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# SEX DIFFERENCES IN HIPPOCAMPAL O-GLCNACYLATION OF THE ADULT MOUSE BRAIN

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# SEX DIFFERENCES IN HIPPOCAMPAL O-GLCNACYLATION OF THE ADULT MOUSE BRAIN

by

Makenzie Johnson

Submitted to the School of Honors Committee

in partial fulfillment

of the requirements for University Honors Scholars

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2021

# Dedication

This project is dedicated to those struggling with anxiety, depression, loneliness, and fear. You are not alone and you are loved, for our Father says, “Are not five sparrows sold for two pennies? And not one of them is forgotten before God” (Luke 12:6 ESV).

Acknowledgements

First of all, I would like Dr. Franklin for being an incredible advisor and mentor, guiding me in this project, and helping me find the next steps in my career and life path. Secondly, I would like to thank my parents for supporting me throughout my college journey. I would also like to thank Dr. Salvatore, Dr. Abraham, and Dr. McConchie for the opportunities, guidance, and aid they have continuously offered me. In addition, I would like to thank all the mice that gave their lives to allow for the completion of this project. Finally, I would like to thank God for never abandoning me, constantly loving me, and providing me so many wonderful opportunities. I would not be here today without His grace and love.

## Abstract

The hippocampus is a structure in the brain crucial for learning and memory. This occurs by synaptic remodeling known as long term potentiation and long term depression. Modifications of proteins in the hippocampus can affect its function. One of these modifications is the addition of *O*-linked β-*N*-acetylglucosamine, also known as *O*-GlcNAc. This is a sugar produced from glucose by the hexosamine biosynthetic pathway that is reversibly added onto serine and threonine residues of proteins by *O*-GlcNAc Transferase, or OGT. It is reversibly removed from these residues by *O*-GlcNAcAse, or OGA. This modification has been implicated in diabetes, cardiac dysfunction, and neurodegenerative diseases. Furthermore, as the gene coding for OGT is located on the X chromosome, the two copies of this chromosome in females may lead to differential expression of OGT. Connectivity, structure, and hormone differences in male and female brains may likewise impact expression in the brain between sexes. A transgenic, or mutated, mouse model can be generated that prevents OGA expression when the mouse is taking a medication known as doxycycline. This is hypothesized to increase *O*-GlcNAc levels. Due to the prevalence of *O*-GlcNAc in various diseases of the brain, this study aimed to validate how levels of OGA, *O*-GlcNAc, and OGT are related in the hippocampus of mice both on and off doxycycline, and if expression differed between sexes. This was accomplished by western blot analysis. No significant differences were found between wild type and mutant mice, whether male or female and whether on or off doxycycline. These results indicate that the amount of time off doxycycline needed to impact OGA levels was not reached, and that male and female hippocampi express similar amounts of OGA and OGT. Future experiments may investigate how OGA, OGT, and *O*-GlcNAcylated protein levels differ by sex in other regions of the brain and subregions of the hippocampus. Other future studies may examine if longer time off doxycycline would change OGA and *O*-GlcNAc levels in the mouse hippocampus.


KEY WORDS: *O*-GlcNAcylation, Hippocampus, Western Blot, Tet-off System

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## Introduction

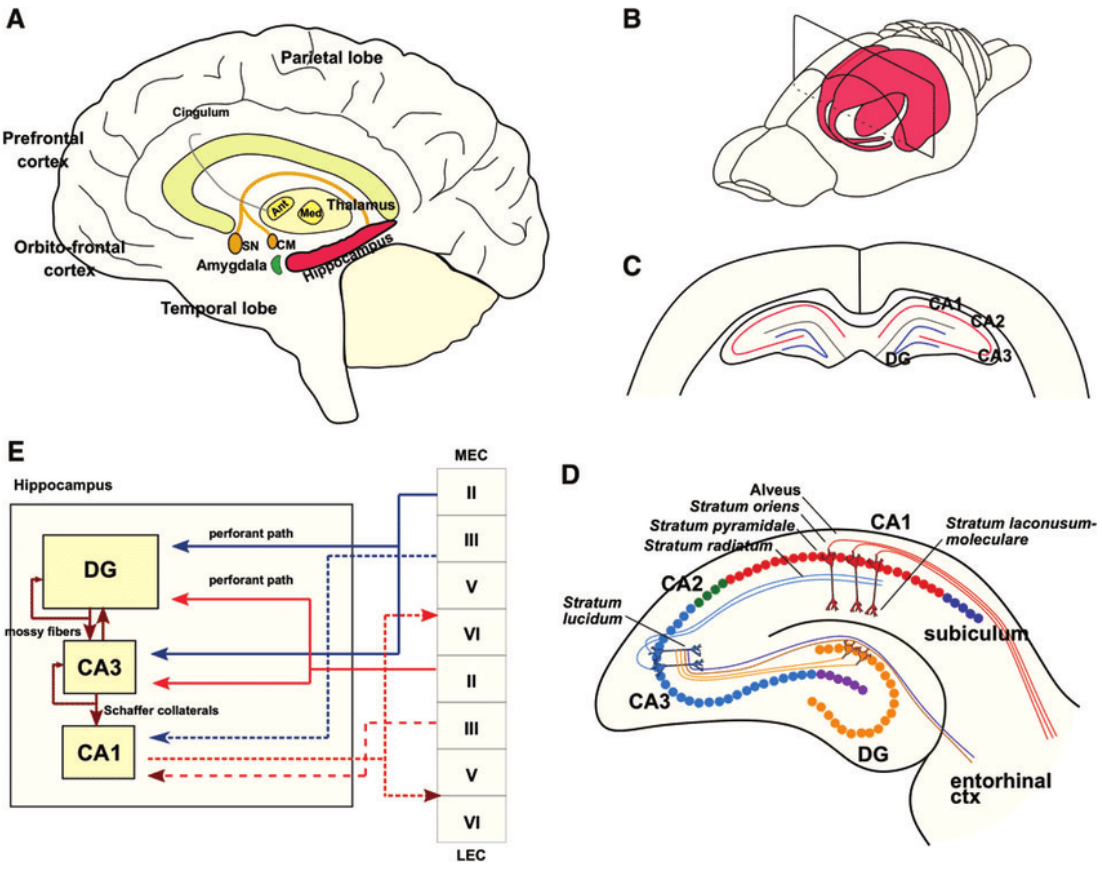
The brain is a highly plastic structure that goes through frequent shifts. Learning and memory take place in this structure, specifically in the hippocampus. This is a region with many excitatory pathways that is crucial in the processes of long-term potentiation and depression.[1] This region exhibits sexual dimorphisms resulting from differential responses to glucocorticoids and sex hormone modulation.[2, 3] It is one of the areas of the brain most highly modified with *O*-linked β-*N*-acetylglucosamine, or *O*-GlcNAc.[4] This modification is akin to phosphorylation and indicates the metabolic state of the body.[5, 6] It is implicated in many diseases, especially those related to glucose metabolism. The enzymes *O*-GlcNAc Transferase, or OGT and *O*-GlcNAcAse, or OGA, regulate the addition and removal of this sugar.[7] This modification may also differ between males and females as the gene encoding for OGT is located on the X chromosome.[6] Therefore, this study will present a background on the hippocampus, learning, and memory in this structure, sexual dimorphisms in brain, *O*-GlcNAcylation, and implications for disease pathology. This will precede an investigation into specific differences in hippocampal *O*-GlcNAc, OGT, and OGA levels. Mice with pharmacologically regulated levels of these products and wild type mice of both sexes were analyzed. Western blot analysis was used to perform this investigation.

