dots represent individual samples; \(n = 4\) mice each for both genotypes; area, \(* P = 0.0328\); thickness (30%) \(P = 0.224\), thickness (70%) \(P = 0.268\); length, \(* P = 0.0026\). P values derived using Student’s t-test for d, e and h and using one-way ANOVA for f. Error bars represent mean ± s.e.m.
Figure 2 Chd8<sup>+/del5</sup> mice exhibit cognitive deficits but no ASD-relevant social or repetitive phenotypes. (a,b) Fear conditioning. Chd8<sup>+/del5</sup> mice exhibit deficits in learning and memory, including reduced freezing after tone-shock conditioning in both (a) context (t<sub>1,37</sub> = 2.7064, *P = 0.0104) and (b) cued assays (t<sub>1,37</sub> = 2.010, *P = 0.049). (c) Novel object. Chd8<sup>+/del5</sup> mice fail to show significant difference in exploration between a novel and familiarized object (WT: F<sub>1,19</sub> = 11.503, *P = 0.0031; Chd8<sup>+/del5</sup>; F<sub>1,18</sub> = 3.2825, *P = 0.0867). (d-f) Three-chamber social approach. Chd8<sup>+/del5</sup> mice do not exhibit differences relative to WT littermates in (d) time spent in chamber with a novel mouse (WT: F<sub>1,19</sub> = 16.31, *P = 0.0007; Chd8<sup>+/del5</sup>; F<sub>1,18</sub> = 9.744, *P = 0.0059), (e) time sniffing a novel mouse (WT: F<sub>1,18</sub> = 7.00369, *P = 0.0164; Chd8<sup>+/del5</sup>; F<sub>1,17</sub> = 12.8051, *P = 0.0023) or (f) chamber entries (F<sub>1,37</sub> = 0.11, *P = 0.73). (g-i) Male–female social interactions. Chd8<sup>+/del5</sup> mice exhibit no differences between WT littermates in (g) time sniffing (t<sub>1,17</sub> = 0.9409, P = 0.35999), (h) time following (t<sub>1,17</sub> = 0.5785, P = 0.5705) or (i) ultrasonic vocalizations with an estrus female (t<sub>1,17</sub> = 0.1634, P = 0.8722). (j,k) Repetitive behavior. Chd8<sup>+/del5</sup> mice do not exhibit any differences in (j) time spent self-grooming (t<sub>1,38</sub> = 0.8552, *P = 0.3978) or (k) number of marbles buried (t<sub>1,38</sub> = 1.0151, P = 0.3165). (l) Open field. Chd8<sup>+/del5</sup> mice do not exhibit any differences in distance traveled (t<sub>1,38</sub> = 1.1795, P = 0.2455). All data shown from first cohort. Male mice were used in g-i; males and females were used in all other panels. Unpaired t-tests used for a, b and g-i; repeated-measures ANOVA used for c-e; one-way ANOVA used for f. Error bars represent mean ± s.e.m.
indicating that Chd8+/del5 mice exhibited deficits in learning and memory.

Behaviors relevant to diagnostic symptoms of ASD were assessed using corroborative assays of social and repetitive behaviors\(^7\). Normal sociability was detected in both genotypes (Fig. 2d–i). Time spent in the chamber with the novel mouse was greater than with the novel object, meeting the definition of sociability in this assay, for both WT and Chd8+/del5 mice (Fig. 2d; WT: \(F_{1,19} = 16.31, P = 0.0007; \) Chd8+/del5: \(F_{1,18} = 9.744, P = 0.0059\)). No sex differences were detected (\(F_{1,37} = 2.16, P = 0.149\)). Time spent sniffing the novel mouse was greater than time spent sniffing the novel object in both WT and Chd8+/del5 mice (Fig. 2e; WT: \(F_{1,18} = 7.00369, P = 0.0164; \) Chd8+/del5: \(F_{1,17} = 12.8051, P = 0.0023\)), with no sex differences (\(F_{1,35} = 0.9985, P = 0.7555\)). The number of entries into the side chambers was not affected by genotype in the social phase (Fig. 2f; \(F_{1,37} = 0.11, P = 0.73\)) or in the habituation phase (\(F_{1,37} = 0.30, P = 0.584\)), indicating normal exploratory activity in both genotypes during the assay. No deficits were observed in social parameters in male Chd8+/del5 mice during male–female reciprocal social interaction (Fig. 2g–i). WT and Chd8+/del5 males spent similar amounts of time sniffing (Fig. 2g; \(t_{1,17} = 0.9409, P = 0.3599\)) and following (Fig. 2h; \(t_{1,15} = 0.5785, P = 0.5705\)) the estrous WT female. Ultrasonic vocalizations during male–female interaction showed no genotype difference in number of emitted calls (Fig. 2i; \(t_{1,17} = 0.1634, P = 0.8722\)). No spontaneous stereotypies or repetitive behaviors were observed in the self-grooming assay (Fig. 2j; \(t_{1,38} = 0.8552, P = 0.3978\)) or numbers of marbles buried (Fig. 2k; \(t_{1,38} = 1.0151, P = 0.3165\)). No sex differences were detected (self-grooming: \(t_{1,36} = -0.504, P = 0.619; \) marble burying: \(t_{1,38} = 1.4883, P = 0.1449\)). Open-field locomotor activity did not differ between genotypes (Fig. 2l; \(t_{1,38} = 1.1795, P = 0.2455\)), indicating normal exploratory and motor abilities. No substantial differences were observed in body weight or other relevant measures of general health in adult Chd8+/del5 mice.

### Analysis of Chd8+/del5 adult brain structure via MRI

Intact brains were collected from the same mice that comprised the first cohort of behavioral phenotyping. Structural MRI was performed to identify changes in absolute (mm\(^3\)) and relative regional brain volume and connectivity. In regional analysis, cortex was most affected, with a 7.5% increase in absolute volume in Chd8+/del5 mice (false discovery rate (FDR) = 1%). Similarly, cerebral white matter and cerebral gray matter were larger in Chd8+/del5 mice at 5.4% (FDR = 3%) and 6.1% (FDR = 2%), respectively. We assessed 159 independent brain regions with divisions across the cortex, subcortical areas and cerebellum (full results reported in Supplementary Table 1). Chd8+/del5 mice showed robust increases in absolute volume across cortical regions, hippocampus (+10.3%, FDR < 1%) and amygdala (+11.0%, FDR < 1%; Fig. 3a).
Figure 4 Differential gene expression in Chd8<sup>+/del5</sup> neurodevelopment. (a) Schematic of our experimental pipeline. DPC, days postconception. (b) Volcano plot showing that most DE genes (red) exhibit relatively subtle fold changes (FC). (c) Chd8 is the top differentially expressed gene; panel shows relative expression in Chd8<sup>+/del5</sup> and WT littermates across brain development. DPC, days post conception. (d) Example expression patterns in Chd8<sup>+/del5</sup> forebrain of three DE genes across developmental stages. DE FDR shown for c and d. Solid lines, means; dashed lines, ±1 s.e.m. (e) Validation of DE expression of Hnmp2b1 RNA (left; n = 6 WT, 4 Chd8<sup>+/del5</sup>) and protein (right; n = 5 WT, 5 Chd8<sup>+/del5</sup>) in Chd8<sup>+/del5</sup> (HT) forebrain at P0 (Student’s t-test; RNA, *P = 0.0248; protein, **P = 0.0055). Error bars represent mean ± s.e.m. (f) Chd8 ChIP-seq at Hnmp2b1 and Adnp loci. (g) Representative significant GO terms for DE genes and for genes whose promoters are bound by Chd8. (h) Enrichment of autism-associated genes (Q < 0.3 from Sanders et al.,<sup>10</sup> P = 2.8 × 10<sup>−10</sup>) in our DE gene set, based on randomly sampled gene sets (bars) vs. observed number (red line). (i,j) Comparison of DE down- and upregulated genes identified here with autism- and Chd8-associated genes. Directionality on x-axis represents test gene set used in analysis (left of zero: downregulated DE genes; right of zero: upregulated DE genes); z-scores generated via permutation test. Vertical lines are at z = ±2.

After correction for total brain volume, relative volumes were still significantly larger, though cortex failed to surpass the FDR < 5% cutoff (Fig. 3b). Chd8<sup>+/del5</sup> mice also displayed increased cortical thickness, particularly along the cingulate cortex (Fig. 3c). Deep cerebellar nuclei showed decreased relative volume (~1 to ~3%, FDR < 0.3%). Voxel-wise differences showed similar trends (Fig. 3d). Diffusion tensor imaging revealed no significant differences in fractional anisotropy or mean diffusivity in either the regional or voxel-wise measurements, indicating that white matter organization and long-range connectivity in Chd8<sup>+/del5</sup> mice was not grossly different from that in WT littermates (Supplementary Fig. 3). Performing behavioral and structural MRI analyses on the same set of mice allowed us to test correlations between brain volume and behavioral performance. Increased absolute volume of cerebral cortex (f-stat = 33.6, FDR < 0.1%), hippocampus (f-stat = 29.0, FDR < 0.1%) and amygdala (f-stat = 38.6, FDR < 0.1%) were correlated with deficits in learning and memory, as assessed by the context conditioning task (R<sup>2</sup>: cortex = 0.1855, hippocampus = 0.148, amygdala = 0.1352; Fig. 3e).
This relationship was driven by Chd8<sup>+/del5</sup> mice with increased brain volume. Similar correlations were present between other brain regions and cued and context responses, suggesting that the correlation between fear conditioning performance and brain volume was a general rather than region-specific relationship.

**Differential gene expression across neurodevelopment in Chd8<sup>+/del5</sup> mice**

We applied RNA-seq in forebrain dissected from four developmental stages (E12.5, E14.5, E17.5 and P0) and adult mice (age > P56; Fig. 4a). This strategy was designed to capture changes during embryonic neurodevelopment but has decreased sensitivity for changes limited to postnatal or adult brains. After quality filtering, we analyzed 26 Chd8<sup>+/del5</sup> and 18 WT littermates (sample details in Supplementary Table 2). Using a statistical model that accounted for sex, developmental stage and sequencing batch, we tested for differential expression across 11,936 genes that were robustly expressed in our data sets. At significance cutoffs corresponding to FDR < 0.05 (<i>P</i> < 0.0021), FDR < 0.1 (<i>P</i> < 0.0088) or FDR < 0.2 (<i>P</i> < 0.0369), we found 510, 1,040 and 2,195 genes, respectively, that were differentially expressed (DE; Supplementary Table 3).
The majority of significant expression changes in Chd8^{+/del5} were relatively small (99.5% < 1.5 absolute fold change across phases, Fig. 4b), indicating that changes in neurodevelopmental gene expression were widespread yet subtle. The top DE gene was Chd8 (log \_fold change = 0.59, P = 2.20 \times 10^{-27}, FDR = 3.18 \times 10^{-23}). Chd8 expression declined across development, with substantial reductions in Chd8^{+/del5} mice at each stage (Fig. 4c). We validated expression changes at P0 for a set of genes via qRT-PCR (Supplementary Fig. 4; primers are listed in Supplementary Table 4), including Hnrnpa2b1, for which we additionally validated differential protein levels in P0 Chd8^{+/del5} forebrains (Fig. 4d,e). Gene set enrichment analysis of Gene Ontology (GO) terms and Reactome pathways identified strong enrichment among DE genes for annotations associated with RNA processing, chromatin remodeling and cell cycle, with numerous additional annotations enriched at lower levels (Supplementary Tables 5 and 6). Similar enrichment was observed for DE genes at FDR cutoffs of 0.05, 0.10 and 0.20.

