Title: Embryonic Knockdown of Chromodomain Helicase DNA Binding Protein 8 (Chd8) in Cortex Causes Autism Spectrum Disorder (ASD)-like Behavior

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Abstract

Autism Spectrum Disorder (ASD) is an increasingly prevalent neurodevelopmental disorder, the the underlying causes of which are not well understood. Chromodomain Helicase DNA Binding Protein 8 (CHD8) has been associated with the disease through exome sequencing studies and is suggested to regulate neurodevelopmental processes whose disruption may underlie the ASD pathology. In the current study, we assess the sociability of Chd8 knockdown mice through the three-chamber social arena assay. Our results show that Chd8 knockdown mice display decreased sociability towards a stranger mouse. Furthermore, we analyzed the morphological changes in upper cortical layer pyramidal neurons following Chd8 knockdown. Neurons from knockdown brains showed a significant decrease in both dendritic branching and spine density. The differences in brain morphology may underlie the social behavioral deficits seen in knockdown mice. In sum, our findings suggest that Chd8 is important for proper cortical development, and causes ASD-like behavior in adult mice when knocked down embryonically.

Keywords: dendritic arborization, three chamber social test, neuronal circuitry

Introduction

Autism Spectrum Disorder (ASD) is an etiologically and clinically complex developmental disorder that is typically characterized by social deficits, communication difficulties, stereotyped behaviors and cognitive delays. The disorder is increasingly diagnosed among children. The CDC reports that the frequency of ASD has risen from 1 in 125 in 2004 to1 in 68 in 2014. Despite its prevalence, the mechanisms causing ASD have yet to be elucidated due to the complexity of neuronal circuitry generation and the variation in genetics contributions.

A hallmark of patients with autism is their lack of executive function (Ozonoff et al, 1991) and “central coherence”, the cognitive ability to bind together a jumble of separate features and

signals into a single object or concept (Frith, 1989). These deficits contribute to an inability to process complex information required for language and behavior and have led to discoveries in abnormal neural connectivity in ASD patients. Though there continues to be disagreement whether ASD patients show increased or decreased connectivity, fMRI imaging studies show differences in brain structure and neural circuitry in autistic individuals (Belmonte et al, 2004).

Neuronal circuitry is determined collectively by synaptic connections between neurons of different brain regions. Evidence for decreased circuitry found in ASD patients can be conceptualized as a disruption of synapses in the brain beginning in the early embryo (Banerjee et al., 2014). Most excitatory synapses occur on small protrusions called dendritic spines on neuronal branches (Penzes, 2011). Development of cortical neurons is a highly orchestrated process where disruptions can result in abnormal neuronal dendritic morphology and synapses- and is likely to contribute to the pathology of the disorder (Courchesne et al., 2001).

The genetics of autism has always been of interest because having one child with ASD increases the chance of ASD in subsequent children (Constantino et al., 2010). However, identifying specific genes has only become possible with advances in genome sequencing. Candidate genes were determined by comparing patient genomes with either the genomes of unaffected parents or siblings. The most recent study identified 64 genes likely to be involved in ASD due to their recurrent disruptive mutations, their presence among networks important to neurodevelopment and their predicted role in chromatin regulation. The study determined that disruptive mutations in Chromodomain Helicase DNA binding protein 8 (CHD8), a chromatin remodeling protein, has the highest association with ASD risk (O'Roark et al., 2012).

CHD8 is an ATP-dependent chromatin remodeler, initially identified as a binding partner and negative regulator of the β-catenin/Wnt signaling pathway (Sakomoto et al., 2000). B -

catenin, a dual function protein that forms part of the extracellular cadherin complex and acts as a Wnt pathway transcription factor. The Wnt/β-catenin pathway is crucial to normal embryonic development of the central nervous system (Ferrari et al., 2006). Disruptions to regulators of this pathway are likely to contribute to abnormal neural development that could contribute to ASD pathology. Chd8 knockdown in zebrafish displayed phenotypes similar to those of ASD patients with CHD8 mutations, further supporting the role of CHD8 in ASD (Bernier et al., 2014).

To provide additional insight on the contributions of CHD8 to ASD, possibly explanations for how altered protein expression leads to behavioral deficits should be explored.

To investigate the role of CHD8 in ASD, we examined the *in vivo* effects of Chd8 disruption on mouse cortical development via the *in utero* electroporation technique. Because the only diagnosis for ASD in humans is behavioral symptoms, we used behavioral analysis to assess social interaction and characterize our mice. We then determined neuronal morphology to establish circuitry differences that may be contributing to behavioral difference. We hypothesized that knocking down Chd8 in mice would disrupt normal social behavior in mice and alter neuron morphology in a way that decreases brain connectivity. Our findings provide insight into the mode of action of *Chd8* mutations leading to ASD, as well as furthering our understandingof the mechanisms regulating mammalian cortical development.

Material and Methods

DNA Constructs and Antibodies.

Control non-targeting shRNA (Sigma, SHC002) and shRNA targeting Chd8 were obtained from Sigma (Chd8 shRNA, Clone ID: NM_201637.2-3342s21c1). The plasmid vector for the non-targeting shRNA can be found in Figure 1 and the sequence of Chd8 shRNA can be

found in Table 1. The following primary antibody was used in this study: chicken anti-GFP antibody (GFP-1020, Aves Labs). Primer sequences for β-actin, CHD8, β-catenin and E-cadherin were used. The forward and reverse primers are listed in Table 2.

