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The Therapeutic Potential of HDAC Inhibitors for Dopamine Deficiency in Alzheimer's Disease: A Research Protocol

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Abstract

Introduction: Histone deacetylases (HDACs) are enzymes with epigenetic down-regulatory functions that are linked to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease. Dopamine deficiency in the ventral tegmental area (VTA) is associated with memory deficits characteristic of Alzheimer's disease. HDAC inhibitors (HDACi) may counter HDAC downregulation of dopamine, increasing VTA dopamine. This investigation hypothesizes that HDACi can increase net VTA dopamine concentration and thus improve memory in Tg2576 transgenic mice models of Alzheimer's disease.

Methods: Firstly, a promoter for each of the 18 HDACs will be individually delivered into mice VTA to identify those decreasing local dopamine concentrations, which will be measured using microdialysis and a Paired-Samples T-Test. Secondly, an HDACi corresponding to each HDAC will be selected and longitudinally injected into the mice while measuring VTA dopamine concentration using microdialysis and a Paired-Samples T-Test to indicate the HDACi countering HDAC-induced dopamine downregulation. Lastly, the dopamine receptor antagonist flupentixol will be injected into the VTA as mice undertake the Novel Object Recognition Test. Performance will indicate whether HDACi-induced increases in VTA dopamine improve memory and learning function.

Results: Lower post-measurement scores are expected for HDACs that decrease VTA dopamine concentration relative to baseline means. Greater post-measurement scores are expected for HDACi that increase VTA dopamine concentration relative to pre-measurement scores. The lowest mean exploration times are expected for HDAC delivery, then HDACi injection with flupentixol, no treatment, and finally HDACi alone. Tg2576 mice are expected to have lower mean exploration times than healthy B6.SJL mice.

Discussion: The first two experiments identify HDACs that decrease net VTA dopamine concentration and HDACi that increase it through Paired-Samples T-Tests. The final experiment investigates whether dopamine rehabilitation caused by HDACi can produce tangible memory improvements. A two-way ANOVA test will determine if exploration times between the four treatment groups are statistically significant, concluding whether learning and memory improved by manipulations alone.

Conclusion: The memory and learning of Tg2576 Alzheimer's mice models are expected to improve through the inhibition of dopamine-decreasing HDACs in the VTA. This protocol offers a preliminary strategy towards identifying the HDACs and HDACi relevant to dopamine deficiency in Alzheimer's disease.

Keywords: Alzheimer's disease; histone deacetylase; HDAC inhibitor; epigenetic regulation; dopamine; memory; ventral tegmental area

Introduction

Biomedical research has yielded previous success in elucidating the molecular hallmarks contributing to the pathogenesis of Alzheimer's disease, though current treatments have been relatively ineffective [1]. This research protocol recognizes a potential path for future therapies by targeting histone deacetylase (HDAC) enzymes, which have down-regulatory effects in numerous metabolic pathways [2]. Various HDACs have been shown to contribute to the pathogenesis of Alzheimer's disease by decreasing synapse plasticity in the brain (such as HDAC2) [3] and promoting the accumulation of incorrectly folded and clustered proteins (such as HDAC6) [4]. Conversely, the usage of HDAC inhibitors (HDACi) has been linked to positive regulatory effects that protect dopaminergic neurons and reduce the accumulation of neurotoxic molecules such as tau protein [4], though specific molecular mechanisms are not yet well understood. This protocol proposes that a novel frontier of research with potential to yield treatments for Alzheimer's disease can be found in the application of behavioural epigenetics to neurotransmitter rehabilitation, especially in the ventral tegmental area (VTA), where the degeneration of

dopaminergic neurons is a harbinger to Alzheimer's pathology [5]. Seeing as dopamine deficiency in the brain has been linked to the cognitive impairment and memory deficits exhibited by Alzheimer's patients [6], the application of HDACi to reverse HDAC downregulation of dopamine metabolic pathways may increase dopamine levels and have a positive behavioural effect on memory. This is a relatively novel hypothesis that unites the studies of neurodegeneration, neurotransmitter homeostasis, and behavioural neuroscience.