To identify direct regulatory targets of Chd8, we used ChIP-seq to map regions of Chd8 genomic interactions in adult mouse forebrain. After merging two independent samples with similar binding patterns, we identified 708 peaks using stringent enrichment criteria (Fig. 4f and Supplementary Table 3). Chd8 binding occurred nearly exclusively at gene promoters in our adult forebrain data set. We observed strong concordance in enriched functional annotation terms between DE and Chd8-bound genes (Fig. 4i). In contrast, we found no enrichment among upregulated DE genes for Chd8 promoter binding. We did not find evidence of a primary sequence motif in Chd8 ChIP-seq peak regions, suggesting indirect genomic recruitment, but we found weaker, yet significant, enrichment of secondary motifs such as YY1 (e = 4.2 \times 10^{-31}), NRF1 (e = 8.5 \times 10^{-24}) and NFYB (e = 1.3 \times 10^{-17}; Supplementary Fig. 5a).

In agreement with other studies of transcriptional changes associated with reduction of Chd8 expression, autism risk genes such as Kdm5b and Bcl11a were DE in Chd8^{+/del5} mice (Fig. 4d). We tested for overlap between DE downregulated and upregulated genes...
(FDR < 0.20) and published gene sets relevant to autism genetics and Chd8 regulation. Of 141 ASD risk genes based on case mutations expressed in our data, 37 were DE at FDR < 0.20 and downregulated, representing strong enrichment (permutation test, \( P = 2.8 \times 10^{-10} \); Fig. 4h). We similarly observed enrichment among downregulated DE genes with autism risk genes identified by other studies\(^{20,21}\) (Fig. 4i) and with FMRP (fragile X mental retardation protein) targets\(^{22}\) (\( P = 0.04 \)). Finally, we observed enrichment (\( P = 0.012 \)) between DE upregulated genes and genes associated with immune response that were upregulated in cortex from postmortem ASD brains\(^{23,24}\). We did not identify enrichment with genes downregulated in postmortem ASD cortex.

Next, we asked whether our DE data was consistent with differential expression in independent studies of Chd8 mutation or knockdown\(^{11-13,15}\). We observed consistent enrichment among up- and downregulated DE genes in Chd8\(^{+/del5}\) forebrain and up- and downregulated DE genes identified in previous studies (Fig. 4j). We also used the same methods used here to reanalyze neurodevelopmental RNA-seq data from independent studies of germine heterozygous Chd8 mutation\(^3\) and in utero knockdown via Chd8 shRNA delivery to E14.5 brain ventricle\(^{15}\) (Supplementary Fig. 5b,c and Supplementary Table 3). Chd8 was more strongly downregulated in the knockdown model\(^3\) and upregulated in the independent Chd8 mouse model\(^15\). Nonetheless, we observed significant overlap between genes identified here and in the other two studies (Fig. 4j). For overlapping DE genes from our data and the other two studies, DE sensitivity and effect sizes were generally larger in the knockdown data\(^{15}\) and smaller in the independent germine model\(^15\) compared to our data (Supplementary Fig. 5b,c), suggesting differences in transcriptional consequences after knockdown and across different alleles or genetic backgrounds. We noted a reversal in direction of some specific DE effects in the knockdown data versus our data, including for neuronal differentiation genes (downregulated here but upregulated after knockdown), further suggesting differential impact of knockdown versus germine mutation.

We next explored how DE genes are organized into expression trajectories during brain development toward identifying perturbation to stage-specific processes. We used weighted gene co-expression network analysis (WGCNA\(^{25}\)) to identify five discrete expression trajectory modules across forebrain development (Fig. 5a–c and Supplementary Table 7). DE genes assigned to specific modules were enriched for stage-specific annotation terms (Fig. 5d and Supplementary Table 5). Two modules (M.1 and M.3) were enriched for downregulated genes, while the other three modules (M.2, M.4 and M.grey) were enriched for upregulated genes (Fig. 5e). M.1, characterized by decreasing expression across neurodevelopment, was strongly associated with chromatin organization, RNA processing and cell-cycle regulation, and it included the largest number of DE downregulated autism risk genes (Fig. 5f). M.1 was the only module enriched for targets of Chd8 binding. M.1 downregulated DE genes overlapped genes in early expressed ASD-relevant networks\(^{26}\) (Parikshak.DEV.M2 and Parikshak.DEV.M3; Fig. 5g). M.2 was characterized by early low expression that gradually increased. Downregulated genes in M.2 were enriched for FMRP targets\(^{22}\), GO and Reactome terms that are hallmarks of mature neurons (Supplementary Tables 5 and 6) and include synaptic genes (Supplementary Fig. 5d) and autism risk genes such as Cers4 and Gria1. DE genes from M.2 were enriched for later developmental ASD-relevant modules\(^{26}\) (Parikshak.DEV.M13, Parikshak.DEV.M16 and Parikshak.DEV.M17) linked to synaptic development and homeostatic processes, consistent with developmental timing (Fig. 5g).

M.3 genes exhibited rising expression from E12.5 to P0, with lower expression in adult brain, were enriched for GO terms associated with transient development processes (for example, axon guidance) and include autism risk genes involved in neuronal maturation (for example, Bel11a). Compared to genes identified as DE in postmortem ASD cortex\(^4\), we identified enrichment with two modules characterized by increased expression of genes linked to immune function and cell identity of astrocytes (Parikshak.ASD.M9, enriched for upregulated genes in our M.2 and M.4) or microglia (Parikshak.ASD.M19, enriched for upregulated genes in our M.1; Fig. 5g). These results show that the impact of Chd8 haploinsufficiency reaches across stages and biological processes.

We further focused on M.1, which showed the strongest enrichment for autism risk early developmental modules\(^{26}\) and for Chd8 binding targets in mouse brain (Fig. 5f,g). M.1 showed a general trend of decreasing expression (Fig. 6a), had the greatest number of DE genes and was significantly enriched for downregulated genes (\( P = 8.8 \times 10^{-26} \)). We found 865 genes in M.1 that were DE at FDR < 0.20 (641 downregulated, 224 upregulated), accounting for ~39% of all DE genes identified in our study. Upreregulation of M.1 genes peaked at E14.5; downregulation peaked from E14.5 to E17.5 (Fig. 6b). Analysis of protein–protein interactions (STRING\(^{27}\)) showed that DE (FDR < 0.10) genes in M.1 had more interactions than expected by chance (observed edges = 1,479, expected edges = 512, enrichment = 2.89, STRING \( P < 0.0001 \)). Interacting genes in M.1 were enriched for GO terms.
including RNA processing, chromatin modification and cell cycle (Fig. 6c). While protein–protein interaction databases have biases, these results highlight interconnectedness among these three processes at the level of Chd8 regulation and gene expression. M1 DE genes include 38 autism-relevant genes, including many annotated to the highlighted GO terms (Fig. 6d).

**Increased prenatal proliferation of neural progenitors in Chd8del5 mice**

To examine whether alterations in developmental genes play a functional role in neuronal development that could lead to megalencephaly, we performed 5-ethyl-2′-deoxyuridine (EdU) proliferation assays at E13.5. After a 1.5-h pulse, we observed a 15.9% increase in EdU+ cells in the germinial cortical ventricular and subventricular zones (VZ and SVZ, respectively) of mutant animals (P = 0.0388; Fig. 6e), indicating perturbed neurogenesis in the Chd8del5 mutants. Additionally, since a number of genes associated with brain development and cortical structure were DE, we examined the cortical cytoarchitecture via analysis of layer-specific markers Tbr1, Ctip2 and Brn2 by immunostaining at P0 and P7 (Supplementary Fig. 6). We observed no gross alterations to lamination and found no evidence for focal cortical lesions. To further delineate proliferative changes in
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**DISCUSSION**

Modeling how constitutive heterozygous germline mutations impact mammalian brain development is critical to understanding the neurobiology of disorders like ASD and ID, which are strongly associated with single-copy loss-of-function mutations. After validation that germline 5-bp and 14-bp deletion mutations in Chd8 exon 5 resulted in haploinsufficiency, we present here an initial integrative picture of the consequences of Chd8 haploinsufficiency on neurodevelopment. The presence of genomic and neuroanatomical phenotypes in our Chd8+/del5 mice paralleled the clinical signature of human CHD8 mutations, suggesting similar neurodevelopmental pathologies between species. Our study revealed NDD-relevant phenotypes and mechanistic insights into why haploinsufficiency of a general chromatin factor produces neurodevelopmental phenotypes.

We report behavioral outcomes in our heterozygous Chd8+/del5 mice, specifically learning and memory impairments, but no atypical sociability or repetitive behaviors. There are three recent publications associated with downregulation of Chd8 in mice. Durak et al. reported an in utero knockdown of Chd8 expression, including restricted knockdown of Chd8 in upper cortical layer neurons. Katayama et al. generated two lines of Chd8 mutant mice via embryonic stem cell targeting, while Platt et al. used CRISPR/Cas9 targeting to generate indel mutations in Chd8. The obvious differences in biological consequences and mechanisms between in utero knockdown and our germline heterozygous model likely explain many differences and make cross-approach comparisons a challenge. Our data on the absence of repetitive behaviors agree with those reported in Katayama et al. and Platt et al.; however, these studies also report specific social phenotypes in heterozygous Chd8 mice. We focus our comparison on the Katayama et al. study as we were able to directly compare neurodevelopmental gene expression changes to our model. We observed differences in strength of expression changes that may be due to allele or genetic background. Katayama et al. report phenotypes relevant to social deficits but report normal acquisition learning using the Barnes and T-maze assays. Discrepancies in cognitive phenotypes could be attributed to the tasks conducted, since spatial maze tasks are primarily hippocampal-dependent, while components of fear conditioning and novel object recognition require other brain regions, including amygdala. Additionally, our Chd8+/del5 mutation was generated and maintained on a C57BL/6N background, while Katayama et al. harbored a different mutation on C57BL/6J. Background strain differences in behavioral phenotypes are common, and C57BL/6N generally show higher basal freezing during contextual fear freezing, which allows for a larger signal and detection of genotype differences.