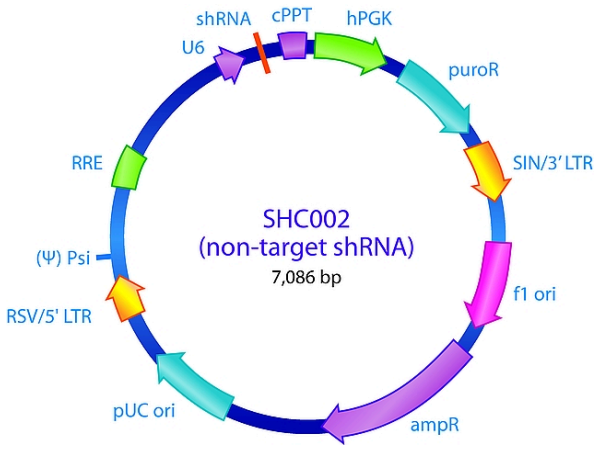

Figure 1. Non-targeting shRNA control vector used as the control plasmid in transfection of N2A cells and injection of shRNA for Chd8 knockdown mice.

ShRNA	Sequence	Targeted Sequence
Chd8 shRNA	5'CCGGGCAGCCTTTAAGCTCAAAGAACTCG AGTTCTTTGAGCTTAAAGGCTGCTTTTTTG-3'	5'-GCAGCCTTTAAGCTCAAAGAA-3'

Table 1. Sequence for the C*hd*8 shRNA used in cell transfections and for generating knockdown mice

Gene specific primers	Forward primer (5'-3')	Reverse primer (5'-3')
β-catenin (*Ctnnb1*)	GCTATTCCACGACTAGTTCAGC	AGCTCCAGTACACCCTTCTAC
β-actin (*Actb*)	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
CHD8 *(Chd8)*	AGCCTTTCAATCCAGACTACG	CATCCTCTTTTAGCTCCCACG
E-cadherin *(Cdh1)*	AGAGAAGCCATTGCCAAGTAC	AACGAATCCCTCAAAGACCG

Table 2. Forward and reverse gene specific primers used in qPCR

Cell culture

Mouse Nueroblastoma-2a cells (N2A) purchased from ATCC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin/streptomycin (100U/mL and 100 mg/mL)) and maintained in 37C incubator at a maximum confluency of 80%.

Cell transfection

48 hours prior to transfection, N2A cells were plated at a 1:10 ratio onto 6-well ($10cm^2$) plates. Cells were transfected with *Chd8* shRNAand control plasmid pLKO.1 by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 1ug/uL DNA plasmid and Lipofectamine 2000 were each diluted in DMEM. The two dilutions of DNA plasmid and Lipofectamine 2000 were combined, incubated for 20 minutes then added to each well containing cells and medium. Cells were incubated at 37°C and medium was changed 5 hours after start of incubation.

RNA Isolation from cultured cells and creation of cDNA Library

48 hours after transfection, total RNA from cells was collected from the cells with the Qiagen RNeasy Plus mini kit (cat. no. 74134) according to manufacturer's protocol for <6mL plates. cDNA was made from isolated RNA with RNA to cDNA EcoDry Premix (oligo dT) bought from Clontech (cat. no. 639543) according to manufacturer's protocol. Prepared samples were placed in a C1000 Thermal Cycler from Bio-rad laboratories and incubated at 42°C for 60 minutes and then at 72°C for 10 minutes.

QPCR

10uL of Ssofast qPCR supermix (Bio-rad) was mixed with 0.5 uM of each primer and 0.05uM of cDNA prepared from total RNA of N2A cells. The solution was diluted to 20uL. The

primers used corresponded to targeting genes of CHD8, β -catenin, E-cadherin and beta-actin as a control (table 2). Samples were placed in a C1000 Thermal Cycler from Bio-rad laboratories and cycled 45 times for 10s at 95°C and then 30s for 60°C. After the 45 cycles, the samples are incubated for 5s at 65°C then for 5s at 95°C.

***In utero* electroporation of mouse**

Pregnant Swiss Webster mice used for in utero electroporation were purchased from Tacomic (Hudson, NY, USA). In utero electroporation was done by grad student Omer Durak (Tsai Lab). Briefly, pregnant Swiss Webster mice were anesthetized by intraperitoneal injections of Ketamine 1% / Xylazine 2 mg/ml, the uterine horns were exposed, and the plasmids mixed with Fast Green (Sigma) were bilaterally microinjected into the lateral ventricles of embryos. Five pulses of current (50 ms on / 950 ms off) were delivered across the head of the embryos. A voltage of 32-35V was used for E15 mice (15 days post fertilization). In the DNA mixture, the shRNA plasmid concentration was 2 to 3-fold higher than that of the control.

Behavioral Analysis

The three-chamber behavioral test for mice was done by grad student Omer Durak and lab technician Anthony Martorell (Tsai Lab) as shown in Figure 2. I hand-scored the data by playing videos of chambers and mice taped during test sessions. The time subject mice spent in each of the three chambers was timed with separate timers for both habituation and the experiment. A separate set of scoring was done to time how long mice subjects spent interacting with either stranger mouse or inanimate object but using the same methods.