## Review of Literature

### Brain and hippocampus

The hippocampus is a structure of the brain that, along with the dentate gyrus and subiculum, is a part of the hippocampal formation. This structure is located above the lateral ventricle and next to the temporal lobe. However, it comes to reside in this position by migration from an embryological placement dorsal to the thalamus. It is crescent-shaped and divided into four zones deemed CA1, CA2, CA3, and CA4.[1] Division along the lateral axis distinguishes the dorsal, medial, and ventral functional parts.[8] Along the longitudinal axis, place cells can be found that increase in size moving from the dorsal to ventral areas. The transverse axis is the location of the trisynaptic loop. Further, hippocampal activity moves in the direction of dorsal to ventral.[9]

**Figure 1.** The hippocampus and its synaptic connections[10]



The hippocampus has three layers. The first is the pyramidal cell layer, made of the somas, or bodies, of pyramidal cells. These cells are efferent projecting neurons. The second layer is composed of axons and the pyramidal cell dendrites and is called the molecular layer. Interneurons make up the final layer, which is called the polymorphic layer.[11] The hippocampus is the only recipient of dentate gyrus projecting neurons. The septal nuclei and contralateral hippocampal formation likewise project to the hippocampus. The inputs from the septal nuclei to the hippocampus release the neurotransmitter acetylcholine at synapses. These form excitatory pathways, as do the numerous glutamatergic inputs to the hippocampus.[1] The efferent neurons in the hippocampus are particularly susceptible to the effects of aging that decrease synaptic plasticity and dendritic spine density.[11]

In contrast to these afferent projections, efferent projections from the hippocampus typically utilize the neurotransmitters gamma-aminobutyric acid, or GABA, an inhibitory neurotransmitter.[1, 12] Efferents from the hippocampus largely extend from the CA1, with dorsal area projecting to the subiculum and entorhinal cortex while the ventral area goes to the nucleus accumbens, amygdala, and prefrontal cortex, or PFC.[8] Projections to the PFC terminate in the medial area and are unidirectional. These afferents interact with projections from the limbic thalamus to the medial PFC.[12, 13]

A crucial role of the hippocampus is in learning and memory. Studies of hippocampal damage initially led to this conclusion and were later verified through studies. Spatial memory disorders and anterograde amnesia have been correlated to damage of the CA1 region.[1] Excitatory inputs from the basolateral nucleus of the amygdala are crucial in spatial memory formation. Place cells in the dorsal hippocampus encode associations to a particular location. Additionally, the dorsal area is linked to episodic memory. Social memory, however, is correlated

with the ventral area's projections to the nucleus accumbens.[8] New memories are processed by the hippocampus before long term storage in the cortex. Processing in this structure is also crucial to the formation of declarative, explicit, and recent memories.[10]

The hippocampus's role in episodic and spatial memory means that it is critical in determining context. The default in the hippocampus is to assume contextual sameness until a threshold of significant change is established. This is a result of the maintenance of patterns with slight changes of input. This is accomplished by dampening signals as they move from one place field to the next within the hippocampus. Small movements through the world can therefore establish smooth hippocampal activity and recognition of sameness. Nevertheless, inputs over a particular threshold allow for a different pattern to be transmitted. Because the ventral medial PFC is particularly concerned with context, it displays key communication with the ventral hippocampus.[9]

**Long term potentiation and memory in the hippocampus**

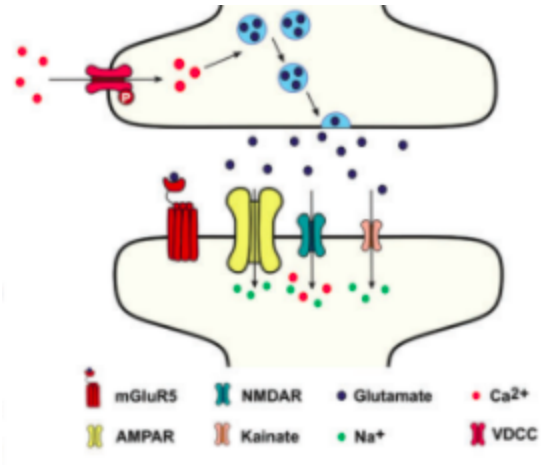
In the brain, activity can modulate the strength of synaptic transmissions, or signals between neurons. This concept is known as synaptic plasticity. Long-term plasticity is a result of activity causing change in transmission that lasts for days, weeks, or years. Shifts in brain plasticity are thought to be linked to memory formation and retention. The most well studied forms of long-term plasticity are long-term potentiation and long-term depression, or LTP and LTD.[10]

LTP and LTD in the hippocampus in particular are thought to underlie memory formation. Quick, repeated excitatory inputs to a neuron that are linked to synaptic changes cause this potentiation or depression. Typically, these excitatory inputs are glutamate neurotransmitters that bind *N*-methyl-*D*-aspartate, or NMDA, receptors in the target neuron.[1] These types of

receptors are ionotropic glutamate receptors that are particularly prevalent in the hippocampus.[1,2] Furthermore, the high concentration of NMDA receptors allows for the processing of two inputs simultaneously, which is thought to be linked to associative memory formation. Binding of glutamate allows calcium to flow into the target, leading to significant depolarization.[1] Flow of calcium ions in response to the binding of NMDA receptors has been specifically recorded in inputs to the PFC from the hippocampus, causing excitatory postsynaptic potentials.[13]

The potential change resulting from NMDA calcium flow regulates the addition in LTP or removal in LTD of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or AMPA, receptors into the synapse.[5] AMPA receptors are another ionotropic glutamate receptor that mediates fast excitatory transmission.[2] They are frequently undergoing exocytosis or endocytosis and diffusion within the cell membrane. The amount, composition, and properties of these receptors modulate synaptic strength. Four types of subunits in this receptor exist, termed GluA1-4. Further, the receptors can be post translationally modified in several different ways that impact protein interactions. The ligand binding and transmembrane domains are largely the same between receptors, but their cytoplasmic carboxy-termini and intracellular amino-termini differ. Two forms of subunits exist, the first of which is long tailed, including GluA1, GluA4, and a variant GluA2L, which are added to synapses based on activity and often added in the development of LTP. The other subunit type is short tailed. This type, including GluA2, GluA3, and variant GluA4S, undergoes constant cycling in and out of synapses. The relative composition of subunits differs by cell. They assemble into tetramers to form the main ion channel through the receptor.[14] The receptors and ligands involved in this process can be seen in Figure 2.