Our finding of normal sociability is consistent with results reported for other Chd8+/− germline models. However, both other models report abnormalities in social behavior based on failure of Chd8+/− mice to exhibit preference for social novelty between a novel and familiar mouse. Preference for social novelty is dependent on olfactory discrimination, which is driven by a variety of sensory and processing systems. As such, the combination of normal three-chamber assay sociability but failure to discriminate a novel mouse in these models could represent mild deficits in social interaction or could be due to other factors impacting olfactory discrimination. Another difference is that Katayama et al. tested only male mice with a broad age range of 12–50 weeks of age, while our battery was conducted using both sexes at younger ages, between 6–16 weeks, to avoid aging as a confounding variable. We also note that several studies of other mouse models with mutations in genes implicated in ASD have not identified deficits in social

*Chd8+/del5* mice, we assessed neural progenitor populations at E14.5 (Fig. 7). First, we examined Pax6+ radial glial cells, measuring VZ area over the entire cortical hemisphere. The VZ in *Chd8+/del5* embryos was significantly increased, by ~18% (Fig. 7a; P = 0.0165). We observed a ~26% increase in Pax6+ cells in Chd8+/del5 brains (Fig. 7b; P = 0.0032). Subsequently, we labeled Tbr2+ intermediate progenitors, observing a significant decrease by ~24% in Chd8+/del5 mice (Fig. 7c; P = 0.0086), indicating different proliferative trajectories for these two progenitor types. Finally, we performed pulse-chase assays to determine the quiescent (Q) fraction of cortical cells exiting the cell cycle over a defined 20 h, using the proliferation marker Ki67. EdU+Ki67+ cells were significantly increased, corresponding to an increase in Q fraction by ~25% in Chd8+/del5 embryos (Fig. 7d; P = 0.0057). These results indicate that alteration to cortical projection neuron production may represent a cellular substrate for megalencephaly in Chd8+/del5 mice.

**Neurodevelopmental RNA processing is perturbed in Chd8+/del5 mice**

Downregulated DE genes with divergent expression trajectories were significantly overrepresented among genes annotated to RNA processing and mRNA splicing in the Reactome database (Fig. 8a, b and Supplementary Table 6). For example, Dhs9 (M.1) decreases across neurodevelopment and has not been functionally characterized in brain but has been reported in autism risk networks, while Upf3b (M.3) expression increases across development and is a neuron-specific factor required during neuronal differentiation that is implicated in ID. We examined whether Chd8+/del5 mice exhibited aberrant splicing during brain development linked to DE of RNA-processing genes. We used the Mixture of Isoforms (MISO) program to examine our RNA-seq data for differential splicing (DS) between WT and Chd8+/del5 mice and across neurodevelopment in WT mice (Supplementary Table 8). Genes associated with a DS event in Chd8+/del5 mice significantly overregulated downregulated (P = 1.2 × 10⁻⁶) but not upregulated DE genes, raising the possibility that splicing changes explain some proportion of differential expression (Supplementary Fig. 7a). DS genes identified in Chd8+/del5 mouse brain were enriched for DS-associated genes identified in postmortem ASD cortex (P = 1.2 × 10⁻⁷), suggesting that DS in Chd8+/del5 mice is linked to ASD-relevant DS (Supplementary Fig. 7a).

At E17.5, MISO identified 591 DS events between WT and Chd8+/del5 mice, of which 393 (~66%) were also DS between E14.5 WT and E17.5 WT mice (Fig. 8c). These results suggest that differential splicing between WT and Chd8+/del5 mice was linked to developmental changes in splicing. To investigate this, we examined correlation between DS events present in both E14.5 WT versus E17.5 WT and E17.5 Chd8+/del5 versus E17.5 WT comparisons. Percentage spliced in (PSI) values of DS events identified in the E17.5 Chd8+/del5 versus E17.5 WT comparison correlated positively with PSI values in the E14.5 WT versus E17.5 WT comparison (Fig. 8d). This correlation suggests DS in E17.5 Chd8+/del5 mice corresponds to an intermediate developmental state between WT E14.5 and E17.5. We validated developmentally relevant Chd8+/del5 DS using a known neurogenic splicing event, inclusion or exclusion of a ~6-kb Ank2 exon 31. Inclusion of the Ank2 exon increased between E12.5 and E17.5 in WT (Fig. 8e), consistent with expectations. qRT-PCR analysis of the Ank2 exon validated the exon inclusion increase across development in WT and the decrease from E17.5 WT levels in E17.5 Chd8+/del5 mice (Fig. 8f). We also validated developmental splicing changes in Srsf7 detected via MISO analysis (Supplementary Fig. 7b). Our results suggest that perturbed splicing in Chd8 haploinsufficiency may contribute to the neurodevelopmental phenotypes in Chd8+/del5 mice.
behaviors, potentially due to evolutionary divergence between mouse and human neurobiology, allele or genetic background differences, or sensitivity of methods used to evaluate ASD-relevant behavior in mice.

The structural changes in the brain of adult Chd8^{+/del5} mice observed here parallel those found in other relevant mouse models. A recent study examined 26 different mouse models related to autism, clustering these models into three distinct groups. Key aspects of Group 1 included larger cortical structures, particularly the frontal and parietal lobes, and smaller structures in the cerebellum, which is in line with the Chd8^{+/del5} mouse described here. This group of models included Nrnx1a, Shank3, Em2 and Fmr1 mutants. The Chd8^{+/del5} mouse most resembled the differences found in the Fmr1 mutant mice. Further examination may reveal similarities with other mouse models within this group beyond neuroanatomy (for example, excitatory deficits in the Nrnx1a mouse), as suggested by the widespread transcriptional changes present in Chd8^{+/del5} neurodevelopment. Increases in cortical anteroposterior length and developmental neurogenesis appear largely overlapping in Chd8^{+/del5} mice and Wdys3 mutants, another model of megalencephaly in ASD. Future work will be needed to test for causal relationships between structural changes and behavior in Chd8^{+/del5} mice.

Our RNA-seq analysis captured subtle changes in transcription across brain development in Chd8^{+/del5} mice, as well as evidence of differential splicing. Differential expression changes were consistent across developmental stages for perturbed genes highly relevant to ASD-associated networks and strongly correlated with biological pathways and expression modules of interest. Our network analysis, using in vivo data, enabled characterization of the impact of Chd8 haploinsufficiency across neurodevelopment at a level of detail sufficient to capture perturbations across developmental stages and processes. Our results indicate convergent neuropathology connecting principle gene networks identified in ASD case sequencing studies, particularly chromatin remodeling, neuronal differentiation and synaptic pathways, and those identified in other ASD-relevant pathways such as immune response and FMRP binding. Our findings suggest a hierarchy of changes in Chd8^{+/del5} brain development anchored by dysregulation of genes in M.1, which represents a highly interactive network central to control of chromatin state, RNA processing and cell cycle, including numerous genes implicated in ASD. Among these genes, our results suggest direct role and requirement for WT Chd8 expression levels in activating target gene expression. As seen with other NDD genetic models such as Fmr1 (ref. [37]) and Rbfox1 (ref. [38]), disruption to RNA processing, likely driven by indirect transcriptional regulation here, appears to be an important player in Chd8^{+/del5} neuropathology. Further studies are needed to capture neuroanatomical and cellular changes associated with differential expression signatures and to determine stage- and cell-specific patterns and roles of Chd8 binding. This is especially critical given that contrasting Chd8 genomic interaction patterns have been reported in brain11–14.

We report overall increased proliferation during the peak window of embryonic neurogenesis, with different consequences for the progenitor populations involved. The observed overall increase in proliferation in our experiments contrasts with the decreased proliferation reported in an in utero Chd8 knockdown model. These differences could be explained by the timing of in utero knockdown at E14.5 (after initial symmetric expansion of the radial glia pool), by the presence of non-cell-autonomous effects or due to differences in proliferation assay timing. Time-point-specific analysis of cell cycle, proliferation and apoptosis will be needed to understand the comprehensive impact of Chd8 haploinsufficiency on cell populations in the brain. It is possible that contrasting outcomes are due to differences in Chd8 dosage, considering that knockdown resulted in greater Chd8 downregulation. As complete Chd8 loss impedes embryonic development at very early stages, downregulation beyond haploinsufficiency may interfere further with developmental cellular pathways. In support of this, neuronal development genes downregulated in our M.3 module were upregulated after in utero knockdown, suggesting that knockdown caused an early shift from proliferation to differentiation that did not appear to occur with heterozygous germline mutation.

Our initial survey of mice heterozygous for mutation to Chd8 revealed significant findings across genomic, anatomical and behavioral axes of neurobiology. Our experiments link cognitive deficits, increased regional brain volume and perturbations of biological pathways across neurodevelopment, recapitulating traits observed in human individuals who carry mutations in CHD8. The transcription data generated here represent a resource for dissecting the pathways involved in NDD pathogenesis and for prioritizing risk genes from genetic studies. Additional studies will be necessary to replicate and compare findings across mutant Chd8 alleles and genetic backgrounds and to clarify dosage-specific phenotypes. Our results offer insight into neurodevelopmental pathology associated with mutations to CHD8, a genetic model that appears to be a bellwether for mutations affecting chromatin remodeling in autism.

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ACKNOWLEDGMENTS
Sequencing was performed at the UC Berkeley and UC Davis DNA cores. This work was supported by institutional funds from the UC Davis Center for Neuroscience, by the UC Davis MIND Institute Intellectual and Developmental Disabilities Research Center (U54 HD079125) and by NIGMS R35 GM119831, L.S.-F. was supported by the UC Davis Floyd and MarySchwall Fellowship in Medical Research and by grant number T32-GM00679 from NIGMS-NIH. A.A.W. was supported by Training Grant number T32-GM007377 from NIH-NIGMS. R.C.-P. was supported by a Science Without Borders Fellowship from CNPq (Brazil). A.V., L.A.P. and D.E.D. were supported by National Institutes of Health grants R24HL123879, U01DE024427, R01HG003988, U54HG006997 and UM1HL098166. Research conducted at the E.O. Lawrence Berkeley National Laboratory was performed under Department of Energy Contract DE-AC02-05CH11231, University of California. J.E. and J.P.L. were supported by the Canadian Institute for Health Research, Brain Canada and the Ontario Brain Institute.

AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Generation of Chd8 mutant mice. We used Cas9-mediated mutagenesis of C57BL/6N oocytes to generate two mouse lines harboring frameshift deletions (5 bp and 14 bp) in mouse Chd8 exon 5. Guide RNA was designed and synthesized according to described methods.13, pooled with Cas9 mRNA and injected into mouse oocytes. We scanned the Chd8 coding sequence for unique gRNA target sites, identifying one in exon 5 with sequence GAGGGAGGGCGATGTAAC. This sequence maps uniquely to the target site via BLAT (mm9), reducing the likelihood of off-target mutations. Initial Cas9 targeting was performed at Lawrence Berkeley National Laboratory. F0s (induced on C57BL/6N background) carrying mutations were genotyped and bred to expand lines that harbored a mutation. We identified F0 pups carrying 5-bp (mm9: chr14:52,847,259–52,847,263) and 14-bp deletions (mm9: chr14:52,847,249–52,847,262) in Chd8 exon 5 that overlapped the target sequence. The deletions occur at position 1,814 (5-bp deletion) or 1,813 (14-bp deletion) of Chd8 mRNA (uc007tot.1). Both alleles match wild-type Chd8 amino acid sequence through position 604, after which the 5-bp and 14-bp deletion frameshifts are predicted to result in 16 and 14 altered amino acids, respectively, before a stop codon is reached. The full-length Chd8 protein is predicted to be 2,582 amino acids long. Both deletion alleles are predicted to result in nonsense-mediated decay based on reduced frequency of mutant transcripts and decreased Chd8 protein levels. Deletion alleles were subcloned to verify DNA changes, and genotyping was performed using allele-specific PCR with sequence verification.

The colony of animals carrying the 5-bp Chd8 deletion allele was rederived at UC Davis and maintained by crossing male carriers with C57BL/6N wild-type females (Charles River). Extensive crossbreeding of heterozygous mutation carriers to wild-type animals vastly reduces the likelihood that any potential off-target mutations caused by Cas9 targeting would persist in our mutant Chd8 line. Genotypes were identified via allele-specific PCR and sequence-verified for all animals included in analyses, with the primers reported in Supplementary Table 4. We examined Chd8 protein and transcript levels via western blot and qRT-PCR at E14.5 and P0 and compared cortical length in whole-brain P0 brains from Chd8+/del5 mice and matched WT littermates. For all experiments, samples from both males and females were used unless otherwise described. All mouse studies were approved by the Institutional Animal Care and Use Committees at the University of California Davis and the Lawrence Berkeley National Laboratory. Subject mice were housed in a temperature-controlled vivarium maintained on a 12-h light–dark cycle. Efforts were made to minimize pain and distress and the number of animals used.

qRT-PCR. Differential expression of selected gene targets was verified by qRT-PCR at P0, using cDNA libraries prepared following the same protocols listed in the "Genomics" section, below. Primers are reported in Supplementary Table 4. For qRT-PCR analysis, n = 9 WT and 7 Chd8+/del5 forebrains from male and female samples were used. Samples were excluded if technical replicates failed. Cycle counts were normalized to Actb. Unpaired t-tests were performed on normalized relative gene expression between WT and Chd8+/del5 brains using ∆∆CT. To reduce noise, the highest and lowest values from both groups were discarded. To validate differential splicing, we used isoform-specific quantitative reverse-transcription PCR on cDNA libraries prepared from forebrains dissected from Chd8+/del5 and matched WT littermates. We used primers validated for isoform analysis of Ank2 expression during development14 and designed primers specific to a differentially spliced exon of Srsf7. Comparisons of splicing across stages and between Chd8+/del5 and matched WT littermates were analyzed using Welch’s t-test and linear regression.

Western blot analysis. Isolated forebrain from E14.5 embryos (male and female littermates), P0 neonates and P60 adults were lysed in 50 mM Tris HCl, pH 8, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40 and 0.25% Triton with lysis buffer (cortical area). Hemispheric circumferences were measured from the skull, embedded in 2% LTE Agarose/PBS and cut coronally in 50-µm sections on a vibratome (VT 1000S, Leica). Analysis of cortical length measurements was performed with Fiji software (National Institutes of Health).

Morphological analysis. All histological experiments were performed at least in triplicate on embryos/pups from at least two separate litters. Following anesthesia, P7 male and female mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), followed by overnight fixation in the same solution. After fixation, brains were removed from the skull, embedded in 2% LTE Agarose/PBS and cut coronally in 50-µm sections on a vibratome (VT 1000S, Leica). Subsequently, the sections were mounted on glass slides (SuperFrost Plus, Thermo-Fisher), Nissl-stained with a 0.1% cresyl violet solution and mounted with DePeX (Electron Microscopy Sciences, Hatfield, PA). Select sections, approximately corresponding to adult bregma ~2 mm, were aligned across genotypes using subcortical anatomical landmarks for orientation (hippocampal length, thalamic size), and images of entire hemispheres were acquired on a Keyence BZ microscope. We measured individual morphological parameters using the Keyence BZ analyzer (hemispheric/neocortical circumference, neocortical thickness) or Fiji software (cortical area). Hemispheric circumferences were measured from the dorsal to the ventral midline. Neocortical circumferences were measured from the dorsal midline to a line perpendicular to the midline originating from the dorsal endopiriform nucleus.

EdU labeling and immunofluorescent analysis. Litters for neuroanatomy analysis were generated by breeding male Chd8+/del5 mice with WT females. Brains were perfused before isolation, embedding and sectioning. Experimenters were blinded to genotype. Both male and female samples were used in the following assays.

Time-pregnant females were intraperitoneally injected at E13.5 with 50 mg/kg body weight EdU. After 1.5 h (proliferation assay) or 20 h (Q-fraction analysis), brains were anesthetized and embryos transcardially perfused with 4% PFA/PBS. After 2 h of further fixation in the same solution, embryo brains were immersed in 15% and then 30% sucrose in PBS, placed in OCT compound (Fisher HealthCare, Houston, TX), frozen in dry-ice-chilled methanol and sectioned at 16 µm (1.5-h pulse) or 18 µm (20-h pulse) on a cryostat (Leica Biosystems, Buffalo Grove, IL). EdU detection was performed with the Click-IT EdU Alexa Fluor 594 imaging kit protocol (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Q-fraction analysis followed previously established practices.16 In brief, midcortical 200-µm-wide sections were imaged and EdU Ki67+ cells positioned basal to the SVZ counted, followed by the count of all EdU+ cells. The ratio of the two counts represented the Q-fraction. One sample each from WT and Chd8+/del5 was removed from Q-fraction analysis due to difficulties in selecting the VZ. All immunolabeling was carried out on slide-mounted cryosections (18 µm) following standard protocols and using primary antibodies directed against Pax6 (ref. 42) (PRB-278P-100; rabbit, 1:100, Covance, Princeton, NJ), Tbr2 (http://www.abcam.com/TBR2–Eomes-antibody-EPR19012-ab183991.pdf; ab183991; rabbit, 1:400, Abcam, Cambridge, United Kingdom) or Ki67 (ref. 43) (#12202; rabbit, 1:200, Cell Signaling, Danvers, MA). Alexa Fluor secondary antibodies (488 and 594) were used at 1:200 concentrations (Thermo Fisher Scientific, Waltham, MA). All quantifications of labeled cells were carried out at equivalent anteroposterior positions between genotypes. To determine the dorsoventral position of cortical segments for cell type counts, we measured the cortical ventricle from the corticostriatal boundary to the apex, defined the 50% position and centered at this position a 200-µm-wide box perpendicular to the ventricle. All imaging was carried out on a Nikon A1 laser scanning confocal microscope.

Lamination assay. P0 or P7 brains were fixed and sectioned as described for “Morphological analysis,” above. Slides were incubated for 24 h at 4 °C in a
blocking solution containing normal donkey serum (5% v/v) diluted in PBS-T (1× phosphate buffered saline and 0.01% (v/v) Triton X-100), rinsed in PBS-T and incubated for 24 h at 4 °C in primary antibody solution containing anti-Chip2 (ref. 44) (ab18465; Abcam), anti-Tbr1 (ref. 44) (ab31940; Abcam) and anti-Brn2 (ref. 45) (sc-6029; Santa Cruz Biotechnology) antibodies, each diluted 1:500 in PBS-T. These antibodies have been previously used for IHC analysis in brain56,47. The slides were then rinsed in PBS-T and incubated overnight at 4 °C in fluorophore-conjugated secondary antibodies (711-545-152 and 712-165-153; Jackson ImmunoResearch). Slides were rinsed in PBS-T, counterstained for 2 h in DAPI (D1306, Thermo-Fisher), rinsed in PBS-T and coverslip-mounted with Fluormount-G (SouthernBiotech). Images were imported into FIJI ImageJ (v. 1.50e) and similar sections from each brain were identified based on anatomical landmarks. Within each genotype, all brains were selected randomly for histological processing without taking morphological criteria into account. All histology was done blind, by investigators who were unaware of group allocation. No data points were excluded. Male and female samples were used. All antibodies used for this study were validated and their use widely reported.

Behavioral testing. Subject mice were housed in a temperature-controlled vivarium maintained on a 12-h light–dark cycle. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize pain and distress and the number of animals used. No previous analyses were performed on animals used for behavioral testing. We used mixed genotype home cages with 2–4 animals per cage and used experimenters and video scorer/processors blinded to genotype during testing and analysis. All tests were conducted during the light cycle.

Chd8+/del male and female mice and WT littermates, ages 2–4 months, were evaluated in a standard battery of neurobehavioral assays relevant to the core diagnostic and associated symptoms of autism56. We performed the testing on two independent cohorts of adult Chd8+/del mice (first cohort: 9 male, 10 female; second cohort: 11 male, 11 female) and WT littermates (first cohort: 11 male, 10 female; second cohort: 11 male, 9 female). One animal in the first cohort died during behavioral testing. This happened during the three-chambered social approach, in the middle of the behavioral battery. Adult Chd8+/del and matched WT littermates were tested in the following sequence: open field, general health, self-grooming, marble burying, three-chambered social approach, male–female social interactions, novel object recognition and fear conditioning. Testing was performed at the UC Davis MIND Institute Intellectual and Developmental Disabilities Research Center Mouse Behavior Core.

Open field locomotion. General exploratory locomotion in a novel open field environment was assayed as previously described49,57. Open field activity was considered an essential control for effects on physical activity, for example, sedation or hyperactivity17,33,52, which could confound the interpretation of results from the reciprocal interactions, self-grooming, fear conditioning and social approach tasks. The testing room was illuminated at ~40 lx.

Adult general health and neurological reflexes. General health and neurological reflexes were evaluated in adult mice as previously described33,54. General health was assessed on a ranking scale of 0–3 based on fur condition, whisker condition, skin color, and body and limb tone. Body weight and basal temperature were measured, using a hand held portable scale (Ohaus, Parsippany, NJ) and a mouse thermometer probe with lubricant and gently inserted 2 cm into the rectum, respectively. Righting reflex and any occurrences of physical abnormalities were noted. Neurological reflex tests included trunk curl, wire hanging, forepaw reaching, righting reflex, corneal reflex, whisker twitch, pinnae response, eyelid response and auditory startle. The reactivity level of each mouse was assessed with tests in measuring responsiveness to petting, intensity of a dull bellowing response and level of vocalization during handling55.

