Inhibition of TET1 Protein Prevents DNA Demethylation to Suppress Memory Formation

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Introduction

The epigenome serves as a signal transduction pathway that encodes for experience in the past and melds current experience, which establishes the forms of molecular memory.[1] Exploration of epigenetic mechanisms has displayed their role in the formation, storage and retrieval of memories in the mammalian brain. Modification in genome sequences affect gene expression which is required for long-lasting forms of neuronal plasticity, cognition, and memory. Epigenetics has been defined as "self-perpetuating, post-translational modifications of DNA and nuclear proteins that produce lasting alterations in chromatin structure as a direct consequence, and lasting alterations in patterns of gene expression as an indirect consequence." More simply, epigenetics involves mitotically and meiotically heritable alterations in DNA expression that are not encoded in the DNA sequence.[2]

The post-translational modification of histone proteins and methylation of DNA are the most common epigenetic mechanisms that have a proposed role in long-term memory formation. DNA methylation has been proposed as one of the underlying pathways by which memories are formed and stored in the mammalian brain. DNA methylation is the process by which a methyl group is added to the 5' carbon on the pyrimidine ring of a cytosine. The methyl group donor is S-adenosyl-methionine. These cytosine residues are immediately followed by a guanine residue and are called CpG sites. Normally, they are found in clusters in DNA sequences.[3]
This reaction is catalyzed by a DNA methyl transferase (DNMT) and the methylation of the complementary DNA strand is catalyzed by a maintenance DNMT.

Recent studies have proposed multiple mechanisms for how DNA methylation induces long term structural changes that promote the consolidation and storage of memories. One theory supported by literature postulates that DNA methylation alterations take place temporally to regulate genes that that encode structural proteins; these proteins are said to stabilize memories. It has been shown in numerous studies that both memory consolidation and the synthetic in vitro model, long term potentiation (LTP), require a cascade of signaling events that activate many proteins and lead to changes in gene transcription. LTP is is the increase in synaptic strength over time. DNA methylation has been shown to regulate gene expression by modifying the structure of chromatin, which is support for its role in the memory process. (Miller, 2008). Inhibition studies of DNMTs have been the primary source of evidence to display the prominent role DNA methylation plays in memory consolidation. Indeed, DNMT inhibition has been shown to prevent LTP induction when associated with changes in DNA methylation patterns observed at certain gene promoters in the hippocampus. Studies have also shown that DMNT inhibition diminishes the prevalence of miniature excitatory postsynaptic currents (mEPSCs). DNA methylation's affect on these mEPSCs changes cell excitability and synaptic plasticity, both of which have been shown to contribute to the consolidation of memories.[4] These two pieces of evidence further support the notion that DNA methylation is a significant mechanism in terms of forming and consolidating memories.

In this study, we take a backwards approach to validating DNA methylation's role in the memory process. We design an inhibitor of TET1, an enzyme that catalyzes the demethylation of DNA. Through this inhibition study, we hope to validate and elucidate the role of DNA methylation in memory storage and consolidation. In addition, we hope to demonstrate the effect that demethylation has on memory and the potential role it plays in memory suppression.

The ten-eleven translocation (TET) family of dioxygenases is involved in active DNA demethylation, which is a phenomenon known to alter the activity of a DNA segment without direct alteration to the sequence[1]. This family of enzymes are highly expressed in the cerebellum,

cortex, and hippocampus. When DNA methylation occurs within the gene promoter, this modification typically acts to repress gene transcription and translation[1]. In previous studies, methylation at CpG islands found within the promoter region that correlated with learning, demonstrated a significant difference between memory impaired rats and those with intact learning.

These CpG islands are tandem, housekeeping repeats of cytosine and guanine base near the start of replication sites and are arranged in a way to be methylated to regulate gene expression. Specifically, ten-eleven translocation methylcytosine dioxygenase 1 (TET1) is an enzyme encoded by the TET1 gene that catalyzes the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in the hippocampus[5]. Enhancing the activity of this gene has been shown to drive active DNA demethylation in the nervous system which is beneficial in memory formation. Therefore, testing the inhibition of the enzyme will ideally result in the deformation of memories. 5-mC was originally believed to serve as a stable transcriptional silencer, but recently discovered, 5-mC levels are dynamic and rapidly reversible at memory and synaptic plasticity-associated genes, suggesting that DNA demethylation results in a decrease in neuronal activity[6]. The deletion of TET1 has lead to the impairment of hippocampal neurogenesis and also spatial memory deficits in mice[5]. If adult neural progenitor cells lack TET1, the group of genes involved in the progenitor proliferation are hypermethylated and downregulated[6]. Thus, a loss of TET1 is believed to lead to transcriptional repression of neurogenesis-related genes in the promoter and indicate that the methylation of the genes.