**Figure 2.** Receptors and ligands in synaptic transmission[10]




AMPA channel permeability to calcium depends on the concentration of the subunit GluA2, which prevents the flow of the ion.[2] Because calcium ions are charged, their concentration differences create a charge imbalance across the cell known as an electric potential, or voltage. Thus, blockage of calcium ion flow can affect the voltage. Those without GluA2, however, allow calcium to pass. These types are often found early in development and have very high conductance, which leads to higher synaptic transmission.[14]

The amount and composition of AMPA receptors can result in long-term changes in synaptic strengthening, shown by endplate synaptic potential recordings.[5] These changes are bidirectional, with increases resulting in LTP and a decrease in strength resulting in long-term depression. Often, the particular mode of conditioning that is applied will change which of these occurs. An example of this variation with conditioning includes frequency of stimulation, with high frequencies inducing potentiation and low frequencies inducing depression. cAMP levels also affect how the synapse changes, with higher concentrations resulting in potentiation.[12] The composition and modifications of particular AMPAs added to or removed from synapses impact the particular change in synaptic strength. Certain forms of AMPA receptors are correlated with particular forms of potentiation.[5] An example of this includes calcium permeable AMPAs, whose

high levels of transmission make them particularly suitable to prime synapses for LTP.[14] As differential amounts of AMPA receptors are associated with synapse strength, their density is directly related to the formation of memory by LTP. This is because strengthened synapses are associated with the formation of memory engrams.[5] These strengthened synapses are also reactivated with the recall of these memories.[12]

Due to the role of NMDA receptors in generating an increase in synaptic strength, blocking these receptors can inhibit potentiation.[12] Additionally, a decrease in protein kinase A is related to decreased LTP.[15] On the other hand, an increase in stimulation of cyclic adenosine monophosphate, or cAMP, concentration inside the neuron can increase LTP.[12] Shifts in potentiation of the hippocampus may be particularly influenced by inputs from the basolateral amygdala.[8] These shifts in LTP facilitate the plasticity of the hippocampus.[1] Importantly, this plasticity may depend upon the medical history of the individual.[12] Whether depression or potentiation take place in learning may vary throughout the learning process as conditioning and stimulation change.[13] Although both LTP and LTD may play a role in learning, decreases in LTP have been shown to lead to a decrease in the capability to form long-term memories.[15]

**Other functions of the hippocampus**

In addition to learning and memory, other behaviors can be modulated by the hippocampus. Projections to the PFC are linked to anxiety behaviors, and ones targeting the nucleus accumbens are implicated in task-oriented activities. Anxiety behaviors have been suggested to be linked to synergistic action between the hippocampus and the basolateral amygdala.[8] Epileptic and psychotic impairments have also been linked to interruptions between hippocampal communication with the PFC by inactivation of the intervening mediodorsal thalamus.[13] This link is supported by reported neuronal loss and epilepsy resulting from lesions

of hippocampal CA fields. NMDA receptor antagonists suppressed the mediodorsal thalamus, likely dysregulating the inhibitory-excitatory balance in the downstream PFC.[13]

Neurogenesis, or the production of new neurons in the central nervous system due to mitosis, occurs in fetal stem cells. However, evidence suggests that multipotent cell precursors exist in the brain in the hippocampus. Adult hippocampal cell regeneration is hypothesized to occur in humans and has been shown to replace aged cells in rats. Further, human hippocampal precursor cells have developed into differentiated cells in vitro. However, neurogenesis has reportedly been repressed in individuals with post-traumatic stress disorder.[1] Additionally, dysregulation of neurogenesis in the hippocampus has been linked to several neurological pathologies.[16]

**Sex differences in the brain**

Structurally, the male brain contains a larger number of granular neurons in the dentate gyrus. Additionally, mossy fiber synapses are prevalent in the male hilus and the female CA3. However, dendritic branch count and spine count in pyramidal basal neurons are increased in female CA3.[3] Dendritic arbors have been observed to be 20% larger in female mice as opposed to males. This increase in size is observed to spike significantly with the application of glucocorticoids. Females also have a higher number of corticosteroid-binding globulin levels and a different ratio of glucocorticoid receptors.[2] Additionally, certain parts of the hippocampus exhibit differential volumes between males and females. The posterior region is larger in females. While hippocampal volume can change due to early life stress, these changes occur more frequently in males than females. Connectivity also differs between the sexes. Females display greater inter-hemispheric connectivity, while males, on the other hand, exhibit greater intra-hemispheric connectivity.[3]

The endocannabinoid system is a key point of difference between the male and female brain. Cognition, emotion, stress response, and pain are controlled by endocannabinoid lipid based neuromodulators. When released from a postsynaptic cell, they modulate the amount of neurotransmitter released.[17]

A key aspect of the sex difference in the endocannabinoid system involves 17β-estradiol, a neurosteroid synthesized in the hippocampus. It has been shown to modulate synaptic transmission by potentiating excitatory hippocampal synapses and suppressing inhibitory ones. Particularly, endplate synaptic potentials at CA1 pyramidal cells are amplified by this steroid.[18] This occurs by activation of NMDA receptors and may play a role in synaptogenesis and the development of LTP in the hippocampus.[1, 18] Furthermore, early and late LTP has been observed in larger amounts in male rats compared to females, particularly in CA1 and CA3. Males also have been shown to have increased CA3 synaptic connections.[3]

cAMP activated protein kinase A, or PKA, is implicated in the 17β-estradiol potentiation of female hippocampal neurons. Several other kinases are likewise linked to potentiation; however, these all were likewise required by male hippocampal neurons. cAMP PKA, on the other hand, was shown to be required solely by females for potentiation to occur. Further, two calcium sources are implicated in the development of potentiation in these neurons, including L-type channels and internal stores. However, either of these sources was sufficient to generate potentiation from E2 in males, whereas females required both. Other types of synaptic plasticity have been shown to require PKA only in females. This suggests a crucial difference in molecular signaling and neuromodulation mechanisms between males and females.[18]

Further differences surrounding E2 in the hippocampus involve estrogen receptor 2. It can suppress inhibition at this receptor specifically in females. Nevertheless, this requires the

activation of the endocannabinoid metabotropic glutamate receptor, mGluR. The result is LTD of inhibition. This differential inhibition in females is due to differential regulation of these signaling pathways. Likely, gonadal hormones cause differential signaling of mGluR1 and estrogen receptor alpha in males and females. Further, once these alternate signaling mechanisms have been established, they retain their male or female dimorphism even without the continued presence of gonadal hormones.[16]

Females exhibit higher levels of estradiol in the brain than males. This may have a protective effect against brain damage. A higher incidence of damage after ischemia in males versus females supports this conclusion. Increased resistance to damage in females may be applicable to the effects of increased glucocorticoids, as well. The release of these compounds is correlated to high stress, and excessive elevation may lead to neuropathological changes. Occurrences of such changes have been implicated in depression and post-traumatic stress disorder. One of the most commonly observed shifts is neural atrophy in CA3, with a particular decrease observed in hippocampal dendrites. The length and branch point number significantly decrease.[2] Atrophy at CA1 is likewise observed.[3]

Although atrophy is observed in both sexes, females exhibit much greater atrophy at CA1 and increased resistance to CA3 atrophy than males.[2, 3] Similarly, males exhibited a larger decrease in synaptophysin protein after applied stress, which is crucial to synaptic reorganization. Further, AMPA receptor GluA2 concentration increased in females with high glucocorticoid levels, but not males. This indicates a lower influx of calcium in females as opposed to males, which may result in higher resistance to excitotoxicity. Specific kainate receptors, another type of receptor implicated in synaptic transmission, namely KA1, were found in higher amounts in females following stress. The result of these factors indicates that males

respond more acutely to stress, leading to higher postsynaptic activity and excitotoxic damage. The increased protection in females is thought to be correlated with the prevention of nervous system changes during pregnancy when corticoids are present in higher amounts.[2]

