The effect of SAHA on the expression of genes in wild type and Hdac2 knockout mouse models and its potential use as treatment for schizophrenia

Maryum K. Ijaz
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The effect of SAHA on the expression of genes in wild type and Hdac2 knockout mouse models and its potential use as treatment for schizophrenia

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology & Biophysics at Virginia Commonwealth University.

By

Maryum Ijaz
Bachelor of Science, Virginia Commonwealth University, 2014

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Virginia Commonwealth University
Richmond, Virginia
September 2017
List of Publications & Manuscripts in Preparation


2. **HDAC2 is responsible for the restricted antipsychotic-like effects of the mGlu2/3 agonist LY379268 after chronic clozapine treatment in mice.**

   Manuscript in preparation

3. **Inhibition of HDAC2 induces antipsychotic-related synaptic and behavioral phenotypes in mice**

   Manuscript in preparation
Acknowledgement

I would like to give thanks to my parents, Mohammad Khalid Ijaz and Sadaf Sultana Ijaz for their ongoing support in all of my endeavors. I want to show appreciation to my siblings Kulsoom, Fatima, Omar, and Ali. I have sincerely enjoyed working with Dr. Javier Gonzalez-Maeso and greatly appreciate his guidance, kindness, and genuine character throughout my research project. To my lab colleagues who never failed to assist me from lab techniques to understanding papers, I thank you, Mario De La Fuente Revenga, Ashkhan Hojati, Justin Saunders, Supriya Gaitonde, Doan On, and Kelsey Hideshima. I would like to also thank my cousin, Sarah Faheem, for reading my thesis countless times and for all of her feedback.
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List of Abbreviations

Histone Deacetylase ........................................................................................................ HDAC
Suberoylanilide hydroxamic acid ................................................................................ SAHA
NIM.............................................................................................................................. National Institute of Mental Health
KO................................................................................................................................... Knockout
Abstract

THE EFFECT OF SAHA ON THE EXPRESSION OF GENES IN WILD TYPE AND HDAC2 KNOCKOUT MOUSE MODELS AND ITS POTENTIAL USE AS TREATMENT FOR SCHIZOPHRENIA

Maryum Khalid Ijaz, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Physiology & Biophysics at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Javier González-Maeso, PhD, Department of Physiology and Biophysics

The symptoms of schizophrenia have been categorized into three subsets including positive, negative, and cognitive symptoms. Although atypical antipsychotic use has shown promising reduction in positive and negative symptoms of schizophrenia, such as hallucination, delusions and flat affect, the cognitive subset of symptoms remains and has an adverse impact on those affected. Chronic treatment with the atypical/secondary antipsychotic clozapine is one example that has ameliorated hallucinations and delusions but results in cognitive memory deficits. When chronically given in conjunction with the HDAC inhibitor, SAHA, the cognitive memory deficits of mouse models decline. HDAC2KO mice were bred and used in chronic treatments with either vehicle or SAHA and compared to WT mice, in order to analyze whether differential gene expression was occurring in an HDAC2 dependent manner. The expression of various genes involved in brain function were evaluated using RT-qPCR to determine potential differential regulation. The results showed differential expression
of the following genes: *Abhd16a, Gbf1, Itch, and Ube2g1*. These genes are all involved in various neuronal functions.
Chapter 1: Introduction

1. Schizophrenia

According to the National Institute of Mental Health, schizophrenia is a “chronic and severe mental disorder that affects how a person thinks, feels, and behaves”. Individuals with the disorder may seem that they have completely lost touch with reality. Symptoms typically begin between ages 16 and 30 and in some rare cases, the disorder can manifest in children as well.

1.1. Clinical features of schizophrenia

Symptoms of schizophrenia are typically categorized into three subsets including positive symptoms, negative symptoms, and cognitive symptoms (11, 13).

1.1.1. Positive symptoms

Positive symptoms are the presence of beliefs, behaviors, and sensations that would otherwise not occur normally. These symptoms include disturbances of thought processes, delusions, and hallucinations (11).

1.1.2. Negative symptoms

Negative symptoms refer to the lack of normal behaviors that are otherwise present in healthy individuals. These include differences in feelings, movements, and behavior (11).

1.1.3. Cognitive symptoms

Cognitive symptoms refer to problems such as disorganized thoughts, difficulty understanding, poor concentration and/or memory, or difficulty expressing thoughts, feelings, and behaviors (11).
Figure 1. Demographics of Schizophrenia (Reprinted from: National Institute of Mental Health)
Prevalence

- **12-month Prevalence:** 1.1% of U.S. adult population
- **Severe:** Not reported

Demographics (for lifetime prevalence)

- **Sex:** Not Reported
- **Race:** Not reported
- **Age:** Not reported

Average Age-of-Onset:
Not Reported

Treatment/Services Use

- **12-month Healthcare Use:** 60.0% of adults with schizophrenia
- **Any Service Use (including Healthcare):** 64.3% of adults with schizophrenia
1.2. Treatment of Schizophrenia

1.2.1. First generation/Primary antipsychotics

Some examples of commonly used primary or first generation antipsychotic medications include chlorpromazine, fluphenazine, thioridazine, and haloperidol. These primary antipsychotics generally treat positive symptoms successfully, but have limited efficacy for the other two subsets of symptoms (11). Another negative result of these primary antipsychotics are their extrapyramidal side effects. Extrapyramidal side effects are commonly categorized as dystonia, dyskinesia, parkinsonism, and akathisia. Dystonia is described as abnormality of voluntary muscle tone or an abnormal degree of fixity associated with sustained muscular contraction. Similarly, dyskinesia describes impairment or the abnormality of voluntary movement. Parkinsonism refers to movement abnormalities usually associated with Parkinson’s disease such as tremor, slow movement, or impaired speech. Akathisia is another movement disorder categorized by an inner feeling of restlessness and compulsion to be in constant motion (21).