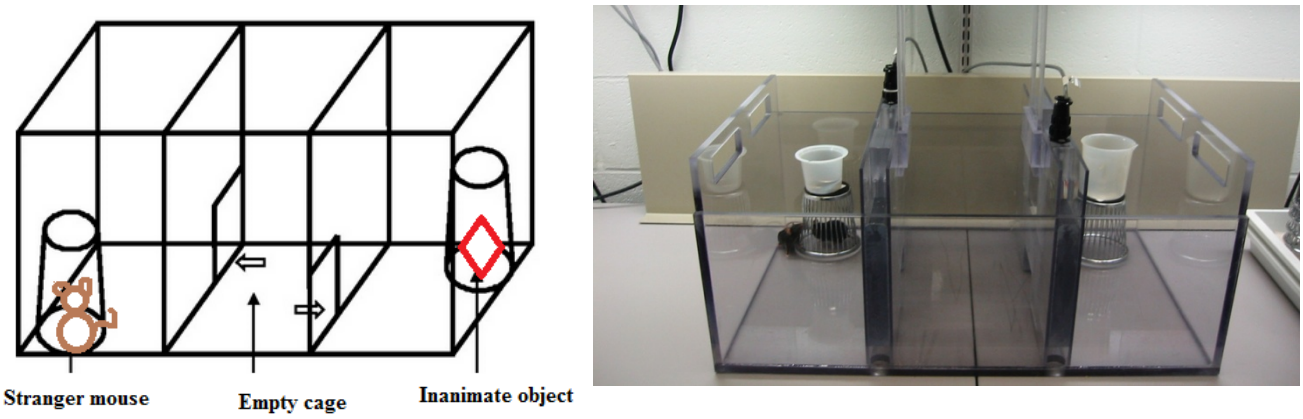

Figure 2. The set up for the three-chamber behavioral analysis. The stranger mouse is placed in the cage in one side chamber whereas an inanimate object is placed into the cage of the other side chamber. The middle chamber is left empty. Mice are placed in the middle cage at the beginning of the experiment. Image from Liu et al., 2010.

Preparation of mouse brain slices

Mice brains were harvested and placed in 4% paraformaldehyde. The brains were mounted and then sliced using a Leica VT 1000S vibratome from anterior to posterior at 40um in thickness and stored in 1X PBS. 96 total slices were collected per brain.

Immunofluorescence

Slices were blocked for 1-2 hours in blocking solution (10% donkey normal serum (DNS), 0.3% Triton X in PBS). The slices were then incubated with primary chicken anti-GFP (1:500) overnight, followed by secondary antibody (1:500) for 1 hour. Slices were mounted onto glass slides using ProLong Gold Antifade Reagent.

Microscopy

Whole brain images were visualized through multiple planes with a Zeiss LSM 710 confocal at 10X. Images were processed with Image J. For quantitative analysis of dendritic

arborization, isolated pyramidal neurons in the cortical layers of the brain were chosen and visualized with Zeiss LSM 510 at 40X. For spine density and dendrite thickness, the secondary branches of the apical dendrite of cortical pyramidal neurons were visualized with Zeiss LSM 510 at 63X and processed with Image J.

Sholl Analysis, spine density and spine thickness

Prior to all analysis, the multiple z stack planes taken by the microscope were consolidated with Image J via the Z Project function. The Z project function takes the maximum intensity of each image slice and compiles them into one image. For dendritic arborization profiling, compiled images were transferred onto a gray scale and a series of equidistantly arranged concentric circles were centered on the neuron body. Radii increments of 5um were used for the circles. The number of intersections of dendrites with these circles was counted and recorded for each neuron. Primary neurites were determined by counting the number of initial branches of the neuron body. Spine density was assessed by counting the number of spine protrusions from secondary branches of the apical dendrite and normalizing to the total length of the spine (in microns). Dendrite thickness was determined by taking the area of a dendrite and dividing by the length.

Results

Coronal whole brain slices of three month old mouse to determine GFP expression

ASD-associated CHD8 mutations are predicted to lead to loss of protein function. Therefore, we utilized *in utero* electroporation technique to knockdown Chd8 expression specifically in cortical neural progenitors at embryonic day 15 (E15) to target upper layer cortex.We performed bilateral *in utero* electroporation at E15 with either control or Chd8 shRNA (Table 1) in addition to membrane bound GFP. The mice were grown to three months after birth.

Bilateral targeting of upper layer cortical neurons was confirmed in test animals following behavioral testing. The majority of brains expressed GFP bilaterally in the upper layer cortex both CHD8 shRNA and scrambled shRNA (Figure 2), meaning that both constructs were incorporated into similar areas of the brain at similar rates. The cortical layer of the brain was specifically targeted because it plays a key role in high-level functions like attention and behavior often disrupted in ASD (Kandel et al., 2000).

The whole brain GFP images (Figure 3) were used to assess whether or not the injected *Chd8* shRNA localized to the cortical brain layers. The co-incorporation rate of two plasmids injected simultaneously using in utero electroporation is 85.4% (Boutin et al., 2008). Because microscopic images show GFP incorporation (Figure 3), it is likely that *Chd8* shRNA was also incorporated in the same brain region. Knockdown assays done on neuroprogenitor cells harvested from Swiss Webster mice embryos using *Chd8* shRNA showed that incorporation of the shRNA corresponded to a knockdown of the gene and decreased protein expression (Unpublished data, Omer Durak, Tsai Lab). These data indicate that areas with GFP fluorescence have reduced CHD8 expression. Mice that did not show GFP fluorescence or only showed unilateral GFP expression in upper layer cortex were excluded from behavioral analysis. Incomplete knockdown could be a confounding factor in for behavior.