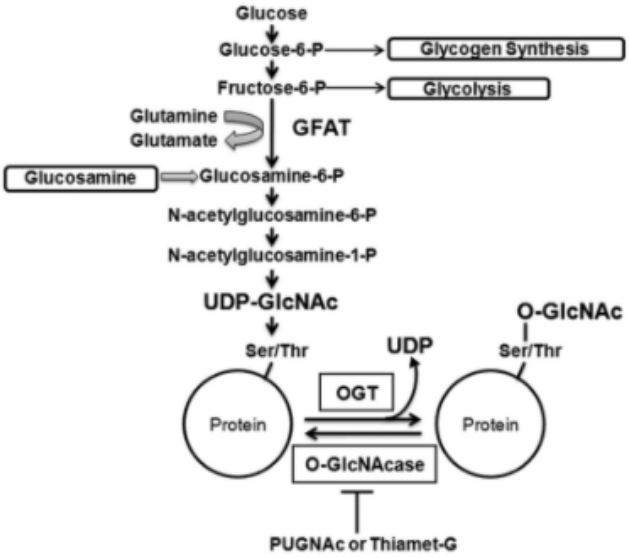
In Alzheimer's disease, an increase in tau protein and amyloid-β-42, or Aβ-42, levels were seen in the female hippocampus compared to males. Further, steeper levels of cognitive and hippocampal volume decrease with disease progression have been observed in females relative to males.[19]

**_O_-GlcNAcylation**

*O*-GlcNAcylation is the addition of an N-acetylglucosamine sugar to serine or threonine residues of intracellular proteins.[7] The sugar uridine-diphosphate-GlcNAc, or UDP-GlcNAc is the most available high energy metabolite behind ATP and is the substrate for OGT, which adds *O*-GlcNAc modifications on to proteins.[7, 20] UDP-GlcNAc is a product of the hexosamine biosynthetic pathway, or HBP, a cycle that utilizes 2-3% of the glucose entering the brain.[6] This path utilizes adenosine-triphosphate, or ATP, glucose, acetyl-Coenzyme A, or CoA, and uridine. Since the availability of UDP-GlcNAc is dependent upon glucose metabolism, as well as fatty acid, amino acid, and nucleotide availability, *O*-GlcNAc is considered a nutrient sensor that quickly reveals changes in metabolism throughout the body, as well as cell-specific metabolism.[4, 7, 20] A full length and a short isoform of OGT exist, and they may act on different proteins.[11] *O*-GlcNAcase, or OGA, removes this modification, making it a reversible addition.[7;4] OGA can be selectively inhibited by treatment with thiamet-G.[4] Regulation between OGA and OGT lead to proper *O*-GlcNAc cycling within the body.[6] The regulation and modification mechanism of *O*-GlcNAc can be seen in Figure 3.

*O*-GlcNAc is a post-translational modification similar to phosphorylation, a key modification in the development of learning, memory, and plasticity.[6, 20] These modifications are mutually exclusive on many proteins. This is supported by observed decreased phosphorylation on ten eleven translocase, or TET, proteins, leading to increased *O*-GlcNAcylation. As TET proteins regulate gene expression, modifications which alter their behavior are critical for regulation.[21] Phosphorylation of proteins is regulated by thousands of phosphatases and kinases.[6,20] *O*-GlcNAc, however, is strictly regulated by OGT and OGA, both of which are located in synaptosomes.[5,6] OGA can also be found in presynaptic terminals and dendritic microtubules.[5]

**Figure 3.** Regulation and modification mechanisms of *O*-GlcNAc[5]




*O*-GlcNAc is involved in many processes within the cell, including autophagy, mitochondrial movement, gene expression, and signal transduction.[4] It is also critical for embryonic development, causing the knockout of the gene *Ogt* to be embryonic lethal. On the other hand, the gene *Oga* knockout can lead to developmental disorders and delay of embryonic differentiation. Further, malfunction of OGA leads to increased *O*-GlcNAc levels and several

typical phenotypes, including high body fat percentage, shortness, and microcephaly. Additionally, neurogenesis may be prolonged in these conditions.[7]

*O*-GlcNAcylation occurs at higher rates in the brain than the rest of the body.[7] This correlates with the high glucose intake of the brain, which is the last structure to suffer a metabolic decrease in starvation and utilizes 20% of the glucose that enters the body. As a result, shifts in glucose levels make the brain particularly susceptible to dysregulated neurogenesis.[22] Once glucose has been converted to *O*-GlcNAc, this modification is added in notably increased levels in the hypothalamus, cortex, and hippocampus compared to other regions.[4] Particular *O*-GlcNAc density is observed at CA1 pyramidal neurons and GABAergic interneurons.[5] This may be due to the high glucose consumption of the brain, totaling 60% of the body's total supply. Several functions, including the growth of axons and dendrites, neuronal metabolism, and neural degeneration, are regulated by this modification.[7] A few critical regulatory proteins are known to be *O*-GlcNAcylated, including Sox2, Nanog, Sox1, Otx2, NR2A, NR2B, PSD-95, and synapsin1.[7, 11] Mouse models have demonstrated that proper *O*-GlcNAc turnover is crucial to maintaining correct levels of these proteins, which go on to regulate the cell's neurodevelopment.[22] Several key receptors involved in the development of LTD and LTP are *O*-GlcNAcylated. This includes the AMPA GluR2 subunit, NMDA, and protein kinase C at CA1-CA3 synapses.[11] *O*-GlcNAcylation of AMPA GluR2 subunits may lead to decreased phosphorylation of the GluR1 subunits.[20]

*O*-GlcNAcylation of these proteins leads to modulation of the flow of cations through voltage gated channels. These include voltage gated potassium channels, leading to an increase in outward current and decreased excitability. Further, *O*-GlcNAcylation leads to a decrease in sodium influx through sodium voltage gated channels. This further depolarizes the cell, and the

reduced flow can lead to endocytosis of AMPA GluR2. Hyperpolarization of cyclic-nucleotide gated channels is also impacted by *O*-GlcNAc levels. The dampening effect of flux through sodium and potassium channels in particular has been specifically observed at CA1 synapses. Current through these channels reduces excitability, a phenomenon increased by more *O*-GlcNAc. The high prevalence of these channels in apical dendrites heightens this effect in this area.[4] As a result, *O*-GlcNAcylation at these proteins induces a particular form of LTD to this hippocampal synapse.[11] This may allow for a break in LTP, resulting in the protective role of this modification discussed previously.[5]

Because LTD is crucial to certain aspects of learning, *O*-GlcNAc has been shown to play a role in the development of hippocampal learning and memory. Further, an increase in this modification has been linked to increased associative-fear memories. Alterations of this modification have been linked to differences in spatial memory, as well.[11] Performance in novel object placement and recognition tasks has reportedly decreased with higher *O*-GlcNAc, however.[20] Memory processing and synaptic efficiency are reportedly modulated by shifts in *O*-GlcNAcylation.[5] Regulation of differentiation and proliferation of neural cells depends on the amount of *O*-GlcNAc present and is key for development of proper neuronal structures. Higher than normal levels of *O*-GlcNAc have been observed to result in lower amounts of hippocampal pyramidal neurons.[7]

**_O_-GlcNAcylation, cognitive decline, and disease**

Neural *O*-GlcNAcylation has been shown to aid in cognitive rehabilitation in aged mice. Age comes with a reduction in synaptic and plasticity changes, as well as a reduction of OGT levels. Similar shifts have been observed in Alzheimer's disease. Further, a decrease in the levels of *O*-GlcNAcylation of NR2A, NR2B, PSD-95, and synapsin1 has been observed with this

reduction. However, studies indicate that restoration of these enzyme levels restore cognitive function, likely due to *O*-GlcNAc's impact on LTP and LTD shifts that are crucial for memory formation.[11]