1.2.2. Secondary/atypical antipsychotics - Atypical antipsychotics or secondary antipsychotic drugs include clozapine and risperidone. These drugs block both 5-HT2A (serotonin) receptors as well as D2 (dopamine) receptors. Clozapine is commonly used because of its effective treatment of negative symptoms. However, in many cases cognitive symptoms persist and may even be heightened (11).
Figure 2. Chart of symptom subsets associated with schizophrenia as defined by the NIMH.
"Positive" symptoms refer to psychotic behaviors that are not otherwise present in healthy people.

- Hallucinations
- Delusions
- Thought disorders → unusual or dysfunctional ways of thinking
- Movement disorders → agitated body movements

"Negative" symptoms refer to a lack of normal behaviors or emotions that would otherwise be present in a healthy individual

- "flat affect" → reduced expression of emotions via facial expression or voice tone
- Reduced feelings of pleasure in everyday life
- Difficulty beginning and sustaining activities
- Reduced speaking

Patients may experience changes in memory and/or other aspects of thinking

- Poor "executive functioning" → ability to understand information and utilize it to make decisions
- Difficulty focusing or paying attention
- Issues with "working memory" → the ability to use information immediately after learning
1.3 Epigenetics & Chromatin Remodeling

The definition of epigenetics has evolved and now refers to changes across the genome that regulate gene transcription without modification of the underlying genome sequence. Epigenetic changes such as histone modifications such as DNA methylation, acetylation, and deacetylation are potentially heritable. However, the new focus of epigenetics is the effect it has on drug treatment and factors that may play in brain function (3, 8, 28, 29).

In eukaryotic cells, DNA is packaged into a nucleoprotein complex defined as chromatin. The basic unit of chromatin is a nucleosome, each of which contains 147 base pairs of DNA wrapped twice around an octamer of core histone proteins, including H2A, H2B, H3, and H4. Histones are proteins that have a globular domain and amino-terminal tail. Chromatin manifests in open and closed structural states. In the open state, the DNA wrapped around histones becomes unwound and is accessible to transcriptional machinery. Conversely, histones in the closed state are tightly wound with DNA, which cannot be transcribed. (8). Histone acetylation results in the open form of chromatin (euchromatin), while histone deacetylation has the opposite effect and suppresses DNA transcription (18, 23).

There are four classes of histone deacetylases (HDACs), referred to as Class I - IV. Each class includes different subsets of HDACs. The focus of this study is HDAC2, which is a Class I HDAC. Class I HDACs, specifically HDAC1 and HDAC2, are believed to be responsible for most changes in histone acetylation. The activity of HDACs are regulated by binding of corepressors and post-translational modifications (5, 7, 23).
Figure 3. Pictorial depiction of the epigenetic mechanisms underlying histone acetylation and deacetylation
Figure 4: Schematic model of improved antipsychotic drug action by HDAC2 inhibition (10)

A. Activation of 5-HT2A by the endogenous neurotransmitter serotonin represses HDAC2 promoter activity, B. Atypical antipsychotic drugs reverse the 5-HT2A-dependent repression of HDAC2, an effect that is associated with increased HDAC2 binding and repressive histone modifications at the promoter regions of synaptic plasticity genes, C. Inhibition of HDAC2 by vorinostat prevents these atypical antipsychotic drug-dependent repressive histone modifications, which improves the therapeutic efficacy.
A

Serotonin

5-HT_{2A} receptor

HDAC2

Naïve

B

Serotonin

5-HT_{2A} receptor

HDAC2

Clozapine

Limited antipsychotic activity

C

Serotonin

5-HT_{2A} receptor

HDAC2

Clozapine

Vorinostat

Active (synaptic plasticity genes)

Grm2

Adam8

GluR1

NR2B

Syt1

...

Improved antipsychotic activity
HDACs are expressed differently in various cell types, including neurons, oligodendrocytes, and astrocytes. However, HDACs have been shown to be expressed primarily in neurons (8, 9).

1.4 Therapeutic potential of HDAC inhibitors

Chronic administration of atypical antipsychotics, such as clozapine and risperidone, have been shown to downregulate the gene that encodes the metabotropic glutamate 2 receptor (mGlu2) via histone deacetylation. Chromatin immunoprecipitation assays have shown that this deacetylation results from HDAC2 binding to the promoter region of the mGlu2 gene thereby decreasing the levels of histone 3 acetylation, an epigenetic modification that is normally pro-transcriptive (11).

The mechanism by which this change occurs begins with a decrease in histone acetylation at the promoter region of the 5HT2A gene caused by clozapine. This ultimately leads to increased HDAC2 expression and the subsequent binding of HDAC2 at the mGlu2 promoter. The net effect of this process is decreased expression of mGlu2. The significance of this finding was further explored using mouse models. Mice that were virally induced to over express HDAC2 in the frontal cortex, thereby decreasing mGlu2 transcription, showed an increase in psychosis-like behavior.

In the same study, researchers investigated the effect of the HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), also known as vorinostat. SAHA was injected into mouse prefrontal cortexes, resulting in an increase in mGlu2 and mGlu3 expression. When SAHA was administered chronically along with clozapine,
Figure 5. Structure of SAHA/vorinostat.