Novel object recognition. The novel object recognition test was conducted in opaque matte white (P95 White, Tap Plastics, Sacramento, CA) open field arenas (40 cm × 60 cm × 23 cm), using methods similar to those previously described10,56. The experiment consisted of three sessions: a 30-min exposure to the open field arena, a 10-min familiarization session and a 5-min recognition test. On day 1, each subject was habituated to a clean, empty open field arena for 30 min. Twenty-four hours later, each subject was returned to the open field arena for 10 min for the habituation phase. The mouse was then removed from the open field and placed in a clean temporary holding cage for approximately 2 min. Two identical objects were placed in the arena. Each subject was returned to the open field in which it had been habituated and allowed to freely explore for 10 min. After the familiarization session, subjects were returned to their holding cages, which were transferred from the testing room to a nearby holding area. The open field was cleaned with 70% ethanol and let dry. One clean familiar object and one clean novel object were placed in the arena, where the two identical objects had been located during in the familiarization phase. Sixty minutes after the end of the familiarization session, each subject was returned to its open field for a 5 min recognition test, during which time it was allowed to freely explore the familiar object and the novel object. The familiarization session and the recognition test were videotaped and scored with Ethovision XT videotracking software (Version 9.0, Noldus Information Technologies, Leesburg, VA). Object investigation was defined as time spent sniffing the object when the nose was oriented toward the object and the nose–object distance was 2 cm or less. Recognition memory was defined as spending substantially more time sniffing the novel object than the familiar object. Total time spent sniffing both objects was used as a measure of general exploration. Time spent sniffing two identical objects during the familiarization phase confirmed the lack of an innate side bias. Objects used were plastic toys: a small soft plastic orange safety cone and a hard plastic magnetic cone with ribbed sides.

Repetitive self-grooming. Spontaneous repetitive self-grooming behavior was scored as previously described37,49. Each mouse was placed individually into a standard mouse cage (46 cm long × 23.5 cm wide × 20 cm high). Cages were empty to eliminate digging in the bedding, which is a potentially competing behavior. The room was illuminated at ~40 lx. A front-mounted CCTV camera (Security Cameras Direct) was placed ~1 m from the cages to record the sessions. Sessions were videotaped for 20 min. The first 10-min period was habituation and was unscored. Each subject was scored for cumulative time spent grooming all the body regions during the second 10 min of the test session.

Repetitive marble burying. Marble burying and digging in the bedding to cover the marbles was measured as previously described49,57–59. Twenty black glass marbles (15 mm in diameter) were arranged in a symmetrical 4 × 5-cm grid on top of 2–3-cm deep bedding in a clean standard mouse cage (27 × 16.5 × 12.5 cm) with a filter top lid. Each mouse was placed in the center of the cage for a 30-min exploration period, after which the number of marbles buried was tallied by the investigator. ‘Buried’ was defined as greater than 50% covered by bedding37,49. Testing was performed under dim light (~15 lx).

Social approach. Social approach was tested in an automated three-chambered apparatus using methods similar to those previously described9,60. Automated Ethovision XT videotracking software (Version 9.0, Noldus Information Technologies, Leesburg, VA) and modified nonreflective materials for the chambers were employed to maximize throughput. The updated apparatus (40 cm × 60 cm × 23 cm) was a rectangular, three-chambered box made from matte white finished acrylic (P95 White, Tap Plastics, Sacramento, CA). Opaque retractable doors (12 cm × 33 cm) were designed to create optimal entryways between chambers (5 cm × 10 cm), while providing maximal manual division of compartments. Three zones, defined using the EthoVision XT software, detected time in each chamber for each phase of the assay. Zones were defined as the annulus extending 2 cm from each novel object or novel mouse enclosure (inverted wire cup, Galaxy Cup, Kitchen Plus, https://www.spectrumsdiversified.com/whs/products/Galaxy-Pencil-Utility-Cup). Direction of the head, facing toward the cup enclosure, defined sniff time. A top-mounted infrared-sensitive camera (Ikegami ICD-49, B&H Photo, New York, NY) was positioned directly above each chamber during a 10 min habituation session. After the end of the habituation session, each subject was returned to the center chamber for 10 min, then allowed to explore all three empty chambers during a 10 min habituation session, then allowed to explore the three chambers containing a novel object in one side chamber and a novel mouse in the other side chamber. Lack of innate side preference was confirmed during the initial 10 min of habituation to the entire arena. Novel stimulus mice were 129SvImJ, a relatively inactive strain, aged 10–14 weeks, and matched to the subject mice by sex. Number of entries into the side chambers served as a within-task control for levels of general exploratory locomotion.
Male–female social interaction. The male–female reciprocal social interaction test was conducted as previously described\(^6\). Briefly, each freely moving male subject was paired for 5 min with a freely moving unfamiliar estrous WT female. A closed-circuit television camera (Panasonic, Secaucus, NJ) was positioned at an angle from the Noldus Phenotype arena (Noldus, Leesburg, VA) for optimal video quality. An ultrasonic microphone (Avisoft UltraSoundGate condenser microphone capsule CM15; Avisoft Bioacoustics, Berlin, Germany) was mounted 20 cm above the cage. Sampling frequency for the microphone was 250 kHz, and the resolution was 16 bits. The entire apparatus was contained in a sound-attenuating environmental chamber (Lafayette Instruments, Lafayette, IN) under dim LED illumination (~10 lx). Duration of nose-to-nose sniffing, nose-to-anogenital sniffing and following were scored using Noldus Observer 8.XT event recording software (Noldus, Leesburg, VA) as previously described\(^8\). Ultrasonic vocalization spectrograms were displayed using Avisoft software and calls were identified manually by a highly trained investigator blinded to genotype.

Fear conditioning. Delay contextual and cued fear conditioning was conducted using an automated fear-conditioning chamber (Med Associates, St Albans, VT, USA) as previously described\(^9\). The conditioning chamber (32 × 25 × 23 cm\(^3\), Med Associates) interfaced with a PC installed with VideoFreeze software (version 1.12.0.0, Med Associates) and enclosed in a sound-attenuating cubicle. Training consisted of a 2-min acclimation period followed by three tone–shock (CS–US) pairings (80 dB–tone, duration 30 s; 0.5-mA footshock, duration 1 s; intershock interval 90 s) and a 2.5-min period during which no stimuli were presented. The environment was well lit (~100 lx), with a stainless steel grid floor and swabbled with vanilla odor cues (prepared from vanilla extract; McCormick; 1:100 dilution). A 5-min test of contextual fear conditioning was performed 24 h after training, in the absence of the tone and footshock but in the presence of 100 lx overhead lighting, vanilla odor and chamber cues identical to those used on the training day. Cued fear conditioning, conducted 48 h after training, was assessed in a novel environment with distinct visual, tactile and olfactory cues. Overhead lighting was turned off. The cued test consisted of a 3-min acclimation period followed by a 3-min presentation of the tone CS and a 90-s exploration period. Cumulative time spent freezing in each condition was quantified by VideoFreeze software (Med Associates).

MRI within brains of the subjects assessed in behavioral assays. Perfusion. Mice from the first cohort (Chd8\(^{+/+}\), 9 male, 10 female; WT: 8 male, 10 female) that had undergone the behavioral assays were anesthetized with isoflurane (4% to effect) and intracardially perfused with 30 ml of 0.1 M PBS containing 2 mM ProHance and 0.02% sodium azide for at least 7 days. After perfusion, a multichannel 7.0 Tesla MRI scanner (Agilent Inc., Palo Alto, CA) was used to image the brains within their skulls. Sixteen custom-built sole plates to image the brains of the subjects assessed in behavioral assays. RI registration and analysis. Structural MRI registration and analysis. To visualize and compare any changes in the mouse brains the images (or \(b = 0\) s/mm\(^2\) images for DTI) were linearly (6 followed by 12 parameter) and nonlinearly registered together\(^6\). Registrations were performed with a combination of mni\_autoreg tools\(^9\) and ANTS (advanced normalization tools)\(^6\). All scans were then resampled with the appropriate transform and averaged to create a population atlas representing the average anatomy of the study sample. Note that the 40-μm anatomical images and the \(b = 0\) s/mm\(^2\) DTI images were registered separately. The result of the registration was to deform all images into alignment with each other in an unbiased fashion. For the volume measurements, this allowed us to analyze the deformations needed to take each individual mouse’s anatomy into this final atlas space, the goal being to model how the deformation fields relate to genotype\(^2\). The Jacobian determinants of the deformation fields were then calculated as measures of volume at each voxel. For the diffusion measurements, the registration allowed us to analyze the intensity differences of all measures (FA, MD, AD and RD) between genotypes. Significant volume changes and intensity differences could then be calculated by warping a pre-existing classified MRI atlas onto the population atlas, which allowed us to assess the volume or mean diffusion measures (FA, MD, AD and RD) of 159 different segmented structures, encompassing cortical lobes, large white matter structures (i.e., corpus callosum), ventricles, cerebellum, brain stem and olfactory bulbs\(^6,7,6\), in all brains. Further, these measurements could be examined on a voxelwise basis to localize the differences found within regions or across the brain. Multiple comparisons in this study were controlled for using the false discovery rate\(^6\). We reported combined sex results in the main text.

Genomics. Bulk forebrains were microdissected from Chd8\(^{+/+}\) and matched WT littermates at E12.5, E14.5, E17.5 and P0 and from adults (represented in plots as >P56). Dissection included whole forebrain hemispheres after removing surface tissue and skull for all ages except E12.5, when the anterior portion of the developing head was collected. Dissections were performed blind to genotype. Samples included males and females of each genotype at each stage; exact numbers are reported in Supplementary Table 2. Total RNA was isolated using Ambion RNAqueous and assayed using an Agilent BioAnalyzer instrument. Stranded mRNA sequencing libraries were prepared using TruSeq Stranded mRNA kits; 6–12 samples per lane were pooled and sequenced on the Illumina HiSeq platform using a single-end 50-bp (E14.5, E17.5, P0 and adult) or paired-end 100-bp (E12.5) strategy. Each library was quantified and pooled before submission for sequencing. E12.5 samples were sequenced at the UC Berkeley Genomics Sequencing Laboratory; all other samples were sequenced at the UC Davis DNA Technologies Center. Reads from RNA-seq were aligned to the mouse genome (mm9) using STAR (version 2.4.2a)\(^7\). Aligned reads mapping to genes were counted at the gene level using subread featureCounts\(^7\). The mm9 knownGene annotation track and aligned reads were used to generate quality control information using the full RSeQC tool suite\(^7\). Samples that exhibited strong 3′ bias using geneBody_coverage.py or poor exon distribution using read_distribution.py were discarded. Unaligned reads were quality checked by FastQC. BLAST\(^7\) was used to identify reads that mapped to either the reference or 5-bp and 14-bp deletion at Chd8 exon 5 to verify genotype and test for deletion allele transcript frequency. We performed quantitative reverse transcription PCR to validate DE and DS analysis using cDNA libraries prepared using the same protocols, with primers reported in Supplementary Table 4. Cycle counts were normalized to ActB and compared via standard methods as discussed in the methods.