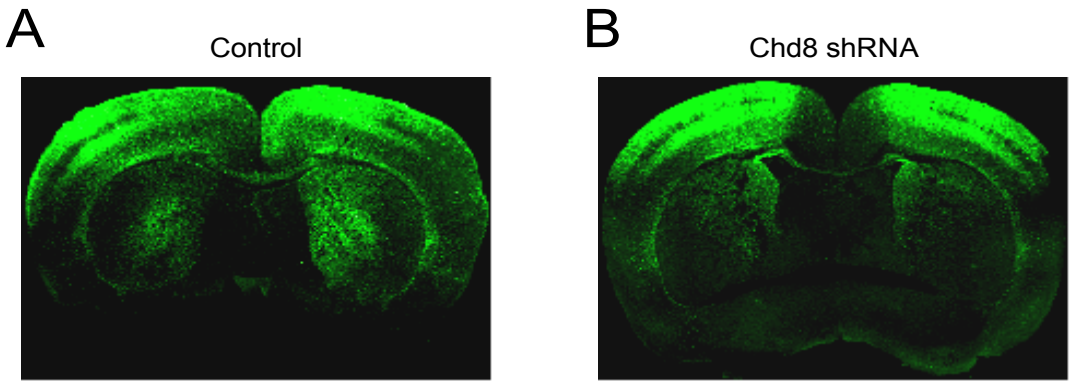

Figure 3. Rostral full brain slices of 3 month old mice electroporated *in utero* at embryonic stage E15 of either A) control shRNA+GFP or B)CHD8 shRNA +GFP. Both control and knock-out brains show bilateral GFP expression on the outer cortex of both right and left hemispheres of the brain.

Three Chamber Social Arena Assay

The three chamber behavioral test assesses two of the diagnostic criteria for ASD: abnormal reciprocal social interactions and anxiety in novel social situations (Silverman et al., 2010). Mice that spend less time in the chamber with the stranger mice and less time interacting with the stranger compared to control mice are considered to have behavioral deficits. This test was conducted on control and Chd8 knockdown mice.

Each mouse was habituated for ten minutes in the chambers containing neither stranger mouse nor an inanimate object. Video analysis of the time mice spent in each of the three chambers showed that neither control nor Chd8 knockdown mice intrinsically preferred one chamber over the other two (Figure 4A). In the next ten minutes, a control stranger mouse was placed inside a cage in one side section of the chamber whereas an inanimate object was placed inside a cage on the other side section of the chamber. The middle chamber was left empty (Figure 2). Video analysis of the time mice spent in each of the three chambers showed that the control mice spent more time in the chamber containing the stranger mouse whereas Chd8 knockdown mice showed

no preference for any of the three chambers (Figure 4B). Further analysis of the time each mouse spent interacting with either wired cages containing the stranger mouse or an inanimate object showed that control mice spent significantly more time interacting with the cage containing the stranger whereas Chd8 knockdown mice showed no preference in interacting with the two (Figure 4C). Experimental mouse and stranger mouse interactions were defined by nose touching through the wired cage containing the stranger mouse. Videos from these experiments are posted on Stellar.

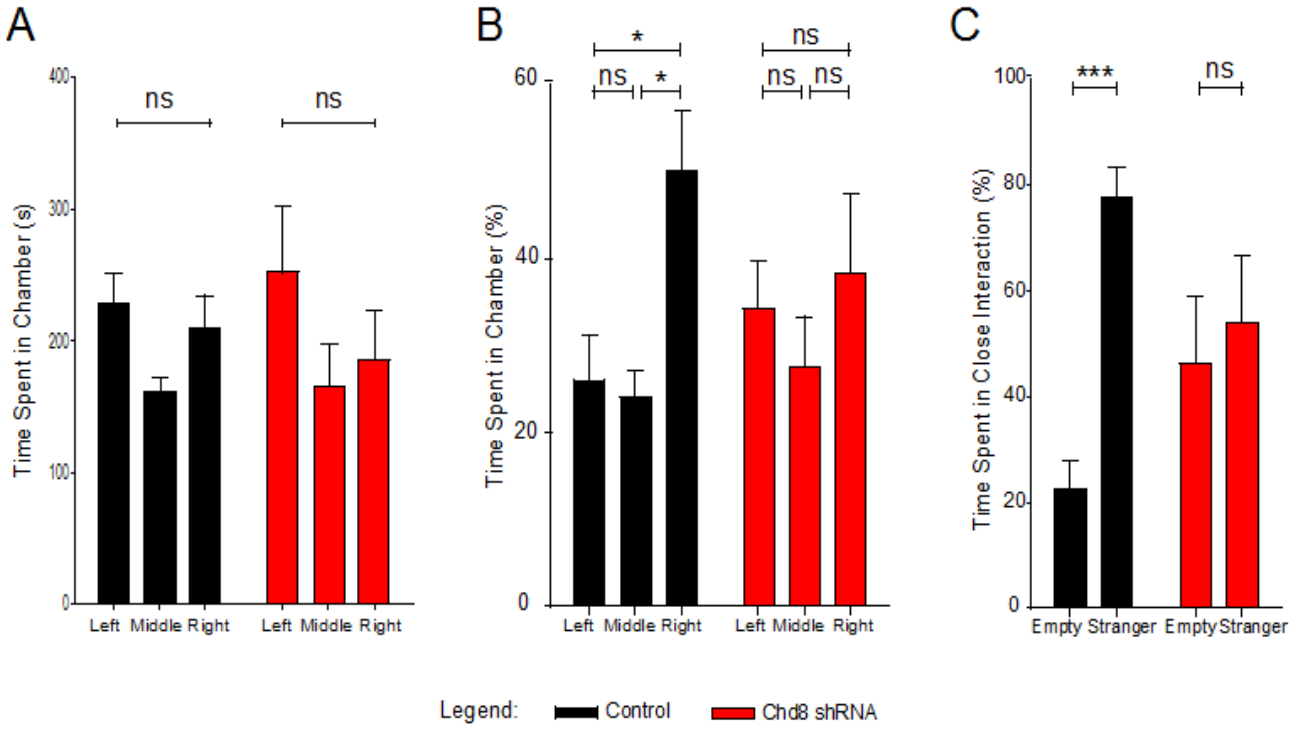

Figure 4. Time analysis of three chamber social arena assay. A) Habituation test where both cages contained nothing.B) Percent time spent in each of the three chambers out of the 10 minute video. C) Percent time spent interacting with nose touching with either stranger mouse or the inanimate object out of total time interacting with both. A total of 14 mice were tested: 6 were control mice and 8 were CHD8 knockdown mice. All error bars represent standard error.