SAHA blocked the downregulating effect of clozapine on mGlu2. Furthermore, it enhanced the ability of clozapine to ameliorate schizophrenia-like symptoms induced by administration of the hallucinogen DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) or another psychotomimetic drug, MK-801. For these reasons, HDAC inhibitors are potentially promising as an adjunctive therapy in the treatment of schizophrenia (11, 12).

2 Previous work in the Maeso Lab

Previous work in the Maeso lab included screening for genes differentially expressed in mouse prefrontal cortex models following administration SAHA (20 mg/kg) or saline for 21 days. Two groups of mice with three independent biological replicates per group were used for the microarray study, totaling 6 microarrays. Mice were sacrificed one day after the last injection. All animals were handled, treated, and sacrificed at the same time and under the same conditions. RNA and array processing was also performed at the same time. RNA was extracted from the mouse frontal cortex using the RNeasy lipid tissue mini kit (Qiagen).

Labeling and hybridization of the samples to Mouse Gene 1.0 ST expression chip (Affymetrix) were performed by the Mount Sinai Microarray Shared Resource Facility using standard methodology. Data quality control was performed using the Affymetrix Expression Console software. Areas under the Receiver Operating Characteristic curve (ROC) discriminate between positive control probesets and negative control probesets (pos.vs.neg.auc). All samples were found to be of very high quality, with pos.vs.neg.auc metric &gt; 0.89 (0.5 being no better than chance and 1.0 being perfect distinction), and no outlier was detected. Probe set
summarization, background correction and normalization were then carried out in the same software using default settings. Normalized data were analyzed in MultiExperiment Viewer software and differential gene list was generated using the Significance Analysis of Microarrays algorithm; Cutoff was chosen so that the false discovery rate does not exceed 12%. As a result, we obtained a final list of 284 probe sets, corresponding to 124 RefSeq genes. Pathway enrichment analysis was performed using the Ingenuity Pathway Analysis, with a score threshold (an adjusted Fisher Exact p-value) of 0.05 (Supplementary Fig. 19). The microarray data discussed in this paper have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo). The data analysis criteria used for our study are recommended by the MicroArray Quality Control project, and these criteria have been validated to provide a high degree of intersite reproducibility and inter- and intraplatform reproducibility.

Microarray analysis showed that animals receiving SAHA treatment had remarkably increased gene expression when compared with controls (Figure 6). Because the method of microarray analysis is not as accurate and can result in false positives. Because the method of microarray analysis is not as accurate and can result in false positives, various genes were chosen to replicate the experiment using RT-qPCR to determine whether in fact there was differential expression (Table 1).
Figure 6: Microarray Heatmap

Analysis of gene expression after chronic SAHA. Heat map shows differentially expressed genes in frontal cortex of mice chronically (21 days) treated with SAHA (20 mg/kg) versus control (three mice per group). Mice were sacrificed one day after the last injection.
### Table 1: Table of genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Lpar2</strong></td>
<td>Lysophosphatidic acids (LPAs) are bioactive lipids that function as lipid-signaling molecules of different chain lengths and phosphorylation and are expressed by a wide array of immune cells (20).</td>
</tr>
<tr>
<td><strong>Ankrd9</strong></td>
<td>Involved in mediating protein-protein interactions in a variety of different families of proteins (NCBI). It is also involved in various cell fxns including intracellular adaptor, trafficking, transcription, signal transduction, and stress response (Wang et al., n.d.).</td>
</tr>
<tr>
<td><strong>Efnb1</strong></td>
<td>The protein that is encoded by this gene may play role in cell adhesion as well as the function and development or even the maintenance of the nervous system (NCBI).</td>
</tr>
<tr>
<td><strong>S100Z</strong></td>
<td>S100 proteins have regulatory effects on a number of different cell types such as microglia, neutrophils, lymphocytes, neurons, astrocytes, schwann cells, and more. Therefore they participate in both innate and adaptive immune responses, tissue development and repair, cell migration, and tumor cell invasion (12).</td>
</tr>
<tr>
<td><strong>Aqp1</strong></td>
<td>Encodes a functioning water channel protein that permits passive transfer of water osmotically (NCBI).</td>
</tr>
<tr>
<td><strong>Abhd16a</strong></td>
<td>Abhydrolase domain containing 15A is included in a cluster of genes localized for tumor necrosis factor alpha and beta. The protein this gene encodes is thought to be associated with immunity as this gene is within the human major histocompatibility complex class III region (NCBI).</td>
</tr>
<tr>
<td><strong>Eif5</strong></td>
<td>Eukaryotic translation initiation factor promotes hydrolysis of GTP to allow joining of 60S ribosomal subunit with 40S subunit resulting in the functional 80S ribosomal initiation complex, which is then active (NCBI).</td>
</tr>
<tr>
<td><strong>Gbf1</strong></td>
<td>Golgi brefeldin A resistant guanine nucleotide exchange factor 1 is involved in regulating proteins to membranes by mediating the GDP to GTP exchange. It also plays a role in vesicular trafficking when in the golgi apparatus and is a host factor for viral replication (NCBI).</td>
</tr>
<tr>
<td><strong>Itch</strong></td>
<td>Itch E3 ubiquitin protein ligase plays a role in cellular processes such as erythroid and lymphoid cell differentiation and further regulates immune responses (NCBI).</td>
</tr>
<tr>
<td><strong>Ssr3</strong></td>
<td>The signal sequence receptor is a membrane receptor that translocates proteins across the ER membrane (NCBI).</td>
</tr>
<tr>
<td><strong>Ube2g1</strong></td>
<td>Proteins with ubiquitin are important for targeting abnormal proteins for degradation (NCBI).</td>
</tr>
<tr>
<td><strong>Homer1</strong></td>
<td>Homer is a scaffolding protein that regulates mGluR signaling.</td>
</tr>
</tbody>
</table>
Long variants of this protein link mGluR and its mediators by their EVH1 domains. The shorter variants are unable to provide this linkage and act as a negative protein, disrupting the crosslink action via mGlur-Homer signaling.

