Determination of the Neurobiological Origins of the CHD8 Phenotype Using Genetic Cre Drivers

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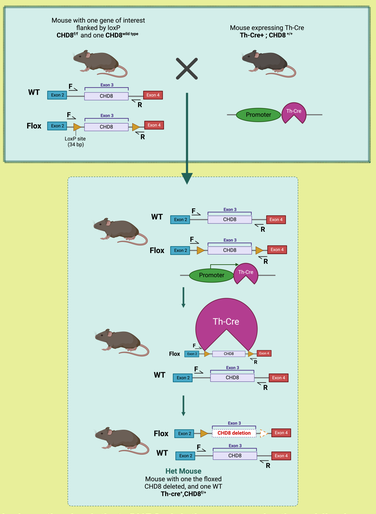
Introduction & Background

- Autism spectrum disorder is a neurodevelopmental condition defined by challenges in social interaction and communication, as well as a narrow focus on particular activities and interests (Faja et al., 2017).
- There is a strong genetic influence that has been demonstrated through twin studies.(Lord et al., 2018; Veenstra-VanderWeele, 2012, Abrahams, 2008).
- Sequencing-based research has successfully identified over 800 risk alleles, one of which is the *Chromodomain Helicase DNA-binding protein 8* or **CHD8 gene** (Muhle, 2018).
- Disruptive mutations causing loss of the CHD8 gene are hypothesized to affect dopamine pathways in the basal ganglia, however, specific genes and mechanisms remain unclear.
- This study aims to confirm the molecular changes and resulting expression associated with cre-mediated recombination of CHD8 via two methods: DNA and RNA analysis, per Luo, 2020.

Goals & Hypotheses

- The purpose of this project is to investigate the role of CHD8 deletions in specific brain regions using a Th-cre driver, thereby exploring how CHD8 gene loss affects neurodevelopmental processes, particularly related to autism spectrum disorder (ASD).
- Familiarize oneself with new techniques such as qPCR, mouse tissue harvesting, mouse colony husbandry, and genotyping.
- Utilize a breeding scheme in order to obtain the needed genotype.



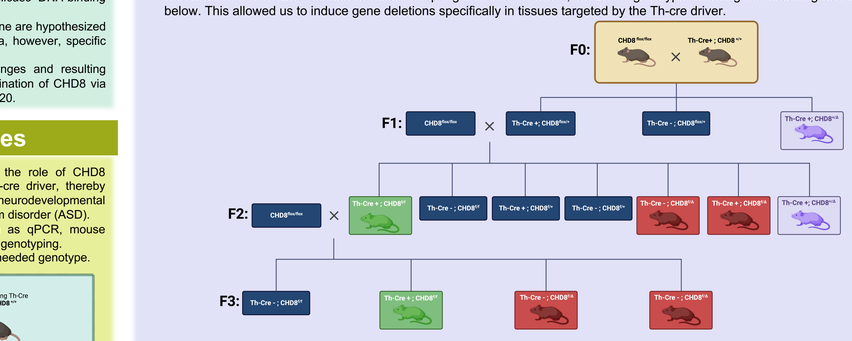
It is hypothesized that CHD8 deletions occur in the midbrain and olfactory bulb, which supports their critical role in ASD-related genetic pathways. This is based on substantial Th-Cre expression in TH-positive neurons in these regions. Additionally, the absence of deletions or recombination in the cortex, tail, and ear suggests that there is region-specific gene expression. The cortex is expected to show no recombination due to minimal Th-Cre expression within that region, and the tail and ear serves as a negative control with no Th-Cre expression.

Methods

To investigate the role of the CHD8 gene in autism spectrum disorders (ASD), a combination of genetic and molecular biology techniques were employed. This study utilized a Th-cre driver to induce gene deletions in specific tissues of the mouse, including the midbrain, and olfactory bulb. Samples of the cortex, tail, and ear served as negative controls. The experimental design includes the following key steps:

1. Mouse Mating: Implementing a Breeding Scheme for the Desired Mouse

A Th-Cre +; CHD8$^{flox/+}$ mouse was bred with a CHD8$^{+/+}$ mouse to obtain offspring with the Th-Cre +, CHD8$^{flox/+}$ genotype following the breeding scheme outlined below. This allowed us to induce gene deletions specifically in tissues targeted by the Th-cre driver.