Differential expression analysis and permutation testing. Raw count data for all samples were used as input along with sample information for differential expression analysis using edgeR\(^1\). Genes with at least 10 reads per million in at least two individual samples were included for analysis, resulting in a final set of 11,936 genes for differential testing. Multidimensional scaling analysis indicated that the strongest driver of variance across samples was developmental stage, with no obvious separation between wild-type and Chd8\(^{+/+}\) samples. Tagwise dispersion estimates were generated, and differential expression analysis was performed with edgeR\(^1\) using a generalized linear model including sex, developmental stage and sequencing run factor-based covariates and using genotype as the variable for testing. Stage-specific differential expression testing was also performed. Normalized expression levels were generated using the edgeR rpkm function followed by removing the sequencing batch effect via the limma removeBatchEffect function. Normalized log\(_2\)(RPKM) values were used for plotting of
summary heatmaps and of expression data for individual genes. We examined DE results obtained with reduced coverage criteria for gene inclusion, which resulted in identification of DE genes with lower CPM but no major differences in overall findings.

Permutation testing was performed by testing for set inclusion between differentially down- or upregulated genes identified in the full model and annotated gene sets, comparing observed with expected overlap. Observed overlap was calculated as the overlapping genes, whereas the expected overlap distribution was generated via iterative random sampling of the same number of genes as in the annotated gene set, followed by testing for overlap of randomly selected genes with DE genes. Random sampling was repeated 100,000 times, enabling us to estimate the mean and standard derivation of the expected distribution. Based on this distribution, empirical z-scores and P values were calculated for the observed overlap. All analysis was performed in R using custom scripts that are available upon request.

ChIP-seq analysis. Adult mouse forebrain was dissected on ice, cross-linked using formaldehyde and lysed with SDS, and the DNA was sonicated on a Covaris instrument using standard ChIP-seq protocols adapted for mouse tissues. Chromatin immunoprecipitation (ChIP) was performed using antibodies for Chd8 (ab114126, Abcam). This antibody has been used for brain or neuronal Chd8 ChIP-seq in a previous publication. DNA libraries of matched input and ChIP samples were prepared using the Nugen Ovation Ultralow Library System V2, indexed for multiplexed runs of four libraries per lane and sequenced on an Illumina HiSeq 4000 instrument using a single-end 50-bp strategy. Resulting reads were filtered to remove artifacts and low-quality sequences, and then mapped to the mouse genome (mm9) using the BWA algorithm.

BWA call: bwa alm -t 6 -I 125 mm9 sample.fastq.gz

We used MACS2 to identify significant peaks, disabling model-based peak identification and local significance testing. Peaks were called for each individual ChIP-seq experiment versus matched input control, as well as for merged ChIP and control data using MACS1.4.

MACS call: macs14 -t chip.bam -control = input.bam -z = 100,000 –step = 100, –bsize = 100, –nomodel –shiftsize = 150 -p 0.001

After peak calling, enriched regions were filtered to remove ENCODE blacklist regions and annotated using custom scripts. As nearly all peaks were at gene promoters, functional annotation was performed on the promoter-bound gene sets using ENRICHR. We performed de novo motif discovery using RSAT with default parameters. We additionally analyzed available Chd8 ChIP-seq data. For comparison, all datasets were run through the same analysis pipeline and are available as UCSC TrackHubs for upload to the UCSC Genome Browser.

WGCNA. We used the WGCNA package in R (version 3.2.3) to construct signed co-expression networks using any gene expressed at an RPKM value of 0.25 or higher in at least one sample. A correlation matrix using the biweight midcorrelation between all genes was computed for all relevant samples. The soft thresholding power was estimated and used to derive an adjacency matrix exhibiting approximate scale-free topology ($R^2 > 0.85$). The adjacency matrix was transformed to a topological overlap matrix (TOM). The matrix $1 - TOM$ was used as the input to calculate co-expression modules using hierarchical clustering. Modules were branches of the hierarchical cluster tree base, using the cutree-Hybrid function in WGCNA, with minimum module size set to 500 genes. Genes with positively correlated expression and high topological overlap were clustered together in these modules. In addition, Pearson’s correlation coefficients were used to calculate correlation between sample traits (for example, genotype and modules). The expression profile of a given module was summarized by the module eigengene, ME. Modules with highly correlated MEs (correlation > 0.98) were merged together. The module connectivity (kME) of each gene was calculated by correlating the gene expression profile with module eigengenes. Four modules were generated using this method and were reordered in descending order by gene set size and named numerically. Genes with no network correlation were placed into the module M.grey. We repeated module generation using only wild-type samples, only Chd8+/+ samples and with/without adult samples, with largely the same results as for the full sample set, which is consistent with the finding that most DE gene expression changes are far smaller than changes for genes across developmental stages.

Gene Ontology enrichment and protein–protein interaction network analysis. Permutation testing was performed to test for overlap between DE genes and published gene sets. Human Gene Ontology (GO) data was downloaded from Bioconductor (org.Hs.egG02ALEFTO, org.Hs.eg.db, GO.db). We used the TopGO program to test for enrichment of GO terms indicating parent:child relationships. For the analysis presented here, we restricted our testing to GO Biological Process annotations and required a minimal node size (number of genes annotated to GO terms) of 20. We used the internal ‘weight01’ testing framework and the Fisher test, a strategy recommended for gene set analysis that generally accounts for multiple testing comparisons. For GO analysis, we examined down- and upregulated genes separately, repeating the analysis on DE genes from the full model (cutoff of FDR < 0.20) for all genes and by expression module and stage-specific gene sets (cutoff of P < 0.05). For all enrichment analysis, the test set of DE genes was compared against the background set of genes expressed in our study based on minimum read-count cutoffs described above. Genes with expected/observed ratios of at least 1.5-fold were considered enriched. Heatmaps showing positive log(expected/observed) values were plotted for GO terms of interest. Protein–protein interaction enrichment and network generation for module-specific DE gene sets was performed using STRING, considering only experimentally defined and database interactions. Only module M.1 exhibited a significant enrichment in protein–protein interactions. We compared TopGO enrichment results with goseq, correcting for gene length, and compared STRING protein–protein interaction with DAPPLE, with no substantial differences observed for either methods comparison.

MISO analysis. The Mixture–of-Isomers (MISO) statistical model was used to identify alternative transcript events in our RNA-seq data. For MISO analysis, individual aligned bam files were sorted and merged based on genotype and developmental stage (for example, E17.5 WT and E17.5 Chd8+/+). One sample (S159) from the E17.5 Chd8+/+ group was discarded from analysis because the sample had lower read coverage compared to the other samples and was significantly impacting MISO results across genotype. Analysis was performed using the standard MISO software package frostmisc (https://github.com/yarden/MISO). Standard GFF alternate-event annotations for mm9 (version 1) were used. MISO was run to compute PSI scores (miso-run) using standard, unfiltered parameters. Comparisons (compare_miso) were run between either genotype for a specific stage (for example, E17.5 Chd8+/+ versus E17.5 WT) or pairwise between WT stages (for example, E14.5 WT versus E17.5 WT). Results were filtered (filter_events) based on the following parameters: num-total = 100, num-sum-inc-exc = 10, -delta-psi = 0.1, -bayes-factor = 100.

Statistical analysis. General. No statistical methods were used to predetermine sample sizes, with the exception of behavioral studies, but our other sample sizes are similar to those reported in previous publications. Data collection and analysis were not performed blind to the conditions of the experiments for ChIP-seq, western blots or qRT-PCR. All other data collection and analyses were performed blind. Data collection was randomized across litters for RNA-seq, western blots and histology assays. Aside from behavioral analyses, data distribution was assumed to be normal and not formally tested. A Supplementary Methods Checklist is available.

Neuroanatomy, biochemistry and immunostaining. All statistical analyses and plots were generated using GraphPad Prism 7.0a. Two-tailed t-tests were used for all analyses comparing two groups. For comparisons of three or more data sets, one-way ANOVA followed by post hoc two-tailed t-tests were used. A minimum of three biological replicates (individual animals) were used for all genotypes and assays. For isoform-specific qPCR, comparisons across stage and genotype were analyzed using two-tailed Welch t-test and linear regression. Behavior. Data were analyzed with Statistics software (Tulsa, OK, USA). Plots were generated using GraphPad Prism 7.0a. Sexes were considered separately, with genotype as the fixed factor. Statistical testing was performed using established assay-specific methods, including Student’s t-test for single parameter comparisons between genotypes, and one-way or two-way repeated-measures ANOVA were used for comparisons across time points and/or between sexes. All significance levels were set at P < 0.05 and all t-tests were two-tailed. Groups sizes indicated were chosen based on past experience and power analyses. Effects of genotype and sex were evaluated using multifactor ANOVA, as previously published. Significant ANOVAs
were followed by Tukey’s honest significant difference test. Behavioral analysis passed distribution normality tests, was collected using continuous variables and thus was analyzed via parametric analysis in all assays. For all behavioral analyses, variances were similar between groups and all data points within 2 s.d. of the mean were included in analysis.

Genomic analyses. Samples for RNA-seq were randomly collected across litters and processed blind to genotype. Samples from RNA-seq were removed if they failed to pass quality scoring and coverage criteria. For differential gene expression analysis, differences were considered statistically significant if FDR and P values calculated by edgeR were < 0.20 and < 0.05, respectively. For ChIP-seq, peaks were considered significant if P values determined by MACS were < 0.001. For permutation testing, enrichment was considered significant if empirical z-scores and P values were > 2 and < 0.05, respectively. For differential splicing analysis, individual events were considered significant if the Bayes factor > 100, as calculated by MISO40.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. DRIIs for all published gene sets used in enrichment analysis:

- Sanders et al.25: https://dx.doi.org/10.1016/j.neuron.2015.09.016
- Parikshak et al.26: https://dx.doi.org/10.1016/j.cell.2013.10.031
- Cotney et al.27: https://dx.doi.org/10.1038/ncomms7404
- Willsey et al.28: https://dx.doi.org/10.1038/nn.2020
- Sugathan et al.29: https://dx.doi.org/10.1038/40526611
- Darnell et al.30: https://dx.doi.org/10.1016/j.cell.2011.06.013
- Hormozdiari et al.31: https://dx.doi.org/10.1101/gr.178555.114
- Katamaya et al.32: https://dx.doi.org/10.1038/nature19357
- Durak et al.33: https://dx.doi.org/10.1038/nn.4400; and
- Parikshak et al.34: https://dx.doi.org/10.1038/nature20612.

Raw, aligned and gene-count data for RNA-seq and raw, aligned and peak call data for ChIP-seq are available on GEO (GSE99331).