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<table>
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<tbody>
<tr>
<td><strong>Hdac1</strong></td>
<td>• Histone deacetylase 1 encodes a protein that interacts with retinoblastoma tumor-suppressor protein and controls cell proliferation and differentiation. This results in a regulation on cell growth and apoptosis (NCBI).</td>
</tr>
<tr>
<td><strong>Hdac2</strong></td>
<td>• Histone deacetylase 2 is part of a family of HDACs that deacetylate lysine residues at the N-terminal regions of core histones. The protein this gene encodes forms transcriptional repressor complexes via association with different proteins (NCBI).</td>
</tr>
<tr>
<td><strong>Hdac5</strong></td>
<td>• The protein encoded by histone deacetylase 5 is part of class II HDACs and functions to repress transcription when it is tethered to a promoter (NCBI).</td>
</tr>
</tbody>
</table>
**Table 2: Table of primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homer1</td>
<td>AGCAGAAGGAAGGCTTGACT</td>
<td>CACGGTACGGCACAATAACTA</td>
</tr>
<tr>
<td>Homer1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hdac5</td>
<td>ACTCCTCTGCACAGCATCC</td>
<td>CTGGGGCTACCTCCACCT</td>
</tr>
<tr>
<td>Hdac5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hdac1</td>
<td>GGCACCAAGGAAAGTCTG</td>
<td>CAAATTTGGAATGTATGAGGA</td>
</tr>
<tr>
<td>Hdac1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hdac2</td>
<td>CATGGCGTACAGTCAAGGAG</td>
<td>TCAACCACGCTCAAGCA</td>
</tr>
<tr>
<td>Hdac2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abhd16a</td>
<td>GCAGCCTCCTCAACCTCAACA</td>
<td>GGTGGTATGATCTCGTCCT</td>
</tr>
<tr>
<td>Abhd16a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gbf1</td>
<td>CCCCAGAGATACCCCTGAAATGG</td>
<td>TCTTGAGCAAGACTCATT</td>
</tr>
<tr>
<td>Gbf1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eif5</td>
<td>AGGAAGAAGATGAAGACGAAAATTTG</td>
<td>TGTTCGAGACTCAGTTC</td>
</tr>
<tr>
<td>Eif5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itch</td>
<td>CATCGAAAAAGTTGGCAAAGAA</td>
<td>GGTCAGAGCTCAGGAAAACAA</td>
</tr>
<tr>
<td>Itch</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ssr3</td>
<td>AGGATTTTCAGCCGCAACCT</td>
<td>CGCGTTTCAGCCGAAGCACG</td>
</tr>
<tr>
<td>Ssr3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ube2g1</td>
<td>CCGGCCTCCTAATGAAATT</td>
<td>GCAAAACATCACTATTTCATCAAC</td>
</tr>
<tr>
<td>Ube2g1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqp1</td>
<td>CTAGTGGGTCGGCCTCAAGGA</td>
<td>GGAGACTGGAGGACCGAAATAA</td>
</tr>
<tr>
<td>Aqp1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
</tr>
<tr>
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<td>---------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Ankrd9</td>
<td>CCTGGCAGTGCGAGATCAG</td>
<td>GCTTCGCTGGCACGTATGT</td>
</tr>
<tr>
<td>Efnb1</td>
<td>TGTGTCGACCCGCACCTA</td>
<td>GGGTGTCACAGCATTGGATCT</td>
</tr>
<tr>
<td>Lpar2</td>
<td>GGGCCAGTGCTACTACAACGA</td>
<td>AGCTCCTTGCGCTGTATTAT</td>
</tr>
<tr>
<td>S100z</td>
<td>CGCATCTCCACCGGTACTC</td>
<td>CCCCTTGTGGAGCTTGAAC</td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

2.1 Wildtype and HDAC2 Conditional Knockout Mice

Treatment mice included wildtypes of the strain C57BL/6 (Taconic) and HDAC2 conditional knockout mice. Animals were housed with a 12h light/dark cycle and were provided with access to food and water. Pups were weaned from their mothers after 21 days and were then housed in cages of 3-5 mice. All mice used were 6-8 weeks of age at the time of treatment and male.

Hdac2 KO mice were obtained from The Jackson Laboratory (stock number: 022625). Heterozygous Hdac2 +/- mice were intercrossed to generate Hdac2-/- mice on a C57BL/6 background. Homozygosity of the Hdac2-null allele resulted in either embryonic lethality or partial lethality during the first few days postnatal as a result of proliferation defects and impaired development (data not shown). These findings are consistent with some but not all of the prior descriptions of global Hdac2 gene deletion. Based on this, we therefore used CaMKIIα-Cre transgenic mice on a C57BL/6 background, in which Cre recombinase is efficiently expressed in combination with conditional loss of function of Hdac2 alleles. In mice, CaMKIIα is expressed postnatally in forebrain glutamatergic pyramidal neurons, beginning 10–14 d after birth. To delete HDAC2 function specifically in forebrain glutamatergic pyramidal neurons, we bred homozygous Hdac2loxP/loxP mice to the CaMKIIα-Cre transgenic line. In contrast to the global deletion of HDAC2, Hdac2loxP/loxP:CaMKIIα-Cre (Hdac2- cKO) mice were viable and did not display any gross histological or developmental abnormality (data not shown). Hdac2loxP/loxP:CaMKIIα-Cre mice were born at near expected Mendelian ratios (data not shown. Genomic DNA was isolated from ears for genotyping by PCR
Figure 7: Flow chart of overall procedure
Chronic treatment with vehicle or SAHA in CTL & HDAC2 KO mice