The goal of this protocol is to introduce a preliminary strategy towards identifying the relevant HDACs and HDACi that are implicated in dopamine rehabilitation and memory. The proposed investigation hypothesizes that the inhibition of HDAC enzymes can upregulate dopamine concentration in the VTA and thus improve memory in Tg2576 transgenic mice models of Alzheimer's disease at an early stage. Evidence from existing literature illustrates that HDACs are conducive to Alzheimer's pathology by inhibiting dopamine synthesis [7]. By inhibiting HDACs, it is thus predicted that dopamine metabolic pathways can be upregulated, increasing net dopamine concentration and thereby increasing memory capabilities. Although past studies have observed the effect of HDAC inhibition on dopamine synthesis pathways [8], this investigation presents a systematic investigation to observe behavioural changes that may arise from the application of epigenetic manipulation to dopamine homeostasis. Rather than focusing on the effects of HDACs on specific genes involved in dopamine synthesis, this research proposal recognizes that HDACs do not act on metabolic pathways in isolation [9], and therefore seeks to observe effects on net dopamine levels in the VTA as a whole along with any changes in behaviour that may follow.

Methods

Animal Models

The experimental group will consist of Tg2576 transgenic mice, a cross between the C57BL/6J and SJL strains and a common animal model of Alzheimer's disease that exhibits molecular and behavioural hallmarks such as elevated amyloid beta levels and age-associated cognitive deficits [10,11]. Tg2576 mice naturally exhibit aberrant HDAC activity [12] and VTA dopaminergic neuronal degradation [13], making them ideal models to study the effect of overexpressed HDAC on VTA dopamine and the potential of HDACi in reversing dopaminergic dysfunction. All experiments will be conducted when the mice are six months old as this is typically when they begin exhibiting memory loss and the accumulation of neurotoxic amyloid beta peptides [14], making them an appropriate model for the early stages of Alzheimer's disease. The control group for this investigation will consist of healthy B6.SJL mice, from which the genetic background of Tg2576 mice is derived [15].

All mice will be female with the purpose of simplifying the methodology to highlight the overall experimental strategy rather than its nuances. Female mice were chosen over male mice to promote the paradigm shift of increasing female inclusion in neuroscience studies, as the misconception of female hormonal fluctuations compromising data has been dispelled [16]. All experimentation will be conducted in accordance with the Canadian Council on Animal Care (CCAC), the institutional Animal Use Protocol (AUP), and supplier guidelines. The housing protocol for mice will be derived from Slater and Cao (2015) to establish environmental enrichment (EE) with the goal of maintaining the mental and physical health of the animals [17]. EE will consist of enlarged living spaces, toys, and novel environmental changes to encourage social, cognitive, and physical stimulation [17].

Measure A Experiment

Before any manipulations, a microdialysis will measure the current dopamine levels in the VTA across all mice to obtain the mean. In addition, the concentration of the amyloid beta 42 (Aβ42) biomarker will be measured using a protein detection method, such as a Single Molecule Array (SIMOA), to track minute changes correlated with Alzheimer's pathology as a second outcome [18]. Neuron-specific expression promoters for each of the 18 HDAC enzymes will be designed according to the protocol of Brown and James (2017), which outlines a strategy to construct reliable cell type-specific synthetic promoters in mammalian cells [19]. Each of the 18 HDAC promoters (the independent variable) will be delivered to the VTA of ten Tg2576 mice by a lentiviral vector inserted via stereotaxic surgery. Stereotaxic injection of a lentiviral vector to target the VTA is a common tactic for in vivo assessment of metabolic pathways [20]. The mean VTA dopamine concentration (the dependent variable) will be longitudinally measured for each of three groups—ten Tg2576 transgenic mice who will receive the HDAC treatment, ten additional Tg2576 controls who do not receive the HDAC treatment, and ten healthy B6.SJL mice who do not receive the HDAC treatment (a control for the mouse strain). A Paired-Samples T-Test will be used to assess pre- and post-measurement scores. Following treatment, the Aβ42 biomarker will be re-measured to track concentration differences correlated with Alzheimer's. The specific HDACs that are observed to decrease VTA dopamine will be identified as potentially having a net effect of downregulating VTA dopamine metabolic pathways (see Figure 1).