Phosphorylation is likewise linked to Alzheimer's disease and *O*-GlcNAc. Because phosphorylation and *O*-GlcNAcylation of tau proteins are mutually exclusive, decreased *O*-GlcNAc is often observed in cases of hyperphosphorylation, regardless of OGA and OGT levels. Triple transgenic mice, meaning three mutations are present, have been created to mimic Alzheimer's patients and have displayed heightened levels of phosphorylation at tau protein Serine residue 396 and Thr residue 205, termed Ser396 and Thr205, in the hippocampus.[23] However, a particular modification that has been shown to have ameliorative effects in Alzheimer's is *O*-GlcNAcylation of tau protein Ser400.[5] Together, these data demonstrate that lower *O*-GlcNAc is considered a crucial aspect of cognitive decline and increase may be a possible treatment for age and neuronal disease-related impairments.[11]

Triple transgenic mice that have been used to model Alzheimer's patients and display a decrease in *O*-GlcNAcylation have led to other pathological considerations. Increased anxiety and depressive behaviors were observed in these mice, indicating a possible link between these mental disorders and *O*-GlcNAc levels.[23] Other cognitive maladies associated with *O*-GlcNAc include Huntington's and Parkinson's disease.[22] Many proteins that aggregate in neurodegenerative disorders are *O*-GlcNAcylated.[22]

Particular focus has been placed on the role of *O*-GlcNAc as a potential treatment for seizures. This is because epileptiform activity is a result of hyperexcitability, meaning the protective aspect of *O*-GlcNAc could ameliorate epilepsy.[20] Studies have found the upregulated neurogenesis with higher *O*-GlcNAc levels to correlate with lower neurodegenerative damage.

These results indicate that lower OGA levels or inhibition of this enzyme may be protective. [7] An increase in OGT may likewise serve this purpose.[11]

*O*-GlcNAcylation has further been implicated in diseases with dysregulated sugar levels, such as diabetes and obesity. [4] Because *O*-GlcNAcylation is dependent upon glucose metabolism, defects in brain glucose uptake that come with these diseases are correlated with lower brain *O*-GlcNAc levels. Insulin and insulin-like growth factor, or IGF, resistance may lead to defective glucose consumption and decreased cycling through the HBP. As a result, the protective effects of *O*-GlcNAc on synaptic activity are decreased. The importance of *O*-GlcNAc neural protection is observed particularly in the hippocampus due to this structure's critical role in learning and memory. Alzheimer's disease pathologies can arise without this protection, including a decrease in hippocampal spatial memory Therefore, a link between diabetes, *O*-GlcNAc, and the development of Alzheimer's disease can be established.[23]

Other pathologies related to diabetes and *O*-GlcNAc include heart disease. Acute hyperglycemia is linked to *O*-GlcNAcylation of Ser279 on calcium/calmodulin-dependent protein kinase II, or CamKII, a regulatory protein in the heart and brain. This causes its activation, which may lead to changes in channel activation, calcium ion flow, and gene transcription. Calcium ion release from the sarcoplasmic reticulum in particular is linked to heart arrhythmias. As diabetic individuals have higher glucose levels in their blood, more substrate is available for *O*-GlcNAcylation. Therefore, they show higher rates of CamKII *O*-GlcNAcylation in the heart and brain and are particularly susceptible to heart arrhythmia pathology. Individuals with diabetes reportedly show twice as much *O*-GlcNAcylation in heart CamKII and are almost three times as likely to develop heart failure.[24] As a result of *O*-GlcNAc's simultaneous role in

cognition, these conditions are often observed along with impaired memory and brain function. In particular, dementia and neurodegeneration are often correlated to diabetes.[4]

**_O_-GlcNAcylation and sex differences**

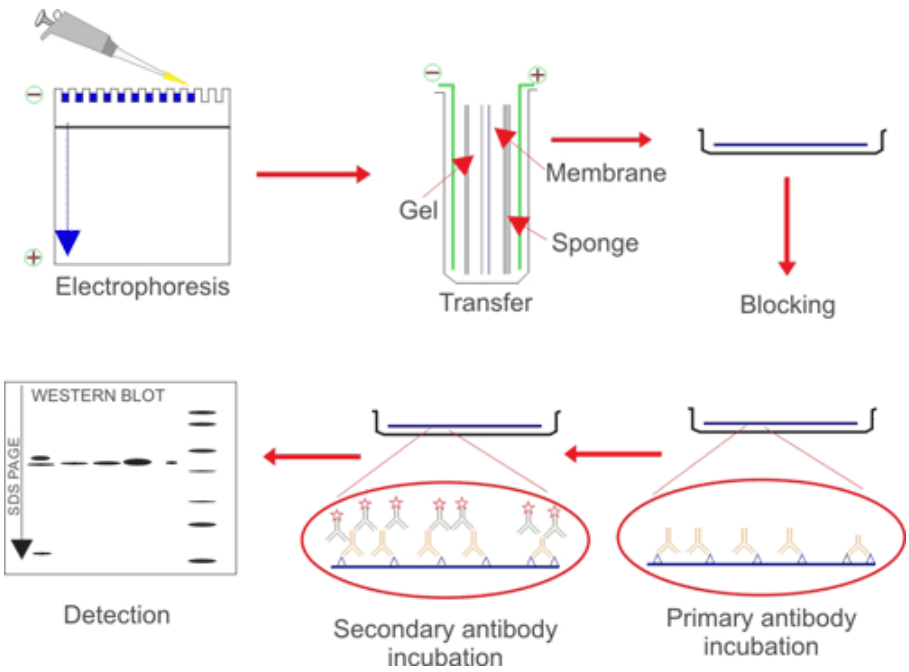
The gene for OGT is located on the X chromosome. Therefore, dosage compensation in females is critical for maintaining proper levels of OGT. Random X-inactivation silences one X chromosome to ensure that the same amount of transcription occurs in females as in males. However, about 15% of genes remain active on the silenced chromosome and are biallelicly expressed. In embryonic tissues, the gene *Ogt* on the paternally inherited X chromosome is preferentially silenced. This is due to epigenetic X chromosome silencing, whereas random X inactivation silences the maternal and paternal X in equal proportions. Outside of embryonic tissues, *Ogt* can escape X inactivation. Therefore, the amount of OGT present and resulting disease phenotypes may be different based on sex. Notably, photoblast stem cells and placentas in females have higher OGT levels than in males. Additionally, lowered placental OGT has been linked to the presentation of neurological disease. Nevertheless, OGT is typically expressed equally in males and females except in the adrenal glands. [6]

In light of the impact of *O*-GlcNAcylation on hippocampus structure and function and potential differences by sex, the regulation and differential expression of this modification may impact key functions of the hippocampus. As learning and memory are included in these functions, studies involving shifts in *O*-GlcNAc and its regulatory enzymes are important in understanding these processes. Furthermore, as OGA regulates *O*-GlcNAc, quantifying how variable expression of this enzyme shifts *O*-GlcNAc levels is crucial. OGT likewise regulates *O*-GlcNAc, and as sex differences with this enzyme are proposed, validation of these differences is necessary.

## Methodology

This study aims to investigate the levels of OGT, the levels of OGA, and the levels of *O*-GlcNAc in the hippocampus of male versus female mice. Western blot analysis, a method of detecting and quantifying proteins in a sample, was utilized to measure OGT, OGA, and *O*-GlcNAcylatyed protein levels in mice with differential OGA expression.[25] This analysis utilizes electrophoresis to push proteins through a polyacrylamide gel and separate proteins based on their respective sizes. Proteins contain a negative charge, resulting in migration from the negative electrode to the positive electrode with lower molecular weight proteins moving faster through the gel and therefore traveling further.[25,26] The basic procedure can be seen in Figure 4.