Sacrificing mice via cervical dislocation and PFC dissection

mRNA isolation

Nanodrop & Normalization of amount of mRNA

RT rxn: mRNA $\rightarrow$ cDNA

qPCR
Figure 8: Strategy for deleting forebrain pyramidal HDAC2 function (h). Western blots showed decreased HDAC2 protein levels in the frontal cortex, but not cerebellum, of *Hdac2-cKO* compared to control littermates (*n* = 4–6 mice per experimental condition) (l, m). (10)
analysis. The primer sequences used were as follows: Hdac2loxP/loxP: Hdac2 wt allele forward (5′-GCACAGGCTACTACTGT GTAGTCC-3′), Hdac2loxP mutant allele: forward (5′GTCCCTCGA CCTGCAGGAATTC-3′), Hdac2loxP mutant allele reverse (5′-CCACCACTGACATGTACCCAAC-3′); CaMKII-Cre, transgene forward (5′-GCGGTCTGGCAGTAAAAACTATC-3′), transgene reverse (5′GTGAAACAGCATTGCTGTCACTT-3′); internal positive control forward (5′CTAGGCCACAGAATTGAAAGATCT-3′), internal positive control reverse (5′GTAGGTGGAAATTCTAGCATC-3′).

2.2 Chronic treatment with saline or SAHA

To test the effect of chronic SAHA treatment on gene expression, both wildtype mice and HDAC2KO mice were injected (i.p.) once a day for 21 days with SAHA (20mg/kg; obtained from Cayman Chemical). SAHA was suspended in a minimal amount of DMSO and brought up to volume with saline on the day of each injection. Control mice were injected chronically with vehicle (saline). On the day after the last injection, the mice were sacrificed via cervical dislocation, and the prefrontal cortices were dissected and stored at -80°C until ready for mRNA isolation.

2.3 Tissue Homogenization & mRNA isolation

The brain tissue was purified using TRIZOL reagent (1 mL per sample) as the lysis buffer and further isolated via the RNAeasy lipid kit (Qiagen). The brain tissue was sheared using an 18-gauge needle attached to a 5-ml syringe. Isolated mRNA was measured using the Nanodrop 1000 software (Thermo Fisher).

RNA ISOLATION PROTOCOL

NOTES
De-contaminate the work area, all work tools, and their surfaces of RNase using 70% EtOH
followed by RNaseZap. Lay out absorbency paper.

Exclusively use filter-tips.

Chill centrifuge to 4°C.

Get a bucket of ice.

**MATERIALS**

- RNaseZap
- 70% EtOH
- benchtop absorbency paper
- RNeasy Lipid Tissue mini kit
- 1.5 ml microfuge tubes
- 18-gauge needles
- 5-ml syringes
- RNase-free DNase kit (Qiagen)
- DEPC-treated water
- Kim wipes

**PROCEDURE**

*Tissue Homogenization & RNA Isolation*

1. Retrieve brain tissue and place on ice.
2. Add 1 ml QIAzol reagent per sample.
3. Homogenize tissue using an 18-gauge needle attached to a 5-ml syringe. Shear tissue upwards of 30 times until no more tissue chunks can be seen.
   - Never recap the needle. Dispose of used syringes in sharps container.
4. Transfer to pre-labeled 1.5 microfuge tubes.
5. In the fume hood (which has been de-contaminated with RNaseZAP), add 200 µl chloroform, secure the cap, vigorously shake for 15 secs, and incubate at room temperature for 3 mins.
6. Centrifuge at 12,000g for 15 mins at 4°C.
7. While waiting, prepare DNase I incubation mix. For each sample, add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.
   - RDD is supplied with the RNase-Free DNase kit.
   - DNase I is sensitive to physical denaturation, so mixing should only be carried out by gently inverting the tube. Never vortex.
8. Transfer the upper, aqueous phase to a new 1.5 ml microfuge tube.
   - Do not transfer any interphase solids.
9. Add 1 volume (about 500 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge.
10. Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 15 secs at 8,000g at room temperature. Discard the flow-through. Repeat until all sample has been applied to the column.
11. Wash the membrane with 350 µl Buffer RW1 and centrifuge for 15 secs at 8,000g (10,000 rpm). Discard the flow-through.
12. Add 80 µl DNase I incubation mix (from Step 7) directly to the RNeasy spin column membrane, and incubate at room temperature for 15 min.
13. Wash with 350 µl Buffer RW1, and centrifuge for 15 secs at 8,000g. Discard the flow-through.
14. Wash with 500 µl Buffer RPE, and centrifuge for 15 secs at 8,000g. Discard the flow-through and replace collection tube.
15. Wash with 500 µl Buffer RPE, centrifuge for 3 min at max speed to dry column.
    a. Any carryover EtOH will interfere with downstream assays.
16. Carefully remove spin column from the collection tube so column does not contact the flow-through.
17. Place the spin column in a new, pre-labeled 1.5 ml collection tube. Add 30 µl RNase-free water (provided in kit) directly to the spin column and incubate at room temperature for 2 mins. Elute by centrifuging for 1 min at 8,000g.
    a. To increase yield, repeat previous step using 20 µl of RNase-free water.
18. Place purified RNA on ice. Measure concentration and purity by NanoDrop.