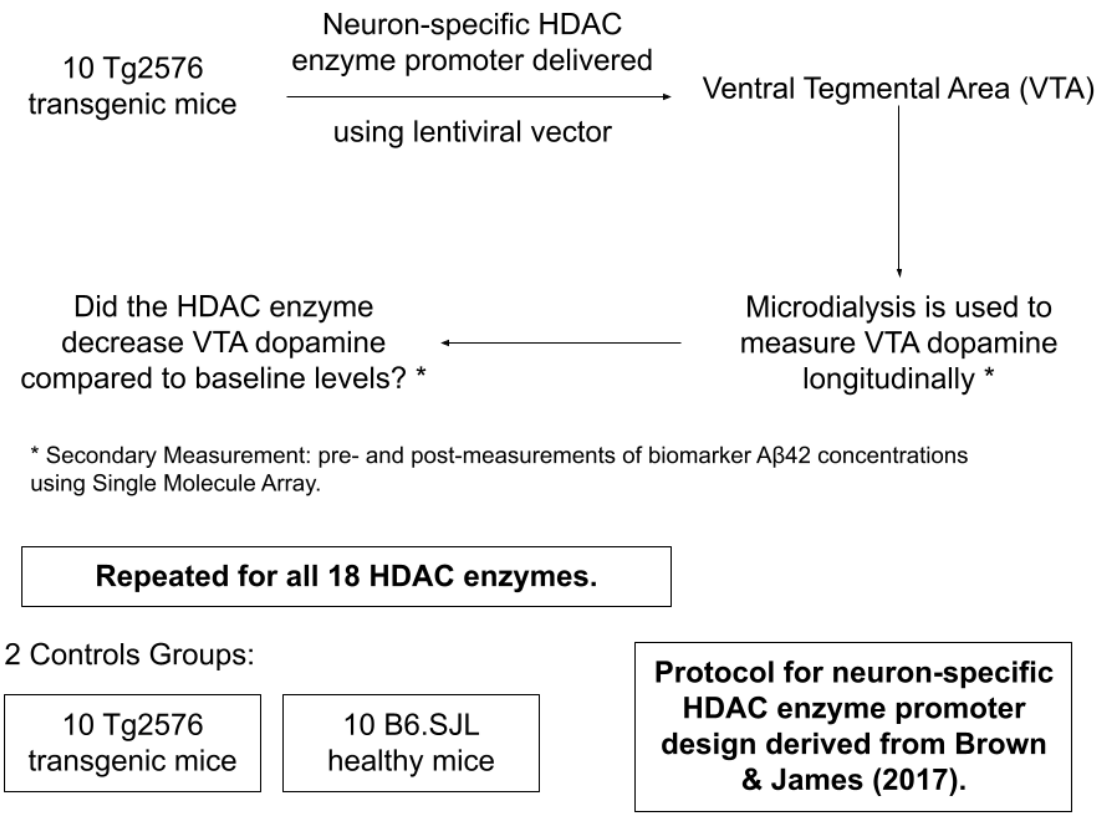

Figure 1. The aim of the Measure A experiment is to determine which HDACs potentially have the net effect of downregulating VTA dopamine metabolic pathways. Pre- and post-measurements of the biomarker Aβ42 help monitor the molecular progression of Alzheimer's disease as a secondary outcome. Created using Google Drawings.