Dendritic Arborization, Spine Density and Spine Thickness

We have shown that knockdown of Chd8 causes defects in social interaction in adult mice. To test whether knockdown of Chd8 also altered the growth and/or morphology of neurons in adult brain, we imaged the complete dendritic arbor of transfected neurons from control and Chd8 knockdown brains. Dendritic arborization was analyzed by drawing a series of equidistantly arranged concentric circles centered on the neuron body and counting the number of intersections between dendrites and each circle. The data shows that the Chd8 knockdown mice had significantly fewer primary neurites (Figure 5A, B) and less dendritic arborization (Figure 5A, C). Microscope images of sample neurons taken from a control mouse brain and a Chd8 knockdown mouse brain also illustrate these differences in morphology (Figure 5A).

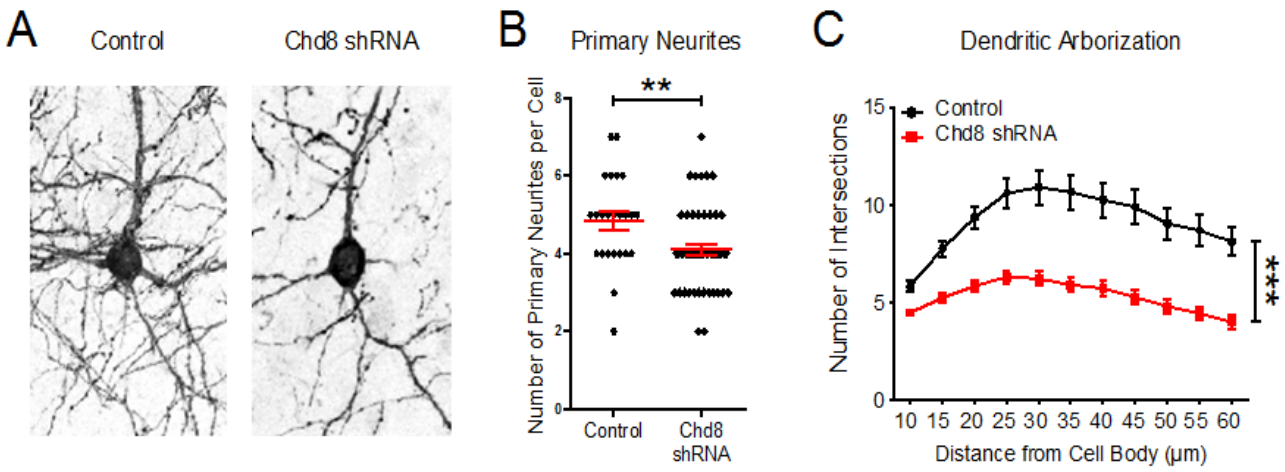

Figure 5. Total dendritic arborization of neurons imaged from Chd8 knockdown mice brains. 79 total neurons were examined: 24 neurons from 5 control brains and 55 neurons from 6 knockdown brains. Error bars are standard error. A) Sample neurons taken from either control or knockdown mice brains display morphological differences in branching. B) Data shows that neurons of Chd8 knockdown brains have reduced number of primary neurites. C) Data shows considerably less arborization for neurons of Chd8 knockdown brains.

Spines are the structures on dendritic branches that form synapses with axons. In the cortical layer, the spines of pyramidal neurons are the junction that receives electrical signals from within the cortex or other areas of the brain. Measuring the spine density of neurons is another way to characterize the connectivity of the cortical layer of the brain Spine density was measured by counting the number of spine protrusions on secondary branches from the apical dendrite and dividing by the length of the dendrite. The data shows that neurons of Chd8 knockdown brains have reduced spine density compared to neurons of control brains (Figure 6A, B). A third piece of data was generated after observing that dendrites from knockdown brains appeared thinner, an observation not captured by the arborization and spine density data. The thickness of dendrites branched from the apical dendrite was determined. Dendrites of knockdown brains are significantly thinner than those of control brains (Figure 6A, C). Microscope images of a dendrite from a control brain and a knockdown brain illustrate the differences shown in our quantitative data (Figure 6A). Overall, these results suggest that Chd8 is necessary for proper maturation of cortical neuron dendritic arborization and cortical neuronal circuitry and its disruption may contribute to deficits in social interaction and behavior in Chd8 knockdown mice.