2.4 Reverse Transcription Reaction

Each sample of the same treatment condition was normalized to the lowest amount of mRNA obtained via the nanodrop software to ensure that the same amount of mRNA was to be compared between samples. The RT reaction was carried out using oligo dt, DTT, and dNTPs, followed by a master mix of 5x buffer, Superscript III, and RNase out.

2.5 RT-qPCR

cDNA obtained from the reverse transcription reaction was diluted to a 1:40 dilution for the RT-qPCR reaction. The QuantFlex Studio 6 machine was used to carry out qPCR for a total of 10 uL including 4uL of cDNA, 1 uL of primer, and 5 uL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Catalog number: A25777) per reaction well. 384 Well plates were used. In Real-Time qPCR, a fluorescent reporter molecule is utilized to monitor the progression of the reaction. For this study, SYBR green was used as the reporter molecule (Figure 7), which allows the quantification of the template to be based on the fluorescence signal during the exponential phase of amplification. As each cycle of amplification is carried out, there
will be an increase in fluorescence intensity, which is proportional to the increase in concentration of the amplicon.

A method of confirmation to verify specific amplification calls for analysis of the melt curve. Each reaction was carried out in quadruplicates, meaning there were 4 wells with the same conditions of cDNA, SYBR green, and primer. The melt curve should show a single homogenous peak, which would indicate specific amplification. We first tested the primers to ensure specific amplification by examining the melt curves. After ensuring that each primer provided a homogenous peak, indicative of specific amplification, we went on to test the primers on the wildtype and HDAC2 KO samples in the experiment.

The amplification plot is graphically depicted as fluorescence vs cycle number. The threshold cycle (Ct) value shows the threshold fluorescence level. Once the plot crosses this threshold level, the subsequent Ct values can be correlated to the starting concentration of the sample. (Figure 8).

2.6 Statistical Methods

Ct Medians obtained from RT-qPCR were processed to determine mRNA level fold change. Significance was tested using a two-tailed t-test (GraphPad PRISM). Values are shown in Tables 3.1 and 3.2 for wildtypes and conditional Hdac2 KO mice, respectively.
Figure 9: Pictorial depiction of SYBR green binding to the amplicon during RT-qPCR
Figure 10: Pictorial description of RT-qPCR amplification plot
Figure 11: Pictorial depiction of Melt Curve to confirm specific amplification
Chapter 3: Results

The results are graph representations of a two-tailed t-test conducted by the PRISM software. Upon analysis, it was found that in wildtypes treated with SAHA vs vehicle, the genes, ITCH and UBE2G1, were downregulated when treated with SAHA (Figure 12 D,E). The genes ABHD16A and GBF1 were both upregulated upon treatment with SAHA in wildtype mice (Figure 12, AB). HDAC2 was significantly downregulated in wildtype mice treated with SAHA as compared to vehicle (Figure 14, C).
### TABLE 3.1: TWO-TAILED T-TEST COMPARING QUANTITATIVE EXPRESSION OF GENES IN WILD TYPE MICE TREATED WITH VEHICLE VS SAHA

<table>
<thead>
<tr>
<th>GENE</th>
<th>Vehicle treated mice</th>
<th>N</th>
<th>SAHA treated mice</th>
<th>N</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABHD16A</td>
<td>1.000 ± 0.08611</td>
<td>12</td>
<td>1.322 ± 0.08362</td>
<td>12</td>
<td>0.0137 *</td>
</tr>
<tr>
<td>ANKRD9</td>
<td>1.000 ± 0.4725</td>
<td>12</td>
<td>2.888 ± 0.8591</td>
<td>12</td>
<td>0.0672</td>
</tr>
<tr>
<td>AQP1</td>
<td>1.000 ± 0.2758</td>
<td>12</td>
<td>1.071 ± 0.1724</td>
<td>12</td>
<td>0.8302</td>
</tr>
<tr>
<td>EFN1</td>
<td>1.000 ± 0.2012</td>
<td>12</td>
<td>0.9913 ± 0.06505</td>
<td>12</td>
<td>0.9675</td>
</tr>
<tr>
<td>EIF5</td>
<td>1.000 ± 0.04188</td>
<td>12</td>
<td>0.8799 ± 0.1456</td>
<td>12</td>
<td>0.4452</td>
</tr>
<tr>
<td>GBF1</td>
<td>1.000 ± 0.06942</td>
<td>12</td>
<td>1.319 ± 0.1291</td>
<td>12</td>
<td>0.0403 *</td>
</tr>
<tr>
<td>HDAC1</td>
<td>1.000 ± 0.1093</td>
<td>12</td>
<td>0.8623 ± 0.09276</td>
<td>12</td>
<td>0.3470</td>
</tr>
<tr>
<td>HDAC2</td>
<td>1.000 ± 0.1148</td>
<td>12</td>
<td>0.6699 ± 0.05243</td>
<td>12</td>
<td>0.0158 *</td>
</tr>
<tr>
<td>HDAC5</td>
<td>1.000 ± 0.1371</td>
<td>12</td>
<td>1.043 ± 0.1291</td>
<td>12</td>
<td>0.8224</td>
</tr>
<tr>
<td>HOMER1</td>
<td>1.000 ± 0.07692</td>
<td>12</td>
<td>1.195 ± 0.1431</td>
<td>12</td>
<td>0.2429</td>
</tr>
<tr>
<td>ITCH</td>
<td>1.000 ± 0.05122</td>
<td>12</td>
<td>0.5699 ± 0.1277</td>
<td>12</td>
<td>0.0040 *</td>
</tr>
<tr>
<td>LPAR2</td>
<td>1.000 ± 0.1155</td>
<td>12</td>
<td>1.130 ± 0.1101</td>
<td>12</td>
<td>0.4258</td>
</tr>
<tr>
<td>S100Z</td>
<td>1.000 ± 0.1862</td>
<td>12</td>
<td>1.181 ± 0.1818</td>
<td>12</td>
<td>0.4943</td>
</tr>
<tr>
<td>SSR3</td>
<td>1.000 ± 0.3114</td>
<td>12</td>
<td>0.6778 ± 0.1556</td>
<td>12</td>
<td>0.3612</td>
</tr>
<tr>
<td>UBE2G1</td>
<td>1.000 ± 0.05237</td>
<td>12</td>
<td>0.7999 ± 0.06986</td>
<td>12</td>
<td>0.0319 *</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. *p<0.05. Student’s t-test.
### TABLE 3.2: TWO-TAILED T-TEST COMPARING QUANTITATIVE EXPRESSION OF GENES IN HDAC2 KNOCKOUT MICE TREATED WITH VEHICLE VS SAHA