Measure B Experiment

A specific HDACi will be selected for each of the HDACs that were observed to decrease VTA dopamine in the Measure A experiment [21]. Before each HDACi is injected, microdialysis will measure current dopamine levels across all mice to obtain the mean and a secondary measurement of the Aβ42 biomarker concentration using SIMOA will be conducted. Each type of HDACi (the independent variable) will be injected into the VTA of ten Tg2576 transgenic mice and ten healthy B6.SJL mice (the experimental groups). There will be two control groups—ten Tg2576 transgenic mice and ten B6.SJL mice who do not receive the treatment. The dosage of HDACi will vary depending on the specific inhibitor and will be derived from past literature, such as 20 mg for selective class 1 HDAC inhibitor tacedinaline [22]. VTA dopamine concentration (the dependent variable) will be measured using microdialysis longitudinally, and Aβ42 concentration will be re-detected as a post-measurement. Before HDACi is applied, VTA dopamine concentrations will be obtained from all Tg2576 and B6.SJL mice for the Paired-Samples T-Test. Dopamine levels will be obtained from all Tg2576 and B6.SJL mice for the Paired-Samples T-Test. Dopamine levels will be obtained after HDACi injection and the differences would be calculated for pre- and post-measurement scores to determine if there was an increase. The HDACi observed to increase VTA dopamine will be identified as potentially promoting VTA dopamine metabolic pathways (see Figure 2).

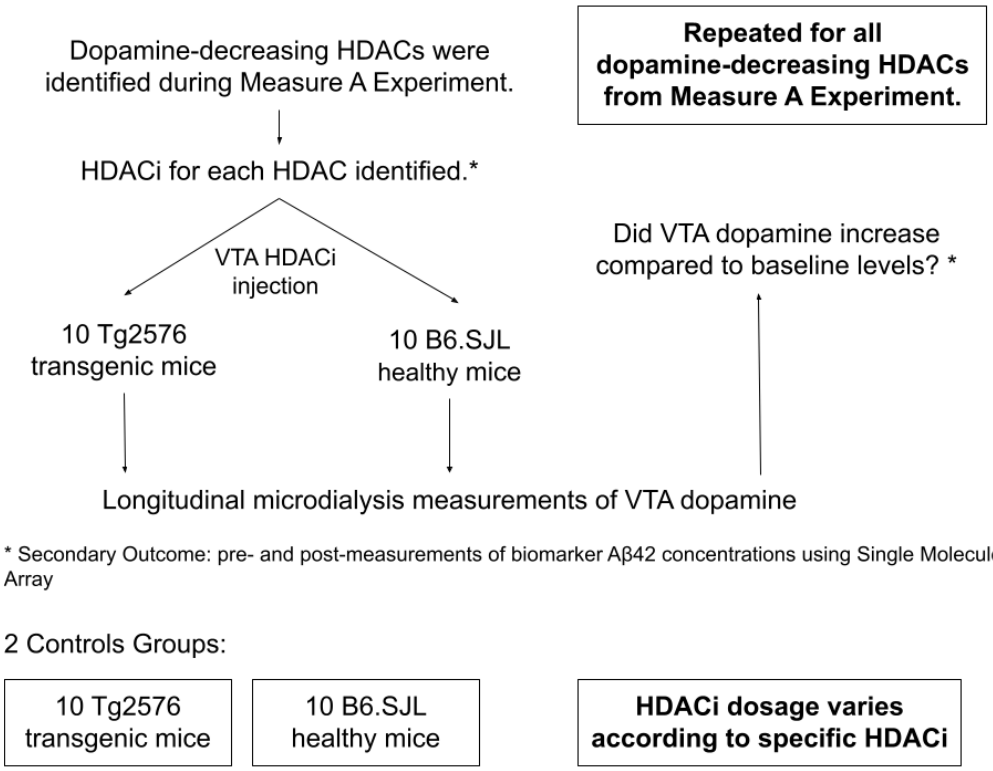

Figure 2. The aim of the Measure B experiment is to determine which HDACi potentially have a net effect of promoting VTA dopamine metabolic pathways. Pre- and post-measurements of the biomarker Aβ42 help monitor the molecular progression of Alzheimer's disease as a secondary outcome. Created using Google Drawings.