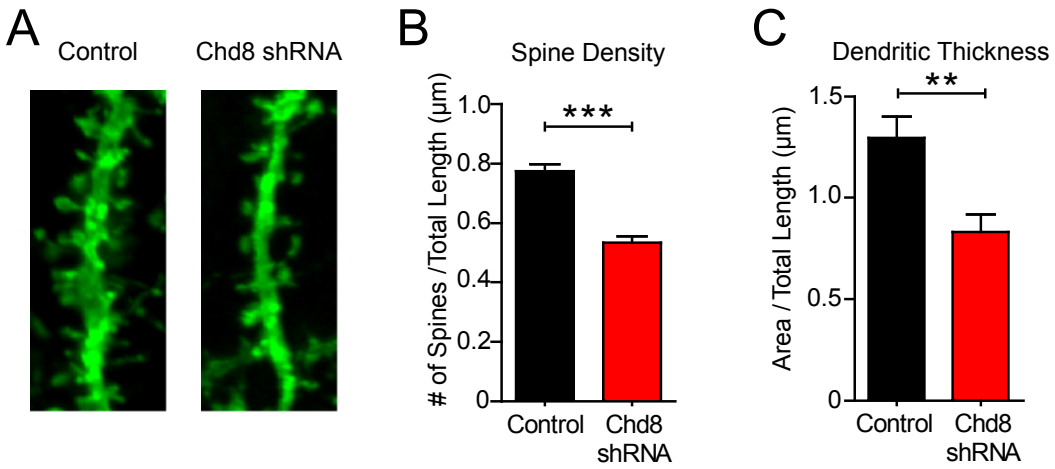

Figure 6. Spine density and spine thickness of dendrites imaged from Chd8 knockdown mice brains. The dendrites are the secondary branches of the apical dendrite of pyramidal neurons. For spine density, 31 total dendrites were examined: 15 from 4 control brains and 16 from 4 knockdown brains. For spine thickness, 32 total dendrites were examined, 14 from 4 control brains and 18 from 4 knockdown brains. Error bars represent standard error. A) Sample dendrites taken from either control or knockdown mice brains display differences in spine density and spine thickness. The data shows A) decreased spine density and B) decreased dendrite thickness in dendrites of CHD8 knockdown brains

QPCR for Chd8, B-catenin, E-cadherin in N2A cells with *Chd8* knockdown

QPCR was done on cDNA generated from whole cell RNA extracts of N2A cells transfected with *Chd8* shRNA to determine the RNA levels of Chd8, β-catenin and E-cadherin. This in-vitro experiment was done to assess the effect of knocking down CHD8 on proteins of interest that could be regulated by CHD8. Fluorescence from each primer condition for knockdowns was first normalized to beta-actin control and then to the amount of cDNA in the control. The QPCR data suggests that knocking down CHD8 *in vitro* is also associated with a decrease in transcription of β -catenin and E-cadherin (Figure 7).

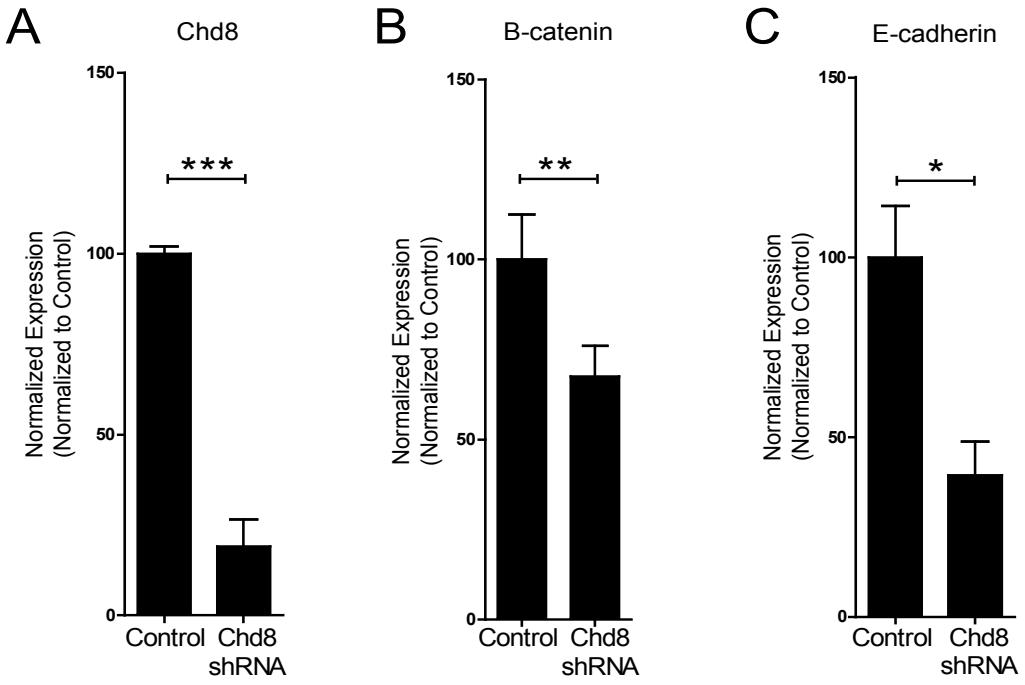

Figure 7. QPCR done on cDNA generated from whole cell RNA extracts of N2A cells transfected with CHD8 shRNA5 show that the shRNA knocks down CHD8 and also decreases the transcription of beta-catenin and E-cadherin.

Discussion

Analysis of adult mice confirms that there are significant differences in behavior and brain morphology between Chd8 knockdown mice and control mice. Our findings from the three chamber social arena assay show that Chd8 knockdown mice are less likely to prefer a stranger mouse over a novel object (Figure 4). This characterization supports that Chd8 knockdown mice display social deficits (Moy et al., 2004), a feature closely associated with ASD. Further analysis of neuron morphology through dendritic arborization, spine density and dendrite thickness demonstrate that knockdown mice display a decrease in all three (Figure 5, 6). The simplest conclusion from our data is that Chd8 knockdown decreases the overall connectivity of

the brain through reduced dendritic branches and spines, which contributes in part to deficits in social behavior.

The relationship between knocking down Chd8 and the morphological phenotypes in mice models may not be analogous in humans with disruptive *CHD8* mutations. The knockdown in mice would be difficult to do at the time of fertilization, yet patients with *CHD8* mutant alleles have constitutive mutations beginning at fertilization. Unlike the knockdown mice that will express Chd8 proteins prior to the knockdown, these patients will never express functional CHD8 proteins. The patients are thus more likely to develop mechanisms to compensate for the absence of CHD8 because the brain has the higher plasticity at the earlier embryonic stages. In addition, patients are also heterozygous for mutations and have at least one functional copy of *CHD8* unlike the knockdown mice. Though our data correlates Chd8 knockdown with aberrant neural circuitry and deficits in social behavior in mice, the same level of correlation may not be identical in patients due to these unknown compensations.