<table>
<thead>
<tr>
<th>GENE</th>
<th>Vehicle treated mice</th>
<th>N</th>
<th>SAHA treated mice</th>
<th>N</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABHD16A</td>
<td>1.000 ± 0.1940</td>
<td>6</td>
<td>1.080 ± 0.1791</td>
<td>6</td>
<td>0.7686</td>
</tr>
<tr>
<td>ANKRD9</td>
<td>1.000 ± 0.2901</td>
<td>6</td>
<td>1.107 ± 0.09678</td>
<td>6</td>
<td>0.7329</td>
</tr>
<tr>
<td>AQP1</td>
<td>1.000 ± 0.3560</td>
<td>6</td>
<td>0.6106 ± 0.04504</td>
<td>6</td>
<td>0.3034</td>
</tr>
<tr>
<td>EFN1</td>
<td>1.000 ± 0.1765</td>
<td>6</td>
<td>1.325 ± 0.3082</td>
<td>6</td>
<td>0.3816</td>
</tr>
<tr>
<td>EIF5</td>
<td>1.000 ± 0.2382</td>
<td>6</td>
<td>0.8960 ± 0.1188</td>
<td>6</td>
<td>0.7042</td>
</tr>
<tr>
<td>GBF1</td>
<td>1.000 ± 0.1797</td>
<td>6</td>
<td>1.210 ± 0.2726</td>
<td>6</td>
<td>0.5344</td>
</tr>
<tr>
<td>HDAC1</td>
<td>1.000 ± 0.07734</td>
<td>6</td>
<td>1.067 ± 0.1578</td>
<td>6</td>
<td>0.7094</td>
</tr>
<tr>
<td>HDAC2</td>
<td>1.000 ± 0.1959</td>
<td>6</td>
<td>2.014 ± 0.8279</td>
<td>6</td>
<td>0.2608</td>
</tr>
<tr>
<td>HDAC5</td>
<td>1.000 ± 0.1329</td>
<td>6</td>
<td>3.972 ± 2.455</td>
<td>6</td>
<td>0.2546</td>
</tr>
<tr>
<td>HOMER1</td>
<td>1.000 ± 0.1217</td>
<td>6</td>
<td>1.156 ± 0.3353</td>
<td>6</td>
<td>0.6717</td>
</tr>
<tr>
<td>ITCH</td>
<td>1.000 ± 0.04153</td>
<td>6</td>
<td>1.193 ± 0.1855</td>
<td>6</td>
<td>0.3339</td>
</tr>
<tr>
<td>LPAR2</td>
<td>1.000 ± 0.2486</td>
<td>6</td>
<td>0.8205 ± 0.1457</td>
<td>6</td>
<td>0.5473</td>
</tr>
<tr>
<td>S100Z</td>
<td>1.000 ± 0.4103</td>
<td>6</td>
<td>0.9759 ± 0.2474</td>
<td>6</td>
<td>0.9609</td>
</tr>
<tr>
<td>SSR3</td>
<td>1.000 ± 0.1564</td>
<td>6</td>
<td>1.325 ± 0.2614</td>
<td>6</td>
<td>0.3116</td>
</tr>
<tr>
<td>TBKBP1</td>
<td>1.000 ± 0.2087</td>
<td>6</td>
<td>2.356 ± 1.239</td>
<td>6</td>
<td>0.3057</td>
</tr>
<tr>
<td>UBE2G1</td>
<td>1.000 ± 0.09328</td>
<td>6</td>
<td>1.343 ± 0.1317</td>
<td>6</td>
<td>0.0598</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. Student's t-test.
**Figure 12:** Differential expression of genes was identified by RT-qPCR in wildtypes and HDAC2 KO mice treated with vehicle vs SAHA. Increased gene expression of ABHD16A (A) and GBF1 (B) in wildtype mice treated with SAHA. Decreased expression of ITCH (C) and UBE2G1 (D) in wildtype mice treated with SAHA.
Figure 13: A trend to increased gene expression of ANKRD9 in wildtype mice treated with SAHA but not in HDAC2 KO mice
ANKRD9

p < 0.0672
Figure 14: Significant decrease in expression of HDAC2 when wildtype mice were chronically treated with HDAC inhibitor, SAHA.
Chapter 4: Discussion

4.1 Implications of this study

The results show the therapeutic potential of SAHA as it is implicated in some genes having neurological functioning. Both ITCH and UBE2G1 showed downregulation when treated with SAHA in wildtypes, but not KO mice. Similarly, both ABHD16A and GBF1 showed upregulation in SAHA treated wildtypes, but not KO mice. There was significant decreased expression of HDAC2 in wildtype mice treated with the HDAC inhibitor, SAHA but not HDAC5 or HDAC1. This provides support that these genes are differentially expressed in an HDAC2 dependent manner. The genes that were deemed insignificant by RT-qPCR that showed upregulation when tested by microarray analysis could be for a number of reasons. The experiments conducted at Mt. Sinai previously were produced in the year 2010. Although the same strain, sex, and age of mice were used in the experiments conducted at VCU, there easily may be individualized differences from sample to sample. Of the genes that previously showed differential expression via microarray analysis ANKRD9 (p<0.0672) showed an upward trend in wildtypes treated with SAHA when compared to vehicle. However, a two-tailed t-test deemed the results insignificant for this gene. Further experiments would need to be carried out to assess whether other genes may possibly be significantly affected.