**Figure 4.** Western blot procedure[27]

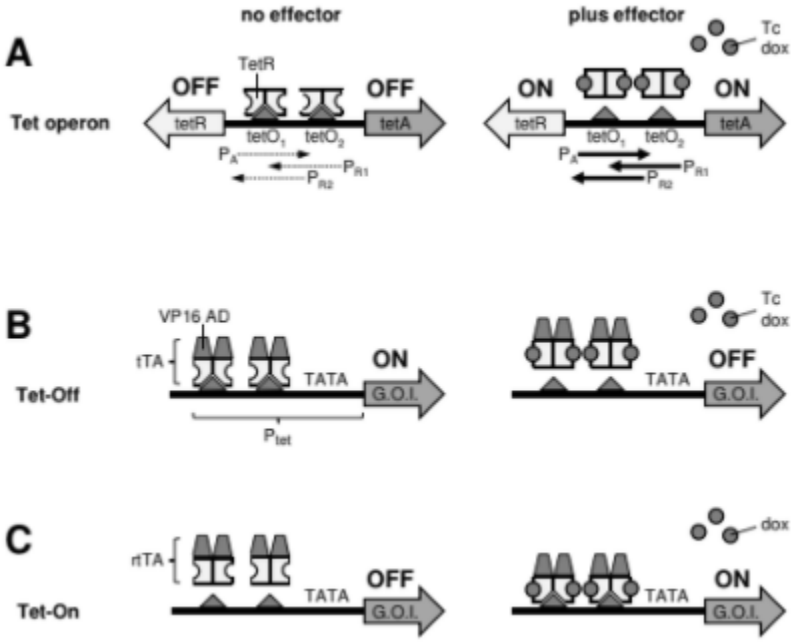


**Genetic model**

*Purpose*

Considering knockout of *Oga* in mice leads predominantly to perinatal lethality, this system is useful for generating differential OGA expression. Tet-On and Tet-Off systems are a method of controlling gene expression based off of the Tet repressor protein, or TetR, and Tet operator, TetO, in the Tet operon of *Escherischia coli*. In Tet-Off systems, transcription activation domain combined to TetR allows for production of transcription activator, or tA. TetO, positioned in front of the gene of interest, binding to tA activates transcription of the gene. Binding of tetracycline or its derivative doxycycline to tA prevents binding to TetO. This in turn prevents transcription of the gene of interest. Without doxycycline, tA can bind TetO, thus allowing gene transcription. The Tet operon, Tet-On, and Tet-Off systems can be visualized in Figure 5. This experiment utilized such a system, administering doxycycline to certain mice to prevent OGA expression and stopping administration to others to allow expression. The mice were identified by a number corresponding to their cage number and ear tag.[28]

**Figure 5.** Tet operon, Tet-On, and Tet-Off systems[28]



**Obtaining and homogenizing the samples**

Mice utilized in this experiment were placed in an isoflurane chamber to render them unconscious. This was followed by sacrifice with a guillotine. rains were then dissected by subfield over dry ice. Hippocampal, cerebellar, and frontal cortex sections were placed in separate labeled test tubes and left to sit in liquid nitrogen for several hours. The samples were then moved to -80°C.[29]

In order to extract proteins and allow storage at -20°C, all tissues were then homogenized. Lysis buffer was prepared by mixing a ratio of one microliter of protease inhibitor and one microliter of phosphatase inhibitor to 100 microliters of Tissue Protein Extraction Reagent. 400 microliters of the prepared buffer were added to each sample tissue followed by 30 seconds in the homogenizer at medium speed. The samples were allowed to sit on ice for 30 minutes. In a 4°C environment, the samples were centrifuged at 3,200G for 15 minutes. The pellets were discarded, and the supernatants were stored in fresh tubes at -20°C.[29]

**Table 1.** Mice Utilized

| ID number | On/Off doxycycline | Genotype | Sex |
|---|---|---|---|
| 1058 | On | WT | M |
| 1038 | On | WT | M |
| 1008 | On | WT | M |
| 1056 | On | CAMK tTA+TREOGNgk | M |
| 1023 | On | CAMK tTA+TREOGNgk | M |
| 1022 | On | CAMK tTA+TREOGNgk | M |
| 1063 | Off | WT | M |
| 1043 | Off | WT | M |
| 977 | Off | WT | M |

| | | | |
|---|---|---|---|
| 1045 | Off | CAMK tTA+TREOGNgk | M |
| 1044 | Off | CAMK tTA+TREOGNgk | M |
| 1026 | Off | CAMK tTA+TREOGNgk | M |
| 1004 | On | WT | F |
| 1033 | On | WT | F |
| 1054 | On | WT | F |
| 969 | On | CAMK tTA+TREOGNgk | F |
| 996 | On | CAMK tTA+TREOGNgk | F |
| 1021 | On | CAMK tTA+TREOGNgk | F |
| 1003 | Off | WT | F |
| 1017 | Off | WT | F |
| 1032 | Off | WT | F |
| 1002 | Off | CAMK tTA+TREOGNgk | F |
| 1018 | Off | CAMK tTA+TREOGNgk | F |
| 1034 | Off | CAMK tTA+TREOGNgk | F |

**Western blot**

Sample buffer was prepared by adding nine parts 4x Laemmli sample buffer to one part beta-mercaptoethanol, or BME, and stirring with a vortex mixer. Each sample was prepared by adding 3.3 microliters of sample buffer solution and 14.7 microliters of de-ionized (DI) water to a micro test tube with 2 microliters of the homogenized tissue. The micro test tubes with prepared samples were then placed in a 70°C for 10 minutes.[29,30]

7.5% polyacrylamide gels were handcast with *Bio-Rad* Mini-PROTEAN® Tetra handcast systems reagents and according to the included guidelines for 1.5 mm glass plates. Gels were placed in an upstand in the electrophoresis chamber. Runner buffer was made by adding 900 mL

DI water to 100 mL of Tris-glycine buffer. Buffer was then poured into the upstand. A micropipette was used to load two microliters of the ladder protein standard solution into the first well of the gel and 20 microliters of sample 1058, 1063, 1004, 1003, 1056, 1045, 969, and 1002 into each successive well. This was repeated on a second gel with samples 1038, 10443, 1033, 1017, 1023, 1044, 996, and 1018 and then on a third gel with samples 1008, 977, 1054, 1032, 1022, 1026, 1021, and 1034. The electrophoresis chamber was closed and run at 200 volts for 40 minutes to push proteins down the gel.[31]

The transfer buffer was prepared by mixing 700 mL DI water, 100 mL Tris-glycine buffer, and 200 mL methanol. A piece of polyvinylidene fluoride, or PVDF, paper was cut to match the size of the gel, rinsed in methanol, and left to sit in the transfer buffer for 10 minutes. Two sponges and two pieces of filter paper were let to sit in the buffer for 10 minutes, as well. The gel was removed from the chamber, the glass plates were separated, and the gel was removed from the plates. One of the sponges and pieces of filter paper were placed in the bottom black side of a cassette followed by the gel, PVDF paper, another filter paper, and another sponge. The cassette was closed and placed in the electrophoresis chamber. Transfer buffer, a stir bar, and an ice pack were added to the chamber, which was placed on a stir plate in a 4°C environment. The chamber was run at 100 volts for one hour. The PVDF paper was then removed from the chamber and allowed to dry.[30,31]

**Specific aim I:** To determine the concentration of OGA relative to actin in male and female mice, both on and off doxycycline

Six male mice on doxycycline, six male mice off doxycycline, six female mice on doxycycline, and six female mice off doxycycline were tested. Three of each group were wild

type mice, and three of each were *O*-GlcNAc knockdown models with the mutation CAMK tTA+TREOGNgk.