Block Experiment

To determine if increased VTA dopamine from HDACi has the behavioural effect of improving memory and learning, a 2×4 (strain × treatment) experimental design (see Table 1) will be employed for HDACi that increased VTA dopamine in the Measure B experiment. The first independent variable will be the mouse strain, of which there will be two—ten Tg2576 transgenic mice and ten healthy B6L.SJL mice for every condition. The second independent variable will be the treatment received by the mice, of which there will be four. The first will consist of HDACi injection and the administration of the dopamine antagonist flupentixol, the second level will be the HDACi injection alone, the third level will be HDAC delivery by lentiviral vector stereotaxic surgery (as was outlined in the Measure A experiment), and the fourth level will have no treatment as a control. Flupentixol was selected as the dopamine antagonist because it blocks D1 and D2 dopamine receptors, which are implicated in memory and learning [23,24]. The Novel Object Recognition Test, derived from the protocol of Lueptow (2017), will be employed to measure memory and learning [25]. Mice will be exposed to a familiar object and a novel object differing in texture, shape, and size. The time spent exploring the novel object, indicative of the degree of object discrimination, will be recorded. The mean exploration time for each of the eight conditions will be used to determine whether blocking dopamine receptors decreases memory and learning, whether the injection of HDAC decreases memory and learning, and whether VTA dopamine increases caused by HDACi improve memory and learning function (see Figure 3).

Table 1. A 2×4 design of the Block experiment

	Mouse Strain	
Treatment	Tg2576 transgenic mice	B6.SJL healthy mice
HDACi injection & flupentixol	D1 and D2 receptors blocked	D1 and D2 receptors blocked
HDACi injection alone	No dopamine blocking	No dopamine blocking
HDAC delivery alone	Positive control	Positive control
No treatment	Negative control	Negative control