4.2 Future directions

Future goals stemming from this study include screening more of the target genes differentially expressed from the microarray analysis. Further, recreating the experiment with a higher sample size of wildtype mice and HDAC2KO mice would be required. It would be worth researching sex differences between male and female mice
as the work conducted so far has been done exclusively using male mouse models.
Additionally, treating two separate cohorts in conjunction with clozapine and SAHA respectively may shed more insight into which exact genes overlap in terms of differential regulation between both treatment types.

Of the genes expressed, UBE2G1 encodes a protein that is a ubiquitin and is important for targeting abnormal genes for degradation. A study showed that olanzapine, another second generation antipsychotic, upregulated the expression of various genes including several ubiquitin-conjugating enzymes, including UBE2G1 (16). The study did not test the effect of clozapine on UBE2G1 levels. It would be worth analyzing the expression of this gene in models treated with clozapine, especially because this gene was down-regulated in SAHA treatment.

Furthermore, the gene ITCH, has been implicated as a regulator for various neurodevelopmental and neurodegenerative disorders. The protein, ITCH, catalyzes the degradation of multiple substrates via autophagy and results in regulating molecular pathways in the brain such as synaptogenesis, synaptic connections, and axon and dendritic branching.

Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1) is involved in regulated proteins to membranes via GDP and GTP exchange. GBF1 typically functions as a transport between the endoplasmic reticulum and golgi apparatus and further recruits other GEFs, such as BIG1 and BIG2 to the trans-golgi network (TGN). A study showed that treatment of neurons with an inhibitor of BIG1 resulted in a significant decrease in GABA_A receptors at the neuronal surface of bovine brain. This suggests the important function of these GEFs in trafficking GABA_A receptors to the cell surface.
Mutations in BIG2 have been previously reported to hinder neural progenitor proliferation and migration in the human cerebral cortex (14). Due to these findings, it would be worth while to test previous samples to examine whether the expression of BIG1 and BIG2 also change along with GBF1.

ABHD16A is the primary brain phosphatidylserine hydrolase, which generates ABHD12. Mutations in ABHD12 are involved in human causes of the neurodegenerative disorder PHARC (polyneuropathy, hearing loss, ataxia, retinosis pigmentosa, and cataract) (15, 19). Because of this implication of ABHD16A's involvement in another neurodegenerative disorder, it further warrants study in relation to schizophrenia.

Ankrd9, the gene that had an upward trend, is involved in lipid metabolism. More experiments would need to be carried out to see if that metabolism has any correlation to the brain, a lipid heavy organ (Wang).

A recent finding implicated epigenetic dysfunction associated with schizophrenia. A study analyzed postmortem brains from patients diagnosed with schizophrenia and found these samples were deficient in an extracellular matrix protein, reelin, which contains several sites for DNA methylation. HDACs increase the expression of this protein, supporting that the regulation of this protein is under epigenetic control (1).

The gene encoding the protein SMARCA2, a part of the SWI/SNF chromatin-remodeling complex has been associated with schizophrenia in genome-wide-association studies. Polymorphisms in this gene produce a change in gene expression or the amino acid sequence (2).

It is reported that histone acetylation increases neuronal activity and may sustain changes in gene expression associated with synaptic plasticity and memory. In mouse
models of Alzheimer’s disease associated cognitive impairment and neurodegeneration, HDAC2 levels were increased in both the hippocampus and prefrontal cortex. This upregulation of HDAC2 corresponded with greater binding of HDAC2 to synaptophysin and Bdnf, genes associated with synaptic plasticity and learning (6).

Acetylation in the prefrontal cortex region was studied in vitro and in mouse models treated with either vehicle, Cpd-60, or SAHA, both of which are HDAC inhibitors. Both of these HDAC inhibitors increased acetylation in the prefrontal cortex by 1.5-2.0 fold, supporting that both of these suppress HDAC activity in the brain following administration (Schroeder).

Dr. Gonzalez-Maeso is in collaboration with a brain bank in Spain, which has samples of post mortem human brain of control subjects, untreated schizophrenics, and treated schizophrenics. Although, we do not have access to samples treated with SAHA, it would be worthwhile to look at the expression of these specific genes in subjects treated with atypical antipsychotics to see if they are expressed in the opposite direction as what we have found in mouse brain models treated with SAHA.

Furthermore, Dr. Gonzalez-Maeso, is in collaboration with VCU School of Medicine for an ongoing clinical study. Currently, they are in the stage of recruiting patients diagnosed with schizophrenia who are taking atypical antipsychotic medications. These patients will receive either SAHA or placebo. To look at the expression of various genes that have been implicated as differentially expressed in mouse models, an option would be to test the peripheral blood tissue of these patients for those specific genes post treatment with SAHA.
References


Slow to Fast Muscle Fiber Type Transition after Olanzapine Infusion in Rats, 1–19.

https://doi.org/10.1371/journal.pone.0123966