Each gel was washed in 1X Tris-buffered saline with 1% Tween, or TBST for five minutes with agitation. The gel was then blocked with 5% milk, made from five grams dehydrated milk mixed with 100 mL TBST, with agitation for one hour. This was followed by three 15 minute washes with TBST. The blot was then incubated in two microliters of OGA primary antibody generated in rabbit diluted in four mL 5% milk in a 4°C environment with agitation overnight. The gel was then washed with TBST for 10 minutes four times with agitation. It was then incubated in two microliters goat-anti-rabbit secondary antibody diluted in four mL 5% milk for one hour at room temperature with agitation. This was followed by three 15 minute TBST washes.[25]

The blot was developed by rinsing in enhanced chemiluminesence solution for 5 minutes with agitation. The blot was exposed for .5-40 seconds with the Bio Rad Chemi Doc Imager.[25]

The blot was washed in a stripping buffer for five minutes, then the process was repeated with actin primary antibody in order to normalize the amount of OGA to actin. This was then repeated with two more blots to enable statistical analysis.[25]

**Specific aim II:** To determine the concentration of OGT relative to actin in male and female mice, both on and off doxycycline.

Six male mice on doxycycline, six male mice off doxycycline, six female mice on doxycycline, and six female mice off doxycycline were tested. Three of each group were wild type mice, and three of each were *O*-GlcNAc knockdown models with the mutation CAMK tTA+TREOGNgk.

Each gel was washed in TBST for five minutes with agitation. The gel was then blocked with 5% milk, made from five grams dehydrated milk mixed with 100 mL TBST, with agitation for one hour. This was followed by three 15 minute washes with TBST. The blot was then incubated in two microliters of OGT primary antibody generated in rabbit diluted in four mL 5% milk in a 4°C environment with agitation overnight. The gel was then washed with TBST for 10 minutes four times with agitation. It was then incubated in two microliters goat-anti-rabbit secondary antibody diluted in four mL 5% milk for one hour at room temperature with agitation. This was followed by three 15 minute TBST washes.[25]

The blot was developed by rinsing in an enhanced chemiluminescence solution for five minutes with agitation. The blot was exposed for 0.5-40 seconds with the Bio Rad Chemi Doc Imager.[25]

The blot was washed in a stripping buffer for five minutes, then the process was repeated with actin primary antibody in order to normalize the amount of OGA to actin. This was then repeated with two more blots to enable statistical analysis.[25]

**Specific aim III:** To determine the concentration of *O*-GlcNAc relative to actin in male and female mice, both on and off doxycycline.

Six male mice on doxycycline, six male mice off doxycycline, six female mice on doxycycline, and six female mice off doxycycline were tested. Three of each group were wild type mice, and three of each were *O*-GlcNAc knockdown models with the mutation CAMK tTA+TREOGNgk.

Each gel was washed in TBST for five minutes with agitation. The gel was then blocked with 5% milk, made from five g dehydrated milk mixed with 100 mL TBST, with agitation for one hour. This was followed by three 15 minute washes with TBST. The blot was then incubated

in two microliters of *O*-GlcNAc primary antibody generated in mouse, diluted in four mL 5% milk in a 4°C environment with agitation overnight. The gel was then washed with TBST for 10 minutes four times with agitation. It was then incubated in two microliters goat-anti-mouse secondary antibody diluted in four mL 5% milk for one hour at room temperature with agitation. This was followed by three 15 minute TBST washes.[25]

The blot was developed by rinsing in enhanced chemiluminescence solution for five minutes with agitation. The blot was exposed for 0.5-40 seconds with the Bio Rad Chemi Doc Imager.[25]

The blot was washed in a stripping buffer for five minutes, then the process was repeated with actin primary antibody in order to normalize amount of *O*-GlcNAc to actin. This was then repeated with two more blots to enable statistical analysis.[25]

## Data Analysis

A representative Western blot demonstrating OGT, OGA, and *O*-GlcNAc relative to actin is shown below in Figure 6. One sample of each type is included, with the genetic makeup, sex, and doxycycline status listed in Table 1.

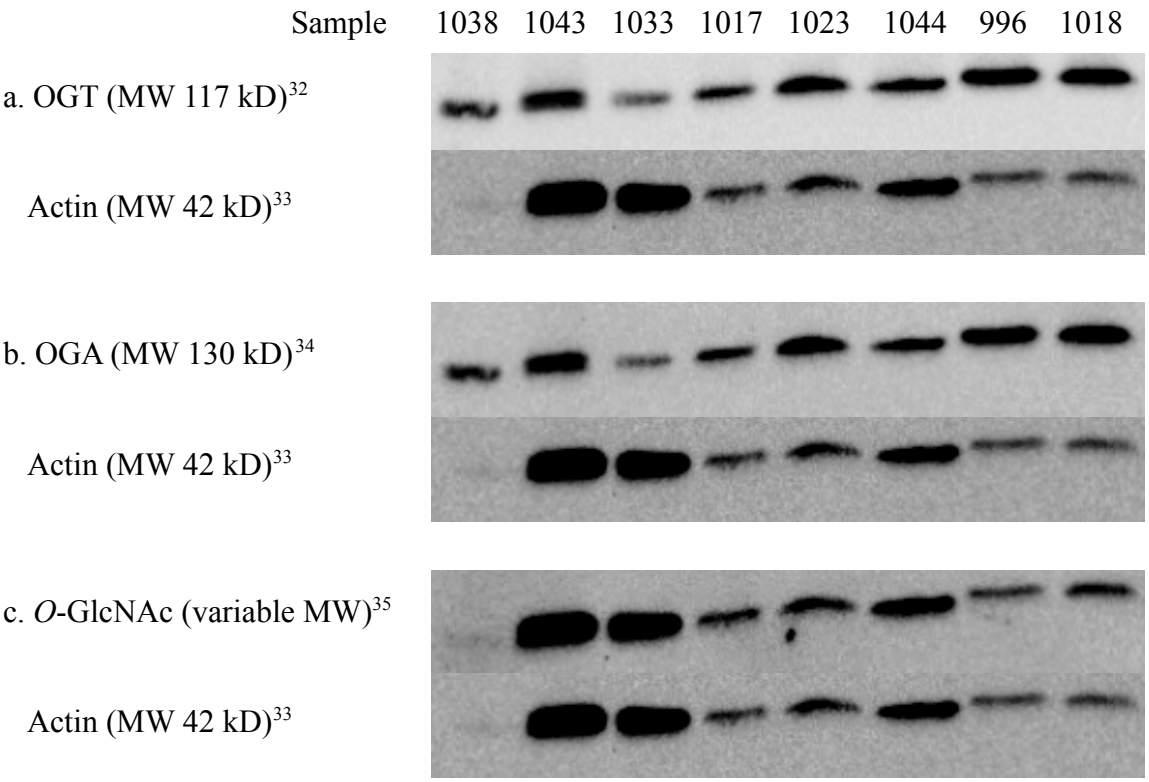



**Figure 6.** a. OGT representative western blot, b. OGA representative western blot, c. *O*-GlcNAc representative western blot

BioRad ImageLab software was used to analyze the pixels in each sample, and pixels of each antibody were normalized relative to actin. Three Western blots were run, with one sample of each type in each blot, creating an n=3. The first blot was run twice, and the second and third blots were run three times. The separate runs were used to calculate the averages of normalized levels for each blots. As each blot contained one sample of each type, the means and standard errors of each type were determined from these averages. These are displayed in Figure 7-9, with

WT M On indicating wild type males on doxycycline, WT M Off indicating wild type males off doxycycline, WT F On indicating wild type females on doxycycline, WT F Off indicating wild type females off doxycycline, Mut M On indicating OGA knockdown mutant males on doxycycline, Mut M Off indicating OGA knockdown mutant males off doxycycline, Mut F On indicating OGA knockdown mutant females on doxycycline, and Mut F Off indicating OGA knockdown mutant females off doxycycline.