In addition, our knockdown only targeted the cortical layers of the brain because the frontal cortex is the primary brain region that governs social cognition (Amodio et al., 2006). However in ASD patients, the mutated *CHD8* gene is expressed throughout all brain regions. Our research is unable to conclude the contributions of CHD8 knockdown in other brain regions towards additional behavioral phenotypes.

Despite these caveats, the relationship of CHD8 to the disease mechanism of ASD is supported by the ASD-like behavioral deficit displayed by knockdown mice. In addition to the three chamber social arena assay that revealed a deficit in social interaction, behavioral assays that measure anxiety were also performed. The two assays, the light-dark box and the elevated

plus maze, showed that Chd8 knockdown mice were considerably more anxious than control mice (Unpublished data, Omer Durak). Because 39.6% of young people with ASD have at least one anxiety disorder (Van Steensel et al., 2011), evidence of anxiety in knockdown mice provides additional support for the correlation between Chd8 disruptions in mice and symptoms often associated with ASD patients.

Morphological phenotypes quantified by our studies show a reduced ability of pyramidal neurons in the cortical layer of Chd8 knockdown mice brains to receive electrical signals and likely have a direct correlation to the atypical social behaviors. Spinal protrusions on the branched dendrites form the receiving structure of synapses. These synapses receive information from all areas of the brain in the cortical layer, and thus collectively dictate the overall connectivity of that brain region to itself and other brain regions. The decrease in dendritic arborization, spine density and spine thickness quantified by our analysis (Figure 5, 6) collectively indicate that these neurons are less mature, less connected and probably have less neural activity. This conclusion supports the cortical underconnectivity theory for ASD that states that reduced long-range functional connectivity may contribute to a neural mechanism in ASD (Anderson et al., 2011). This theory is further upheld by functional imaging studies done on human brains that have shown that developmental disconnection contributes to ASD disorders (Geschwind and Levitt, 2007).

CHD8 is a chromatin remodeling protein that can bind to DNA and histones and regulate gene expression by controlling the modification process of chromatins. The disruption of CHD8 presumably alters expression of multiple genes associated with its chromatin remodeling process. B-catenin is one such gene (Sakomoto et al., 2000). *In vitro* data presented in this study shows that knocking down Chd8 in mouse N2A cells results in a reduction in β-catenin transcription

(Figure 7). RNAseq done on FACS sorted neurons from embryonice Chd8 knockdown mice confirm our *in vitro* findings and also show a reduction in β-catenin transcription (Unpubished data, Omer Durak). Furthermore, reduction in dendritic branching displayed by knockdown embryonic mice is rescued by co-injection of Chd8 shRNA with β-catenin (Unpublished data, Omer Durak). Collectively these data show that Chd8 regulates the transcription of β-catenin and that this regulation is also crucial to the normal branching of dendrites.

β-catenin has a dual function and disruptions in either or both functions could result in reduced connectivity and contribute to the social behavioral phenotypes that resemble ASD. β-catenin plays a role in the Wnt/ β-catenin signaling pathway and also forms a part of the cadherin/β-catenin membrane complex. The Wnt/β-catenin signaling pathway plays an essential role in the embryonice development of the central nervous system (Ferrari et al., 2006). Accumulation of β-catenin in the cytoplasm leads to its entry into the nucleus. The presence of β-catenin in the nucleus allows it to activate the T cell factor-lymphoid enhancer factor (TCF/LEF) family of transcription factors, many of which control cell cycle and proliferation. Though the disruption of this pathway is unlikely to directly impact reduced dendritic branching and spine density, additional data shows that Chd8 knockdown mice have less cell proliferation in cortical areas of the brain (Unpublished data, Omer Durak). A reduction in the total number of neurons also reduces the number of neuronal circuits the cortical layer can establish and is likely to also contribute to the social deficits observed.

The interaction between β-catenin and members of the cadherin protein group is a critical mediator in dendritic morphogenesis. Cadherin is a membrane-bound protein that tethers the actin cytoskeleton to the cell. Preliminary *in vitro* data shows that knocking down Chd8 in N2A cells decreases the transcription of both β-catenin and E-cadherin (Figure 7). Though the

mechanism is unclear, an increase in β-catenin or other members of the cadherin/catenin complex inside the neuron increases dendritic arborization in rats through the stabilization of dendritic spines and synaptic contacts (Yu and Malenka, 2003). B-catenin would facilitate the motile actin to anchor to membrane cadherin and stabilize dendrites during its grown and development. Interestingly, this process is independent of intracellular Wnt-signaling transcription though extracellular Wnt is observed to stabilize intracellular β-catenin. This information collectively suggests that the disruption of Chd8 could have disrupted the cadherin/β-catenin complex, a complex potentially important to dendritic growth and the formation of neuronal circuitry. It is possible that the disruption of this complex contributed to reduced circuitry and the social deficits.

In conclusion, though the exact mechanism through which Chd8 and β-catenin regulate neuronal circuitry development is unclear, all studies to date strongly support their involvement in these processes. Our data provides insights on the correlations between knocking down Chd8 in cortical mice brain, disruptions in neuronal circuitry and the ASD-like symptoms displayed by these mice. Additional research is needed for a more comprehensive behavioral assessment and better understanding of mechanisms through which Chd8 regulates synaptogenesis and neuronal circuitry. ASD is characterized by a triad of behavioral deficits. Mice assays that assess abnormalities other than social deficits can be used to determine additional effects of knocking down Chd8. For example, monitoring vocalization between mice using ultrasound is one way to assess the quantity of communication (Jamain et al., 2007). Rescue assays that co-inject N-cadherin and Chd8 shRNA can be done to clarify the importance of the β-catenin/N-cadherin complex for regulating dendritic branching. The creation of CRIPR-CAS Chd8 mutations analogous to patient mutations would be useful to assessing if mutating regions of the gene

rather than completely eliminating the protein would give rise to similar behavioral deficits and reduced neuronal circuitry seen in knockdowns.