In a more recent study, contextual fear learning increases methylation of the memory suppressor gene, protein phosphatase 1 (PP1), and the promoter region of reelin, a gene involved in synaptic plasticity, was adjacently demethylated[7]. Although these findings focus on TET1 and the formation of memory via demethylation, previous studies have not been conducted showing that the silencing of the TET1 protein and its immediate effects on the deformation of memories. To better understand TET1 and it's ability to modify memory formation, by competitively inhibiting its enzymatic activity, the results will show the deformation of memories due to the reversibility of the conversion of 5-hmC back to 5-mC. If the inhibition is also successful, then the role of memory formation can be attributed to the TET1 protein. And if the TET1 inhibition is unsuccessful, then the substrate 5-mC will be demethylated and converted to 5-hmC and memory formation will continue.

The inhibition of the TET1 enzyme raises some potential ethical concerns. TET1 is a tumor suppressor. The diminished levels of TET1 and 5hmc suggests its critical role in the upkeep of epigenetic modification. Therefore, the inhibition of this tumor suppressing enzyme can lead to breast and colon cancer.[8] This is one ethical concern of the proposed study. Another potential ethical concern is the use of rats as the test subjects. We will be subjecting these rats to injections, dissections, aversive stimulus tests and other assessments. All protocols involving animal testing must be approved by the Animal Ethics committee at the University of San Diego.

Proposed Research

To inhibit the TET1 protein, we will begin by designing and synthesizing three different inhibitors. These inhibitors either replace the deoxyribose sugar attached to the 5-methylcytosine or replace the methyl group on the 5-methylcytosine with an amine group. The efficiency of the inhibitors will be tested by quantitative measures, such as high performance liquid chromatography analysis of DNA methylation and functional magnetic resonance imaging, and qualitative measures, including three types of mazes. Successful TET1 inhibition will contribute

to the understanding of the role of TET1 and memory formation. Inhibition of this enzyme will result in the deformation of memories. However, unsuccessful inhibition of TET1 will result in demethylation of 5-methylcytosine to 5-hydroxymethylcytosine and memory formation will remain unaffected.

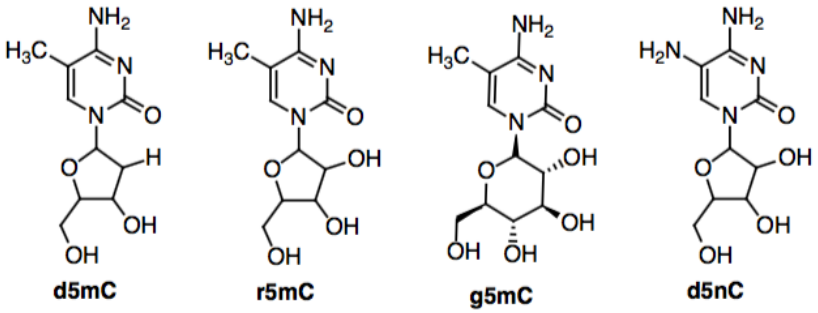

Figure 1. Comparison of the original TET1 substrate d5mC and the three designed inhibitors r5mC, g5mC and d5nC.

To inhibit TET1, the designed inhibitors will differ from a deoxyribonucleic acid (DNA) nucleoside consisting of a 5-methylcytosine, molecule d5mC, by the attached sugar or functional group. TET1 binds to the 5-methylcytosine nitrogenous base attached to a deoxyribose sugar in a DNA strand. By altering the sugar bound to the 5-methylcytosine nitrogenous base, the base will bind in the active site and carry out the enzymatic function, however the sugar prevents the base from participating in phosphodiester bonds in a DNA strand. Alteration of the sugar competitively inhibits the TET1 active site to reduce the rate at which TET1 can act on d5mC. In molecule r5mC, the deoxyribose sugar is replaced with a ribose sugar, which is similar in size and stereochemistry to deoxyribose. The r5mC molecule is not able to participate in phosphodiester bonds within a strand of DNA and therefore will be degraded in the cell by a degradation enzyme. In molecule g5mC, the deoxyribose sugar is replaced with a glucose molecule, which is larger than deoxyribose. If inhibition of TET1 using molecule g5mC is successful, this will indicate the sugar attached to the 5-methylcytosine does not significantly interact with the functionality of the enzyme.