2. Tissue Harvesting: Performing Dissections and Brain Extractions

- **Preparation**: We used Mouse K291-2 and K291-4, preparing three ice buckets to separate different genotypes. Tools included a brain mold, 20 razor blades, 2 scalpels, tooth forceps, a spatula, and 2 scissors for precise dissection. The entire dissection and harvesting process was conducted under a hood to maintain sterile conditions, and all razors and scalpels were disposed of after a single use to ensure safety and accuracy.
- **Dissection**: Mice were quickly decapitated, and the skulls were carefully removed. The brains were placed in a mold, and 10 razors per mouse brain were placed into the mold to make clean slices for collecting specific brain regions.
- **Control Tissues**: The midbrain and olfactory bulb served as the hypothetical positive controls to ensure consistency, while the cortex was collected as an uncertain variable, and the tail confirmed the genotype.
- **Sample Handling**: RNA samples were stored in 1.5 mL tubes on dry ice, while DNA samples were stored in 1.5mL tubes on regular ice. All instruments were kept ice cold and cleaned between mice to prevent contamination. From the collected RNA and DNA samples, various experiments can be conducted.

3. DNA Experimentation

- **Tissue Digesting and NanoDrop:** Mouse ear clippings were digested using 180 µL of ATL lysis buffer and 20 µL of proteinase K per sample, incubated for 24 hours at 56°C. After digestion, genomic DNA purification was performed with Qiagen DNEasy columns. DNA concentration was measured with a NanoDrop. The device was blanked with 1 µL of EB buffer, and 1 µL of each sample was analyzed twice to ensure consistent DNA concentration.
- **gDNA PCR Protocol :** A master mix is prepared using deionized water, primers, and Q5 Hot Start High-Fidelity 2X Master Mix, adjusted according to sample size. Twenty-four µL of the master mix is combined with 1 µL of digested mouse tissue samples, which are then loaded into the Veriti thermal cycler. Cycling conditions are customized based on the target DNA band size. The protocol employs primer pairs for DNA amplification, exponentially increasing sensitivity. Details on specific PCR types and conditions are outlined below.
- **Gel Electrophoresis**: After PCR in the Veriti machine, samples were prepared for gel electrophoresis. The gel was made by mixing 2.50 grams of agarose with 125 mL of 1x TAE solution, heating, and adding 12.5 µL of SYBR Safe DNA gel stain. Once set in the electrophoresis box, a 100bp ladder was added as a size reference. DNA samples, mixed with 5 µL of orange loading dye, were pipetted into the wells. Depending on the experiment's unique needs, a PCR can run for 1 hour on 100V or 3 hours on 70V, then imaged for analysis.

4. RNA Experimentation

- **Preparation:** After RNA isolation and cDNA preparation, a 96-well plate is prepared, using the extracted cDNA, primers to each loci, deionized water, and SYBR Master Mix. Multiple master mixes are made by combining the SYBR Master Mix, and deionized-free water, and cDNA. The master mixes are then dispensed into each well following a 96 well guide, and forward and reverse primers are added to amplify specific targets of interest. A housekeeping gene is amplified to permit quantitative comparison of expression between samples. The plate is sealed, centrifuged for 2 minutes, and loaded into the qPCR machine with unique cycling conditions.
- **Analysis:** Data is loaded is analyzed with the ΔΔCt method using an appropriate reference gene. The average fold changes and standard deviations are calculated, graphed, and further analyzed for results.

Results & Discussion

DNA Results

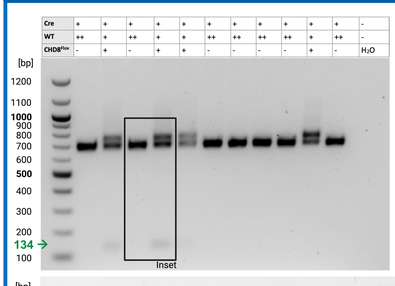


Figure A: Deletion PCR Model
This model demonstrates the successful deletion of the CHD8 gene when it is positive for CHD8^{flox}, indicated by a specific band at approximately 134 base pairs. The presence of this band confirms the targeted gene was knocked out. The inset further highlights each component of the PCR results and their size. This model was utilized in conjugation with Figure B, to analyze the presence of the recombination. Lanes 1-5 harbored the Emx1-cre allele, while lanes 6-11 harbored the Th-cre allele.

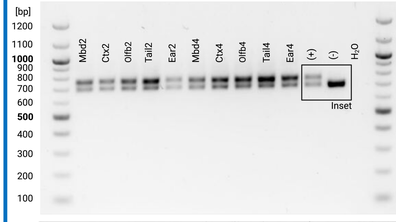


Figure B: Deletion PCR
This figure displays the results of the deletion PCR on the samples from the mice K291-2 and K291-4, showing no evidence of recombination or deletion in the samples. The absence of the expected band at 134 base pairs suggests that the CHD8 gene deletion did not occur in these samples.