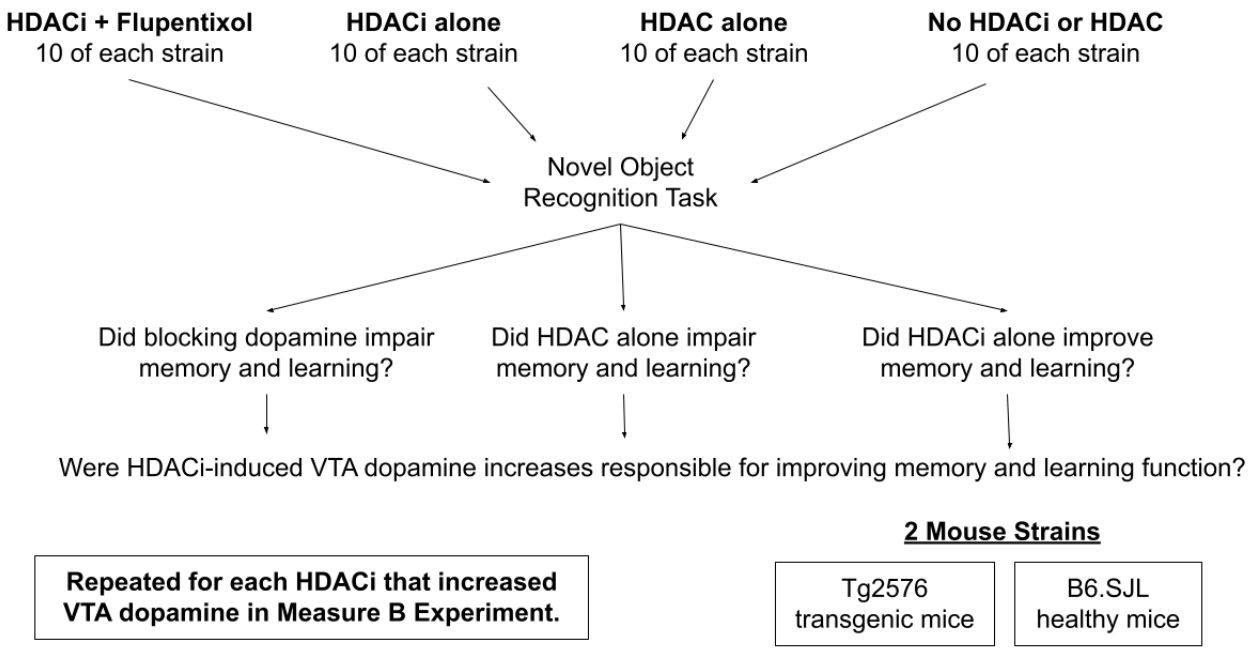

Figure 3. The aim of the Block experiment is to determine whether increases in VTA dopamine caused by HDACi are responsible for memory and learning function. Created using Google Drawings.

Results

For the Measure A experiment, the HDACs identified as potentially having a net effect of downregulating VTA dopamine metabolic pathways are expected to decrease VTA dopamine concentration longitudinally relative to baseline levels for the experimental group (the ten Tg2576 transgenic mice who receive the HDAC treatment) as indicated by a lower post-measurement score in the Paired-Samples T-Test. No changes in VTA dopamine concentration are expected in the control groups (ten Tg2576 mice and ten healthy B6.SJL mice who do not receive the HDAC treatment).

For the Measure B experiment, the HDACi identified as potentially promoting VTA dopamine metabolic pathways are expected to increase VTA dopamine concentration longitudinally relative to baseline levels for the experimental groups (ten Tg2576 transgenic mice and ten healthy B6.SJL mice) as indicated by a greater post-measurement score. No changes in VTA dopamine concentration are expected in the control groups (ten Tg2576 mice and ten healthy B6.SJL mice who do not receive the HDACi treatment).

It is hypothesized that VTA dopamine increases caused by HDACi improve memory and learning function as indicated by longer exploration times in the Novel Object Recognition Test. For the Block Experiment, the mean exploration time is expected to be lowest for the condition of HDAC delivery followed by HDACi injection with flupentixol administration, no treatment, and then HDACi injection alone. The mean exploration times are expected to be lower for Tg2576 transgenic mice than healthy B6.SJL mice overall. Therefore, the condition with the lowest mean exploration time is expected to be Tg2576 mice with HDAC delivery followed by Tg2576 mice with HDACi injection and flupentixol administration, Tg2576 mice with no treatment, Tg2576 mice with HDACi injection alone, B6.SJL mice with HDAC delivery, B6.SJL mice with HDACi injection and flupentixol administration, B6.SJL mice with no treatment, and B6.SJL with HDACi injection alone. Blocking dopamine receptors with flupentixol is expected to block benefits to memory and learning that would have otherwise arisen due to HDACi increasing VTA dopamine. A two-way ANOVA test will be conducted using the exploration times from each level of the independent variable (mouse strain and treatment) to calculate the f-statistic and effect size.

Discussion

Despite past underrepresentation of female mice in biomedical research due to concerns of hormonal fluctuations from their menstrual cycle, recent studies have proven that male and female mice are equally variable physiologically [26]. Including all female mice in this study helps diversify Alzheimer's research among both sexes in mice models, providing identification and promoting further study of variances between adoption of pathological conditions between the sexes, while also furthering current knowledge on the potential to tailor treatments when accounting for hormonal differences [26].

The aim of this study is to investigate HDAC inhibitors as a potential treatment approach to slow the progression of Alzheimer's disease by upregulating dopamine in the VTA.

The usage of HDACi has been linked to positive regulatory effects that protect dopaminergic neurons and reduce the accumulation of neurotoxic molecules such as tau protein [27]. The Measure A and Measure B experiments focus on identifying the HDACs that decrease net dopamine concentration in the VTA and finding the respective HDACi to counter this effect. Each type of HDAC is injected into a sample size of ten female Tg2576 and B6.SJL mice. After, the HDACi associated with the HDAC responsible for dopamine reduction is injected into the VTA to observe which enzymes increase dopamine concentrations.