The designed inhibitor with a different functional group than molecule d5mC has the nitrogenous base 5-aminecytosine. Molecule d5nC has an amine group at the 5' carbon of cytosine rather than a methyl group. Since TET1 adds a hydroxyl group to the 5' methyl group, the amine functional group will compete with the methyl group for the active site of TET1. Nitrogen differs from carbon by one electron, causing a similarity in size, although nitrogen is larger and more electronegative than carbon. The amine functional group may produce more favorable interactions with the TET1 active site than the methyl group and therefore TET1 would have a higher affinity for molecule d5nC. Thus, TET1 would be less available to interact with a d5mC molecule. Unsuccessful inhibition of TET1 using molecule d5nC will indicate information about polarity and size of the TET1 active site. This information will be used to design more inhibitors with a higher affinity for the TET1 active site.

Synthesis of TET1 Inhibitors

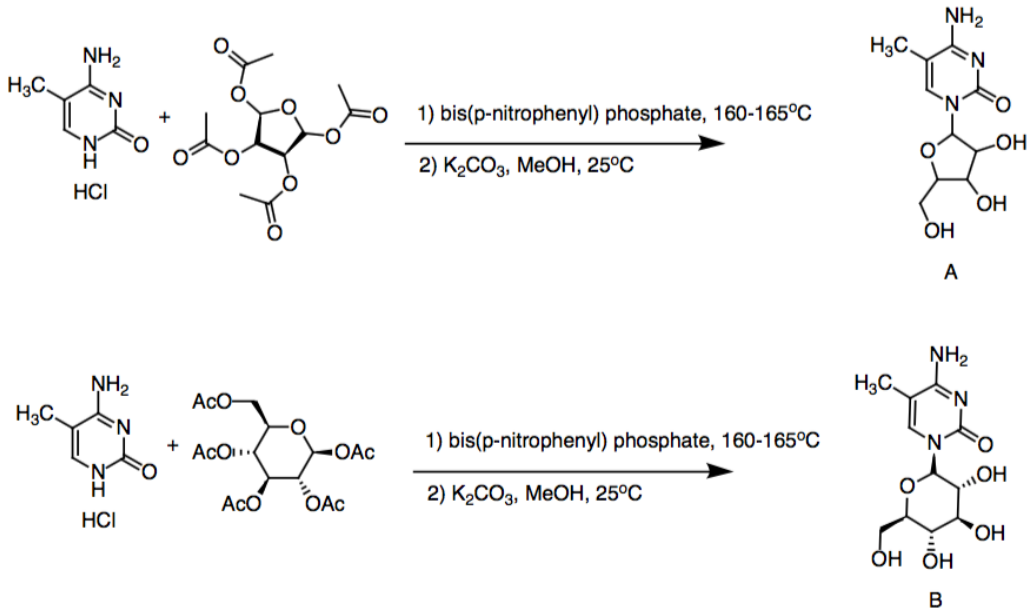

Figure 2. Proposed method of TET1 inhibitor synthesis to produce 5-methylcytosine with a ribose (A) or glucose sugar (B).

To alter the attached sugar to 5-methylcytosine, two different monosaccharides were chosen, ribose and glucose. To attach the 5-methylcytosine base to a ribose sugar, a coupling reaction will be performed following the fusion synthesis method using β-D-Ribofuranose 1,2,3,5-tetraacetate, a protected form of the ribose sugar, and 5-methylcytosine. The reagents are combined under vacuum at low pressure in the presence of bis(p-nitrophenyl) phosphate and held at 160 to 165℃ for 20 minutes.[9] This method of synthesis will be applied to protected glucose, β-D-Glucose pentaacetate.[10] To deprotect the synthesized nucleosides, potassium carbonate and dry methanol will be added to the reaction mixture at room temperature with stirring for 15 minutes.[11]

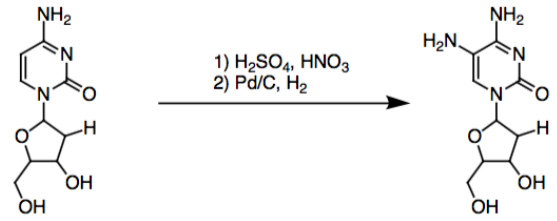

Figure 3. Proposed synthesis of TET1 inhibitor to produce 5-aminecytosine attached to deoxyribose.