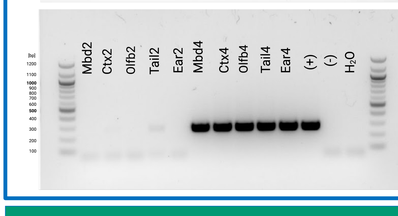


Figure C: General Cre PCR
This figure displays the results of the general Cre PCR on the samples from the mice K291-2 and K291-4. A general Cre PCR tests the presence of any Cre in the samples. The mouse K291-4 indicates that there is Cre detected, while the mouse K291-2 does not have any Cre detected. On both sides of the samples, we have a ladder to help identify band size, where the Cre band is approx. 300 bp. Along with the general Cre PCR, three more PCRs targeted toward a specific Th-Cre identification displayed the same results (data not shown).

RNA Results

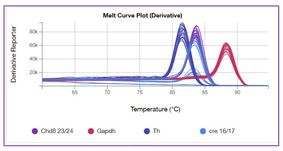


Figure D: Melting Curve Plot for Primers
Melting curve analysis of mCHD8, Gapdh (the housekeeping gene), Th, and Cre primers shows accurate and specific amplification. Each curve exhibits a single sharp peak which indicates that the primers are amplifying a specific target region with high accuracy. This means that the specificity and efficiency of the qPCR process is sufficient to test expression.

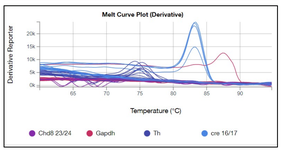


Figure E: Melting Curve Plot of no RT control to detect background DNA.
This melting curve analysis would indicate if any amplification occurred, as there was no reverse transcriptase included in the reaction, and only contaminative genomic DNA would serve as a template. The high peak of cre is concerning but inconclusive due to the amplifications occurring at much higher cycles. Therefore, this can be used as a benchmark that any amplification occurring in samples is precise and specific amplification, that is not hindered by background non-specific amplification.

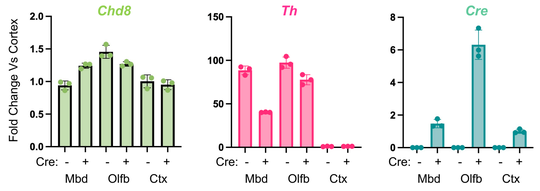


Figure E: Average Fold Change Graphs
The series of graphs displays the average fold change in *Chd8, Th,* and *Cre* RNA expression across various brain regions, with all data normalized to the cortex samples. *Chd8* is expressed in each sample at equivalent or silghtly higher levels than fpund in the cortex, Compared to the level of expression in the cortex, *Th* was expressed at 40-100X greater levels. Interestingly, the midbrain sample harboring the Th-cre allele showed decreased level of expression of *Th.* As expected, *Cre* expression was only observed in the samples from the animals that screened positive by genotype PCR (see left panel), with the highest expression noted in the olfactory bulb. The olfactory bulb shows a substantial level of Cre expression, as expected. However, the fold changes in the cortex and midbrain are similar, which is surprising given that the cortical sample had greatly reduced levels of *Th* expression.. This finding suggests there is an impediment to full expression of the cre allele in the midbrain.

Conclusion and Future Directions

- Despite confirming the expression of Th-Cre, no deletion was observed in the brain tissues, suggesting several possible issues. The accuracy of brain dissections needs to be reassessed to ensure proper sample processing. Alternatively, high levels of Th occupancy might be interfering with the cells, or the Cre recombinase might not be effectively reaching its genomic target.
- These findings imply that the hypothesis of expecting deletion with active Th-Cre via the animal breeding strategies was not supported, and adds a further note of caution to interpreting experiments predicated on recombination mediated by genetic cre drivers.
- Future research will focus on refining experimental protocols, including continuing with the matings and exploring different animal crosses. In one approach, a *Chd8$^{fl/fl}$* mouse will be crossed with a floxed GFP mouse to visualize successful Cre activity through fluorescence.
- Implementing immunohistochemistry and testing different Cre drivers, such as Emx1-Cre, will further enhance our understanding. Taking these future steps will allow for the continued assessment of the role of the *CHD8* gene in Autism Spectrum Disorders and the identification and understanding of the underlying mechanisms.

Acknowledgments

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