Acknowledgements
This work was done in collaboration with Carol Liu, Omer Durak, Anthony Martorell and lab administrators of the Tsai Lab. Carol Liu prepared all brain slices and produced images and raw data for the analysis of neuronal connectivity. Carol also scored the three chamber social areana assay. Omer Durak generated all knockdown mice through *in utero* electroporation and was responsible for performing the behavioral assays with Anthony Martorell. Omer also helped organize the raw data and generate the images. Anthony perfused all the mice. Lab administrators of the Tsai Lab were responsible for purchasing the material, mice, plasmids, kits, and everything else required for these experiments.

References

American Psychiatric Association Task Force on DSM-IV. *Diagnostic and statistical manual of mental disorders: DSM-IV-TR* (American Psychiatric Association, 2000)

Anderson, J.S., Druzgal, T.J., Froehlich, A., DuBray, M.B., Lange, N., Lainhart J.E. (2010). Decreased Interhemispheric Functional Connectivity in Autism. Cerebral Cortex. Online

Armodio, D.M. and Frith, C.D. (2006).Meeting of minds: the medial frontal cortex and social cognition. Nature Reviews Neuroscience. *7*, 268-277.

Banerjee, S., Riordan, M., Bhat, M.A.(2014). Genetic aspects of autism spectrum disorders: insights from animal models. Cell Neurosci. 8:58

Belmonte, M.K., Allen, G., Beckel-Mitchener, A., Boulanger, L.M., Carper, R.A., Webb, S.J. (2004). Autism and abnormal development of brain connectivity. The Journal of Neuroscience. *24,* 9228-9231

Bernier, R., Golzio, C., Xiong, B., Stessman, H.A., Coe, B.P., Penn, O. (2014). Disruptive CHD8 mutations define a subtype of autism early in development. Cell. *158*, 263-276

Boutin, C., Diestel, S., Desoeuvre, A., Tiveron, M-C., and Cremer, H. (2008). Efficient in vivo electroporation of the postnatal rodent forebrain. Plos One. *3,* e1883.

Consantino, J.N., Zhang, Y., Frazier, T., Abbacchi, A.M. and Law, P. (2010). Sibling recurrence and the genetic epidemiology of autism. Am. J. Psychiatry., *167*, 1349-1356

Courchesne, E., Karns, C.M., Davis, H.R., Ziccardi ,R., Carper, R.A., Tigue, Z.D. (2001). Unusual brain growth patterns in early life in patients with autistic disorder: an MRI study. Neurology. 57, 245–254

Ferrari, G.V., Moon, R.T. (2006). The ups and downs of Wnt signaling in prevalent neurological disorders. Oncogene. *25*, 7545–7553

Frith, U. (1989) *Autism: explaining the enigma*. Oxford: Blackwell.

Geschwin, D.H. and Levitt, P. (2007).Autism spectrum disorders: developmental disconnection syndromes. Development., *17,* 103-111

Jamain, S., Radyushin, K., Hammerschmidt, K., Granon, S., Boretius, S., Brose, Nil. (2007). Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. PNAS. *105,* 1710-1710

Kandel, E.R., Schwartz, J.H., Jessell, T.M. (2000). *Principles of Neural Science Fourth Edition.* United States of America: McGraw-Hill.

Liu, P.Y., Erkkila, K., Lue, Y., Jentsch, J.D., Schwarcz, M.D., Abuyounes, D., Hikim, A.S., Wang, C., Lee, P., Swerdloff, R.S. (2010). Genetic, hormonal, and metabolomic influences on social behavior and sex preference of XXY mice. American Journal of Physiology. *299.* E446-E455

Moy, S.S., Nadler, J.J., Perez, A., Barbaro, R.P., Johns, J.M., Magnuson, T.R., Piven, J. and Crawley, J.N. (2004). Genes, Brain and Behavior. *3,* 287-302

O'Roak, B.J., Vives, L., Fu, W., Egertson, J.D., Stanaway, I.B., Phelps, I.G., Eichler, E.E. (2012). Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science. *338,* 1619-1622

Ozonoff, S., Pennington, B., Rogers, S.J. (1991). Executive function deficits in high-functioning autistic individuals: relationship to theory of mind. J Child Psychol Psychiatry. *32*, 1081-1105

Penzes, P., Cahill, M.E., Jones, K.A., Vanleeuwen, J-E., Woolfey, K.M. (2011). Dendritic spine pathology in neuropsychiatric disorders. *14,* 285-293

Sakamoto, I., Kishida, S., Fukui, A., Kishida, M., Yamamoto, H., Hino, S., Kikuchi, A. (2000). A novel β-catenin-binding protein inhibits β-catenin-dependent Tcf activation and axis formation. J Bio Chem., 275, 32871-32878

Van Steensel, F.J.A., Bogels, S.M., Perrin S. (2011). Anxiety disorders in children and adolescents with autistic spectrum disorders: a meta-analysis. Clin Child Fam Psychol Rev. *14*, 302-317

Yu, X. and Malenka, R.C.(2003). B -catenin is critical for dendritic morphogenesis. Nature Neuroscience. *11,* 1169-1177.

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