To synthesize 5-aminecytosine, a nitration reaction of cytosine will be performed followed by a reduction. To perform this synthesis, sulfuric acid, followed by nitric acid, will be added to cytosine at 0℃. The reaction will be held at room temperature for about 5 minutes, heated for 15 minutes, then cooled over ice. To reduce the nitro group to an amine group, palladium on carbon is added to the reaction and held under hydrogen gas.[12]

Inhibition Impact Testing- Methods of Approach

Testing various pharmaceuticals on the brains of animals, typically R. *norvegicus*, is a common method of impact and analysis. The strength and dependability of this method is supported by a substantial body of scientific literature, which draws connections between various regions of the brain and tasks that require specific forms of memory. The most straightforward approach to studying the effect of the inhibition of TET1 on memory formation is to observe the effects of inhibited subjects' ability to perform standard spatial memory tasks; spatial memory is closely associated with both long and short term memory.

There are two final notes to be addressed before proceeding to the detailed outlines of the tests the subjects will be subjected to. The group intends to use rats as test subjects since their intelligence, ease of care, affordability, and applicability to humans. The testing on rats requires ethical considerations and boundaries to prevent mistreatment. They will be addressed individually as they arise. The inhibitor will be introduced painlessly through the application of local anesthesia. Previous studies have used a similar approach to adhere to the proper treatment of these animals and to avoid their ill treatment.[13]

Morris Water Maze/Oasis Maze Testing and Design

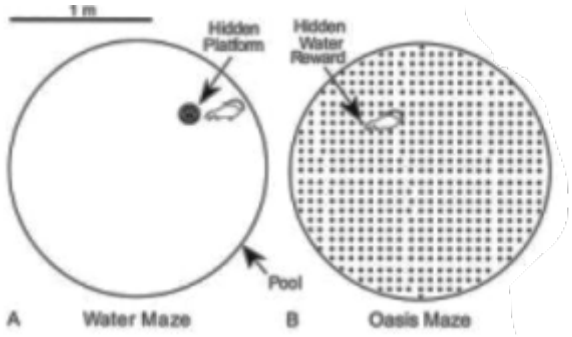

Figure 4. Diagram of Morris Water and Oasis Maze construct for behavioral testing.

The Morris Water Maze is a standard spatial memory test that takes advantage of a rat's natural aversion to water. The length of exposure to this discomfort will not exceed a time interval of two minutes and should the subjects fail to escape they will be removed by the test administrator. The construction of the maze involves a ring shaped exterior that will be filled with water sufficiently to conceal the escape platform and ensure the subject is forced to swim. The escape platform should be large enough for the rat to stand on and should be positioned just below the surface of the water to decrease the probability of visual detection. The platform may also be made of a transparent material to further minimize this possibility. The subjects are trained at regular intervals prior to the actual experiment to use proximal cues (visual stimuli) to locate the escape platform.[13] Initially this cue consists of an object suspended over the concealed platform. Following successful training, the proximal cues will be removed and the subject will be forced to rely on distal cues and memory.[13]

During the experiment, the subjects will be permitted to 'escape' if they are able to maneuver within a specified distance of the platform (20 cm in a 1.8m diameter maze) and remain in that area for a short time interval of 2-3 seconds.[13] The time it takes the subjects to successfully complete this maneuver will be compared to the performance of an unmodified control group. Statistical models will then be applied to determine the degree of impact and this

will subsequently be compared to existing literature results to determine the relative severity of its effect.

The Oasis Maze is sometimes referred to as a dry land version of the Morris Water Maze. As opposed to using an aversive stimulus, the Oasis Maze favors the use of controlled water deprivation in order to allow the subjects to navigate in an environment that is more natural to them. The subjects will have access to food, but will only have access to water in a one hour period.[13] This will add value to the reward system that drives the Oasis Maze-- finding as many hidden reservoirs as possible in the time allotted. Should the subjects begin to exhibit any significant adverse effects attributable to the deprivation, the administrator will increase the amount of water available within a limited margin or remove the subject from the experiment.