Once the HDAC or HDACi are injected, microdialysis will measure VTA dopamine longitudinally. Applying a Paired-Samples T-Test in both Measure A and Measure B allows for pre- and post- measurement comparisons of VTA dopamine concentrations after injecting a specific HDAC or HDACi. The dopamine concentration in both transgenic and healthy strains will be collected before any manipulations. After injecting HDAC and HDACi, the altered dopamine levels are compared to the pre-measurement scores to determine if there were any significant changes. The differences will be calculated for each paired measurement to find the t-statistic, which is compared to the critical value ($\alpha = 0.5$) to determine if the pre- and post- measurement differences are statistically significant.

The two-way ANOVA test will be applied in the Block experiment to determine if there is a relationship present between both levels of the independent variable (mouse strain and treatment group) on the exploration times in the Novel Object Recognition Test. This will also provide information about the interaction effect, specifically if the effect of each treatment is dependent on the mouse strain. In this study, the null hypothesis is that the exploration times are equal among all mice strains in each of the four treatment groups. The alternative hypothesis is that at least one exploration time differs from the rest of the treatment groups. The null hypothesis will be rejected if the f-statistic exceeds the critical value. Lastly, effect size will be calculated for treatment, strain, and the intersection containing both levels using the Eta^2 formula, $\boldsymbol{SSeffect} \div \boldsymbol{SStotal}$ [28]. This is a preliminary step for understanding how HDAC and HDACi influence behavioural outcomes.

A future consideration is to include both female and male mice models when studying the effects of HDAC and HDACi on both the molecular and behavioural level. A study by Clayton (2015) explains that the sex differences in certain proteins differ by several amino acids [29]. Addressing these differences is important for the transparency and reproducibility of this study. Sex differences must be taken into consideration when observing how gene expression is altered through HDAC and HDACi because certain proteins are not the same across all mice.

Conclusions

Experimental groups longitudinally receiving HDACi treatment selective to VTA dopamine-decreasing HDACs should expect to see increased VTA dopamine concentration through countering the effects of HDAC downregulation. HDAC inhibition has been identified to have multiple roles in combating neurodegenerative diseases by restoring deficiencies caused by imbalances in acetylation and transcription levels, thus countering pathological conditions [30]. When considered with memory-impacting dopamine downregulation caused by specific HDAC enzymes, HDACi play a vital role in the interdisciplinary approach to regulate behavioural effects of memory in combination with molecular structures. Enhancement of histone acetylation and promotion of dopaminergic signalling can be accomplished by reversal of chromatin remodelling through treatment with HDACi [31]. This raises the question of the unknown plethora of cascading effects that may be caused by the inhibition of the multi-functional HDAC enzymes within the VTA. Such effects should be considered with caution, as the extent that these unknown physiological changes and affected metabolic pathways can only be identified with further research studying the range of other functional aspects of HDACs, such as their stake in neuroglial development through decreased synaptic plasticity or their delayed feature of arresting growth during the cell cycle [31].

List of Abbreviations Used

Aβ42: amyloid beta 42
AUP: animal use protocol
CCAC: Canadian council on animal care
EE: environmental enrichment
HDAC: histone deacetylase
HDACi: histone deacetylase inhibitor
SIMOA: single molecule array
VTA: ventral tegmental area

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

All experimentation will be conducted in accordance with the Canadian Council on Animal Care (CCAC), the institutional Animal Use Protocol (AUP), and supplier guidelines. Approval will be sought from the institutional research ethics board (REB).

Authors' Contributions

AH: Contributed to the planning, design, and experimental methodology; drafted and revised the manuscript.
JC: Contributed to the planning, design, and data analysis; drafted and revised the manuscript.
SK: Contributed to the planning, design, and theoretical background; drafted and revised the manuscript.

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