Code availability. All custom scripts and TrackHubs used for data processing and analysis are available in Supplementary Software and at the Nord Lab Git Repository (https://github.com/NordNeurogenomicsLab/). A custom sample-processing pipeline was used to align raw sequencing samples to mouse genome mm9 using RNA-seq aligner STAR (version 2.4.2a), features assigned via subreads featureCounts (version 1.5.0) to UCSC mm9 genes.gtf and quality checks performed on individual samples using RSeQC (version 2.6.3). Differential expression analysis was done with a custom pipeline in R Studio using functions from edgeR (version 3.10.5) and limma (version 3.24.15). Permutation testing was performed with a custom R script. Co-expression network analysis was performed with a custom pipeline following the standard WGCNA (version 3.2.3) workflow and functions. Gene Ontology analysis was performed with a custom wrapper using standard the TopGO (version 2.20.0) program. ChIP-seq data was aligned to mm9 via BWA (version 0.6.2) and peaks were called via MACS (version 1.4.2). Peaks were then filtered to remove ENCODE blacklist regions and annotated. Comparison of enriched gene sets between RNA and ChIP-seq was performed using Enrichr (http://amp.pharm.mssm.edu/Enrichr/). MISO analysis (https://github.com/yarden/MISO/) was performed using standard parameters. See above for description and parameters.


Reporting Checklist for Nature Neuroscience

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. For more information, please read Reporting Life Sciences Research.

Please note that in the event of publication, it is mandatory that authors include all relevant methodological and statistical information in the manuscript.

Statistics reporting, by figure

- Please specify the following information for each panel reporting quantitative data, and where each item is reported (section, e.g. Results, & paragraph number).

- Each figure legend should ideally contain an exact sample size (n) for each experimental group/condition, where n is an exact number and not a range, a clear definition of how n is defined (for example x cells from x slices from x animals from x litters, collected over x days), a description of the statistical test used, the results of the tests, any descriptive statistics and clearly defined error bars if applicable.

- For any experiments using custom statistics, please indicate the test used and stats obtained for each experiment.

- Each figure legend should include a statement of how many times the experiment shown was replicated in the lab; the details of sample collection should be sufficiently clear so that the replicability of the experiment is obvious to the reader.

- For experiments reported in the text but not in the figures, please use the paragraph number instead of the figure number.

Note: Mean and standard deviation are not appropriate on small samples, and plotting independent data points is usually more informative. When technical replicates are reported, error and significance measures reflect the experimental variability and not the variability of the biological process; it is misleading not to state this clearly.

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<td>Fig legend</td>
<td>p=0.3978 Sex diff: p = 0.619</td>
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<td>18 HT (8 m, 10 f), 19 WT (9 m, 10 f)</td>
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<td>Cortex: p=0.0001, Amygdala: p=0.0001, Hippocampus: p&lt;0.0001</td>
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<td>Kdm5b: FDR=1.4E-02, Bcl11a: FDR=2.5E-02, Hnmp2a2b1: FDR=1.9E-02</td>
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<td>mRNAProcessing: p=6.62E-14, 1.37E-12, Chromatin mod: p=8.56E-11, 1.57E-12; Reg of Cell Cycle: p=0.0317, 0.0021</td>
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<td>Permutation test: gene set compared for test and random sample (100k) set against DE genes</td>
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<td>Z-scores shown top-to-bottom: 8.2, 6.3, 6.8, 4.8, 2.0, 0.3, 2.5 FMRP p=0.04 DE-up ASD p=0.012</td>
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<td>Z-scores: M1: 10.2, NA M2: NA, 5.0 M3: 11.3, NA M4: NA, 9.7 Mgrey: NA, 10.9</td>
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<td>permutation p=p&lt;8.8e-26</td>
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<td>e17.5: WT=2, HT=7 (591 total events on bar plot, 395 dark grey)</td>
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<td>e12.5=7 e14.5=9 e17.5-WT=7, e17.5-HT=7 P0=11</td>
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<td>e14.5-e17.5: p=0.001 e17.5-e17.5: p=0.03 Dev time: p=4.53E-14 Dev time: R²=0.77</td>
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<td>One way ANOVA, unpaired post hoc t-test</td>
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<td>WT=3 (280), WT=4 (110), HT-5bp=3, HT-14bp=3</td>
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<td>Fig legend</td>
<td>280 kDa ANOVA: p=0.0118 110 kDa ANOVA: p=0.1676 280 kDa: 5bp p = 0.0319; 14bp p=0.0328 110 kDa: 5bp p=0.1086, 14bp p = 0.4509</td>
<td>Fig legend</td>
<td>280 kDa ANOVA: F=10.19, R²=0.7726 110 kDa ANOVA: F=3.33, R²=0.3997 280 5bp t=2.33, df=4 280 14bp t=3.203, df=4 110 5bp t=1.951, df=5; 14bp t=0.8174, df=5</td>
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<td>Unpaired t-test</td>
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<td>Fig legend</td>
<td>280 kDa: p=0.0089 110 kDa: p=0.001</td>
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<td>280 kDa: t=2.977, df=16 110 kDa: t=4.022, df=16</td>
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<td>280 kDa: p=0.9903 110 kDa: p=0.02</td>
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<td>280 kDa: t=2.765, df=10 110 kDa: t=0.01248, df=10</td>
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<td>Error bars are mean +/- SEM</td>
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<td>p=0.1046</td>
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<td>t(1, 40)=1.6607</td>
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<td>p=0.0571</td>
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<td>t(1, 40)=1.9593</td>
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<td>WT=21, HT=20 littermates</td>
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<td>Error bars are mean +/- SEM</td>
<td>Fig legend</td>
<td>WT p=0.0453 HT p=0.3846</td>
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<td>F(1, 20)=4.5583, HT F(1, 19)=0.7921</td>
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<td>Error bars are mean +/- SEM</td>
<td>Fig legend</td>
<td>WT p=0.0042 HT p=0.0008</td>
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<td>F(1, 20)=10.438 HT F(1, 20)=15.470</td>
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<td>Results para 4</td>
<td>42</td>
<td>WT=21, HT=21</td>
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<td>Error bars are mean +/- SEM</td>
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<td>p=0.583</td>
<td>Fig legend</td>
<td>F(1, 40)=0.307</td>
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<td>S2G</td>
<td>Unpaired t-test</td>
<td>Results para 4</td>
<td>22</td>
<td>WT=11, HT=11</td>
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<td>Error bars are mean +/- SEM</td>
<td>Fig legend</td>
<td>p=0.8807</td>
<td>Fig legend</td>
<td>t(1, 20) = 0.1520</td>
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<td>Results para 4</td>
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<td>WT=11, HT=11</td>
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<td>Error bars are mean +/- SEM</td>
<td>Fig legend</td>
<td>p=0.8057</td>
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<td>t(1, 20)=0.2492</td>
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<td>18 HT (8 m, 10 f), 19 WT (9 m, 10 f)</td>
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<td>Effect size</td>
<td>Table S1</td>
<td>All relevant stats (FDR, p) reported</td>
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<td>Unpaired t-test</td>
<td>Results para 9</td>
<td>13</td>
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<td>Fig legend</td>
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<td>Fig legend</td>
<td>t=0.5663, df=7</td>
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<td>Results para 10</td>
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<td>Methods</td>
<td>Predicted sites, logo motif bit scores</td>
<td>Fig</td>
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<td>NA</td>
<td>All relevant stats reported</td>
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<td>Fig legend</td>
<td>NA</td>
<td>NA</td>
<td>All relevant stats reported</td>
<td>Fig legend</td>
</tr>
</tbody>
</table>
### Representative figures

1. Are any representative images shown (including Western blots and immunohistochemistry/staining) in the paper?

   If so, what figure(s)?

2. For each representative image, is there a clear statement of how many times this experiment was successfully repeated and a discussion of any limitations in repeatability?

   If so, where is this reported (section, paragraph #)?

Western blot data is presented in Figures 1E, 4E, and S1.
IHC data is presented in Figures 6E, 7A-D, S6.

Yes to all, and is reported in the above statistics reporting table as well as the following:
- Full western blot data and is displayed in Figure S1
- IHC data is described in figure legends (6E, 7A-D, S6)
Statistics and general methods

1. Is there a justification of the sample size?
   If so, how was it justified?
   Where (section, paragraph #)?
   Even if no sample size calculation was performed, authors should report why the sample size is adequate to measure their effect size.

   Behavioral analysis sample size is justified in Paragraph 3, Statistics.
   No justification of sample size is reported as power analysis is not standardized for RNA-seq. However, sample size per group and condition is equal to or greater than generally accepted standard of three biological replications per group. Our sample size is additionally sufficient for network analysis. This is reported in Para 2, Statistics.
   No justification of sample size for other experiments, though biological replicate numbers are standard in the field and sufficient to capture significance and trends. This is reported in Para 1, Statistics.

2. Are statistical tests justified as appropriate for every figure?
   Where (section, paragraph #)?

   a. If there is a section summarizing the statistical methods in the methods, is the statistical test for each experiment clearly defined?

   Behavioral analysis statistical tests are delineated within the text in Paragraph 3, Statistics.
   For RNA-seq, yes, for each method described, in the relevant Methods sections.
   All statistical methods reported in full in the text or supplement.

   b. Do the data meet the assumptions of the specific statistical test you chose (e.g. normality for a parametric test)?
   Where is this described (section, paragraph #)?

   Behavioral analysis data meets the assumptions of the statistical tests chosen. Data passed distribution normality tests, was collected using continuous variables and thus analyzed via parametric analysis, in all assays. This is described in Para 3, Statistics.
   Yes - QC performed to examine specific parameters and we used tests specifically designed for RNA-seq differential expression analysis. Additionally, we use non-parametric tests where necessary. This is described within the text in paragraph 1, subsection "RNA sequencing," of section "Methods."

   c. Is there any estimate of variance within each group of data?
   Is the variance similar between groups that are being statistically compared?
   Where is this described (section, paragraph #)?

   Behavioral analysis variances are similar within each group and between groups that are being statistically compared. This is described within the text in Paragraph 1 of "Behavioral Testing" in the "Methods".
   For genomics, analysis methods are designed to be robust to sample variance. Further, we perform QC on individual samples to ensure robust comparisons. This is described within the text in paragraph 1 of Genomics in Methods.
   For other analysis (e.g. qPCR and western blot), we used standard approaches (e.g. Student’s t-test and ANOVA) in the field.

   d. Are tests specified as one- or two-sided?

   Always two-sided.
3. To promote transparency, *Nature Neuroscience* has stopped allowing bar graphs to report statistics in the papers it publishes. If you have bar graphs in your paper, please make sure to switch them to dot-plots (with central and dispersion statistics displayed) or to box-and-whisker plots to show data distributions.

4. Are criteria for excluding data points reported?
   - Was this criterion established prior to data collection?
   - Where is this described (section, paragraph #)?

5. Define the method of randomization used to assign subjects (or samples) to the experimental groups and to collect and process data.
   - If no randomization was used, state so.
   - Where does this appear (section, paragraph #)?

6. Is a statement of the extent to which investigator knew the group allocation during the experiment and in assessing outcome included?
   - If no blinding was done, state so.
   - Where (section, paragraph #)?

7. For experiments in live vertebrates, is a statement of compliance with ethical guidelines/regulations included?
   - Where (section, paragraph #)?