The design of the Oasis Maze is similar in that it is circularly shaped and of similar diameter. Small reservoirs are spaced at regular intervals in a grid pattern. The distance traveled by the subject and the number of wells discovered are the variables of interest. The subject is trained on a sequence of wells that is gradually reduced over subsequent training runs. The purpose of this is to promote further exploration in the early runs and efficiency in the later runs. In order to facilitate the reinforcement of this pattern, the entry point of the maze for each individual subject is fixed. After a period of time has elapsed, the subject is removed from the maze and given free access to water.[13] Inhibited subjects should show less efficient behaviors in attempting to locate water than non-inhibited subjects.

Functional MRI Testing

The neural impact of the inhibition of TET1 may also be observed specifically in those regions where spatial activity is located, the hippocampus. Resting fMRIs (rsfMRIs) will be used to establish a functional baseline level of activity under anesthesia. The anesthesia is necessary to ensure the stillness of the subject and to facilitate the monitoring of partial pressure of carbon dioxide. This may reduce hemodynamic responses of stimulus-induced fMRI scans (stfMRI), body temperature, blood pressure, heart, and respiratory rate.[14] Specific types of anesthesia are more desirable than other general ones. For example, while general purpose anesthetics can interfere with the activity that the fMRI measures, an anesthetic like Medetomidine does not impact the spatial reasoning regions of the brain.[14] The stfMRIs will be used to compare neural activity levels of subjects treated with inhibitor and subjects not treated with inhibitor in order to observe differences in the degree of response to physical stimuli. Subjects that are treated with the inhibited should express diminished levels of functionality in the observed regions as compared to the control groups.

The fMRI has an additional advantage in specificity and flexibility. First, fMRI has a higher degree of specificity than behavioral modeling techniques because behavioral modeling cannot entirely isolate proximal and distal variables.[15] They can be emphasized or deemphasized but not eliminated entirely, thus fMRI must be artificially controlled for potential errors. The fMRI allows for the focus on specific regions of the brain individually, which ties into its flexibility. By varying the stimuli, different regions of the brain will be isolated and observed such that if the TET1 inhibition has a neural effect other than the one hypothesized, it might be isolated and followed up in further experiments. Unlike the two previous proposed methodologies, this procedure requires a significantly greater commitment of resources and technical knowledge. It is proposed that this methodology be utilized in the case that the behavioral studies do not exhibit statistically significant data.

HPLC Analysis of DNA Methylation

High Performance Liquid Chromatography with Ultraviolet detection (HPLC UV) will be used to quantitate the level of DNA methylation in a given sample. By testing the level of DNA methylation of inhibited samples in comparison to untreated samples the effectiveness of the inhibition will be determined. Subjects treated with inhibitor should exhibit lower levels of DNA methylation in comparison to subjects not treated with inhibitor. To test the effectiveness of the inhibitors in preventing DNA methylation, four groups of rats will be tested such that three groups are each treated with one synthesized inhibitor and the fourth group will be untreated and remain the control group. Each group will be run through the mazes at least 3 times to allow for sufficient time for memory formation to occur. The rats will then be dissected and the brain cells from the hippocampus will be harvested. The DNA from these cells will be isolated by a QIAgen prepared DNA isolation kit. The isolated DNA will be PCR amplified to ensure enough DNA, at least 50 μg, has been isolated to be analyzed by HPLC UV.

To analyze the samples by HPLC UV, the isolated DNA will be digested by incubating overnight at 37℃ with a mixture of Dnase I and nuclease buffer P1. The digested DNA will then be dephosphorylated using alkaline phosphatase for 24 hours at 37℃. The HPLC will be performed on a Nucleosil SA cation exchange silica 150x4.6 mm x5 column at 30℃ with an acidic mobile phase consisting of ammonium acetate and acetonitrile with a pH of 4.8. A 30 μL sample of digested DNA will be injected onto the column with a flow rate of 0.5 mL/min. The UV detection will be set to 272 nm to detect for DNA cytosine and at 279 nm to detect for 5-methylcytosine DNA.[16] Levels of DNA cytosine and methylated DNA cytosine will be determined from the obtained standard curves.

Utilization of the proposed research methods should result in sufficient evidence to support the role of the TET1 enzyme in memory formation and its effect on DNA methylation. Inhibition of this enzyme will result in the deformation of memories. However, unsuccessful inhibition of TET1 will result in demethylation of 5-methylcytosine to 5-hydroxymethylcytosine and memory formation will remain unaffected.

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