---

**e. Are there adjustments for multiple comparisons?**

- Yes for behavior.
- Yes for RNA-seq.
- Yes for MRI.

**All figures converted to show individual data points and summary/variance measurements.**

**We followed established laboratory protocol in which all data points that lie within 2 standard deviations of the mean are included in analysis. This is described within the text in Para 3 of Statistics.**

No data points excluded in RNA-seq after original quality filtering to remove samples with 3’ coverage bias. This is described in the results and methods. For MISO analysis, one sample (e17.5 S159) was removed before pooling samples; this is reported in Para 1 of Miso Analysis in Methods.

For qPCR, the highest and lowest values for both WT and Chd8+/del5 groups were removed to reduce variation. This was described in qRT-PCR in Methods; actual n’s are listed in the figure legends.

One of each sample from WT and HT in the Q-fraction analysis was removed; this is mention in Paragraph 1 of section “EdU labeling and immunofluorescent analysis” in “Methods”.

**Behavioral analysis was performed on mixed genotype home cages and blinded to experimenter and video scorer/processor. This is described within the text in paragraph 1 of “Nissl staining, EdU labeling, and immunofluorescent analysis” in Methods.**

For RNA-seq: samples were randomly collected across litters from male het x female wt and processed without knowing genotype. This is described in paragraph 4 of Statistics.

Samples for western blot analysis were randomly collected from multiple litters. For all histology, samples were randomly selected for analysis, as described in Statistics, and additionally in methods.

The investigator was blinded to behavioral experiments and analysis and to histology. This is described within the text in the first paragraph of subsections “Behavioral testing” and “Lamination Assay” in Methods.

All experiments were performed blind to genotype (“Results” section, paragraph 1), unless denoted otherwise in Methods.

**A statement of compliance with ethical guidelines and regulations is included in both the methods and subsection “Behavioral testing” of Methods.**
<table>
<thead>
<tr>
<th>Question</th>
<th>Detailed Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>8. Is the species of the animals used reported?</td>
<td>The species of animal used is given and described within the text in Paragraph 1 of section &quot;Mice harboring heterozygous germline Chd8 mutation exhibit megalencephaly&quot; in &quot;Results&quot;.</td>
</tr>
<tr>
<td>9. Is the strain of the animals (including background strains of KO/</td>
<td>Animal strain information is included in Paragraph 1 of section &quot;Mice harboring heterozygous germline Chd8 mutation exhibit megalencephaly&quot; in Results, and in Methods.</td>
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<tr>
<td>transgenic animals used) reported?</td>
<td></td>
</tr>
<tr>
<td>10. Is the sex of the animals/subjects used reported?</td>
<td>The sex of animals used in the behavioral analysis is described within the text in paragraph 1, section &quot;Behavioral phenotyping of adult Chd8+/del5 mice&quot; in Results, and additionally in Methods and Figure Legends.</td>
</tr>
<tr>
<td>11. Is the age of the animals/subjects reported?</td>
<td>The age of animals used in the behavioral analysis is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
</tr>
<tr>
<td>12. For animals housed in a vivarium, is the light/dark cycle reported?</td>
<td>The environmental light conditions of the vivarium in which the animals used in the behavioral analysis is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
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<tr>
<td>13. For animals housed in a vivarium, is the housing group (i.e. number</td>
<td>The number animals per cage used in the behavioral analysis is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
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<tr>
<td>of animals per cage) reported?</td>
<td></td>
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<tr>
<td>14. For behavioral experiments, is the time of day reported (e.g. light</td>
<td>The environmental light of the behavioral testing conditions in which the animals used in the behavioral analysis is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
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<tr>
<td>or dark cycle)?</td>
<td></td>
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<tr>
<td>15. Is the previous history of the animals/subjects (e.g. prior drug</td>
<td>Animals used in the behavioral analysis were not previously subjected to other assays or analysis. This is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
</tr>
<tr>
<td>administration, surgery, behavioral testing) reported?</td>
<td></td>
</tr>
<tr>
<td>a. If multiple behavioral tests were conducted in the same group of</td>
<td>Animals used in the behavioral analysis were subjected to a set sequence of behavioral assays. This is described within the text in paragraph 1 of &quot;Behavioral Testing&quot; in Methods.</td>
</tr>
<tr>
<td>animals, is this reported?</td>
<td></td>
</tr>
<tr>
<td>16. If any animals/subjects were excluded from analysis, is this</td>
<td>No data points were excluded from behavioral analysis. This is described within the text in paragraph 3 of Statistics in Methods.</td>
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<tr>
<td>reported?</td>
<td></td>
</tr>
</tbody>
</table>
a. How were the criteria for exclusion defined?
   Where is this described (section, paragraph #)?
   N/A

b. Specify reasons for any discrepancy between the number of animals at the beginning and end of the study.
   Where is this described (section, paragraph #)?
   One animal died during behavioral testing. This happened during Three-Chambered Social approach, in the middle of the behavioral battery. This is described in paragraph 3 of Statistics in Methods.

### Reagents

1. Have antibodies been validated for use in the system under study (assay and species)?
   Yes, except for Tbr2.
   
   a. Is antibody catalog number given?
      Where does this appear (section, paragraph #)?
      Yes, in methods.
   
   b. Where were the validation data reported (citation, supplementary information, Antibodypedia)?
      Antibodies used in previous studies are cited in the methods. For Tbr2, we cite the Abcam data sheet. The Tbr2 antibody exhibits similar histology staining patterns to other Tbr2 antibodies.

2. Cell line identity
   
   a. Are any cell lines used in this paper listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample?
      NA
   
   b. If yes, include in the Methods section a scientific justification of their use—indicate here in which section and paragraph the justification can be found.
      NA
   
   c. For each cell line, include in the Methods section a statement that specifies:
      - the source of the cell lines
      - have the cell lines been authenticated? If so, by which method?
      - have the cell lines been tested for mycoplasma contamination?
      NA
Data availability

Provide a Data availability statement in the Methods section under "Data availability", which should include, where applicable:

- Accession codes for deposited data
- Other unique identifiers (such as DOIs and hyperlinks for any other datasets)
- At a minimum, a statement confirming that all relevant data are available from the authors
- Formal citations of datasets that are assigned DOIs
- A statement regarding data available in the manuscript as source data
- A statement regarding data available with restrictions

See our data availability and data citations policy page for more information.

Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Microarray data

Deposition is strongly recommended for many other datasets for which structured public repositories exist; more details on our data policy are available here. We encourage the provision of other source data in supplementary information or in unstructured repositories such as Figshare and Dryad.

We encourage publication of Data Descriptors (see Scientific Data) to maximize data reuse.

Where is the Data Availability statement provided (section, paragraph #)?

Data availability reported in methods with DOIs.

Computer code/software

Any custom algorithm/software that is central to the methods must be supplied by the authors in a usable and readable form for readers at the time of publication. However, referees may ask for this information at any time during the review process.

1. Identify all custom software or scripts that were required to conduct the study and where in the procedures each was used.

   Described in methods. All analysis software was previously published.

2. If computer code was used to generate results that are central to the paper's conclusions, include a statement in the Methods section under "Code availability" to indicate whether and how the code can be accessed. Include version information as necessary and any restrictions on availability.

   Code availability:
   All custom scripts and TrackHubs used for data processing and analysis will be available at the Nord Lab Git Repository (https://github.com/NordNeurogenomicsLab/).

Human subjects
<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Which IRB approved the protocol?</td>
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<td>Where is this stated (section, paragraph #)?</td>
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<tr>
<td>2. Is demographic information on all subjects provided?</td>
<td>NA</td>
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<tr>
<td>Where (section, paragraph #)?</td>
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</tr>
<tr>
<td>3. Is the number of human subjects, their age and sex clearly defined?</td>
<td>NA</td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>4. Are the inclusion and exclusion criteria (if any) clearly specified?</td>
<td>NA</td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>5. How well were the groups matched?</td>
<td>NA</td>
</tr>
<tr>
<td>Where is this information described (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>6. Is a statement included confirming that informed consent was</td>
<td>NA</td>
</tr>
<tr>
<td>obtained from all subjects?</td>
<td></td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>7. For publication of patient photos, is a statement included confirming</td>
<td>NA</td>
</tr>
<tr>
<td>that consent to publish was obtained?</td>
<td></td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
</tbody>
</table>

**fMRI studies**

For papers reporting functional imaging (fMRI) results please ensure that these minimal reporting guidelines are met and that all this information is clearly provided in the methods:

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Were any subjects scanned but then rejected for the analysis after the data was collected?</td>
<td>NA</td>
</tr>
<tr>
<td>a. If yes, is the number rejected and reasons for rejection described?</td>
<td>NA</td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>2. Is the number of blocks, trials or experimental units per session and/or subjects specified?</td>
<td>NA</td>
</tr>
<tr>
<td>Where (section, paragraph #)?</td>
<td></td>
</tr>
<tr>
<td>3. Is the length of each trial and interval between trials specified?</td>
<td>NA</td>
</tr>
<tr>
<td>4. Is a blocked, event-related, or mixed design being used? If applicable, please specify the block length or how the event-related or mixed design was optimized.</td>
<td>NA</td>
</tr>
</tbody>
</table>
5. Is the task design clearly described?
   Where (section, paragraph #)?
   NA

6. How was behavioral performance measured?
   NA

7. Is an ANOVA or factorial design being used?
   NA

8. For data acquisition, is a whole brain scan used?
   If not, state area of acquisition.
   a. How was this region determined?
   NA

9. Is the field strength (in Tesla) of the MRI system stated?
   a. Is the pulse sequence type (gradient/spin echo, EPI/spiral) stated?
   NA
   b. Are the field-of-view, matrix size, slice thickness, and TE/TR/flip angle clearly stated?
   NA

10. Are the software and specific parameters (model/functions, smoothing kernel size if applicable, etc.) used for data processing and pre-processing clearly stated?
    NA

11. Is the coordinate space for the anatomical/functional imaging data clearly defined as subject/native space or standardized stereotaxic space, e.g., original Talairach, MNI305, ICBM152, etc? Where (section, paragraph #)?
    NA

12. If there was data normalization/standardization to a specific space template, are the type of transformation (linear vs. nonlinear) used and image types being transformed clearly described? Where (section, paragraph #)?
    NA

13. How were anatomical locations determined, e.g., via an automated labeling algorithm (AAL), standardized coordinate database (Talairach daemon), probabilistic atlases, etc.?
    NA

14. Were any additional regressors (behavioral covariates, motion etc) used?
    NA

15. Is the contrast construction clearly defined?
    NA

16. Is a mixed/random effects or fixed inference used?
   a. If fixed effects inference used, is this justified?
   NA

17. Were repeated measures used (multiple measurements per subject)?
    NA
## Additional comments

Additional Comments

Efforts have been made to report specific methods in the manuscript or in the methods section. We will make our raw data, processed data, and analysis codes available via our GitHub site.