**Figure 7.** Mean OGT relative to actin by sample type

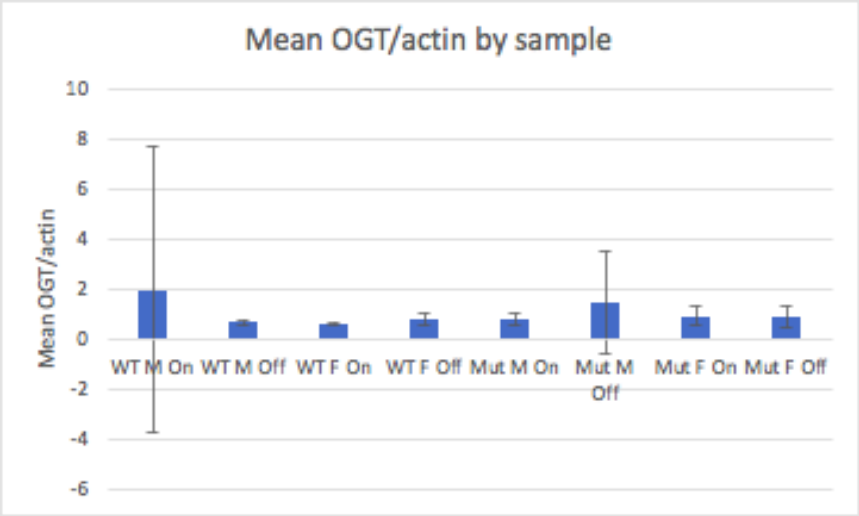



**Figure 8.** Mean OGA relative to actin by sample type

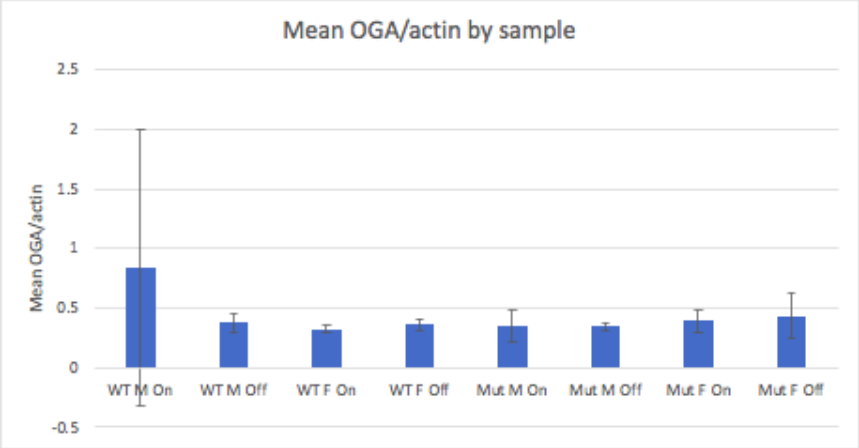
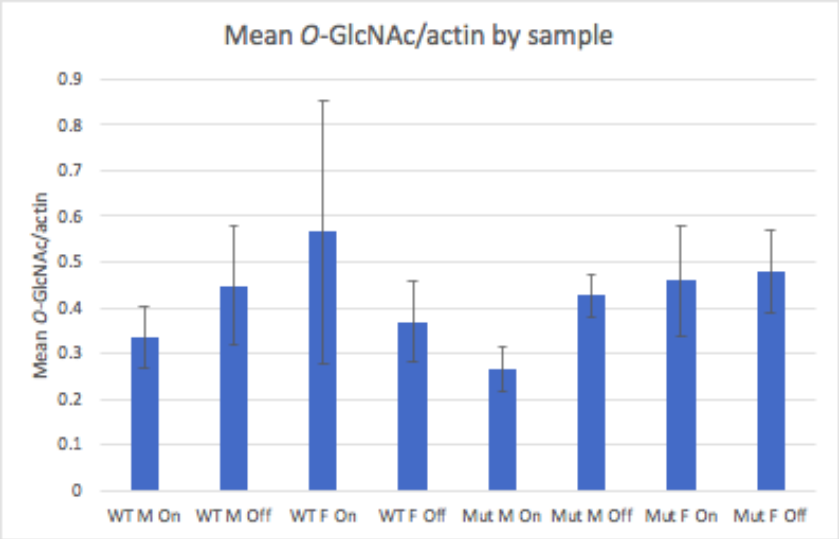


**Figure 9.** Mean *O*-GlcNAc relative to actin by sample type




Relative OGT to actin was found to be 1.95±5.70 in WT M On, 0.63±0.14 in WT M Off, 0.58±0.06 in WT F On, 0.77±0.25 in WT F Off, 0.74±0.24 in Mut M On, 1.43±2.02 in Mut M Off, 0.89±0.39 in Mut F On, and 0.86±0.44 in Mut F Off. Relative OGA to actin was found to be 0.84±1.16 in WT M On, 0.37±0.07 in WT M Off, 0.32±0.03 in WT F On, 0.36±0.05 in WT F Off, 0.35±0.13 in Mut F On, 0.35±0.03 in Mut F Off, 0.40±0.10 in Mut F On, and 0.43±0.19 in Mut F Off. Relative *O*-GlcNAc to actin was found to be 0.33±0.07 in WT M On, 0.45±0.13 in WT M Off, 0.57±0.29 in WT F On, 0.37±0.09 in WT F Off, 0.26±0.05 in Mut F On, 0.43±0.04 in Mut F Off, 0.46±0.12 in Mut F On, and 0.48±0.09 in Mut F Off.

Statistical analysis was performed using a Student's paired t-test for two samples assuming equal variances. Hypothesized mean difference was set to zero for all comparisons. Differences were considered statistically significant if one tailed p values were less than 0.05. However, no samples displayed significant differences for any antibody.

## Conclusion

Wild type mice acted as controls, and were expected to have the same amounts of OGA, OGT, and *O*-GlcNAc relative to actin when on or off doxycycline. This was shown to be true in both males and females. OGA knockdown mice were not expected to display this same trend. When on doxycycline, the drug binds tA to prevent expression of OGA. Therefore, lower levels of OGA were expected to be observed in mutant mice on doxycycline versus mutant mice off doxycycline. However, no statistically significant difference was found in mutant male or female mice. Additionally, the hypothesized decrease of OGA expression in mutant mice on doxycycline would result in an decrease in removal of *O*-GlcNAc from serine and threonine residues. Therefore, an increase in *O*-GlcNAcylated proteins was hypothesized to occur in mutant mice on doxycycline. Nevertheless, no statistically significant difference was found in *O*-GlcNAcylated proteins relative to actin in male or female mutant mice. These findings indicate that mice off of doxycycline had not yet been off of the drug for long enough to create a significant OGA expression difference. Therefore, a longer period of time off of doxycycline is hypothesized as necessary to create a change. As OGT remained unaffected by the administration of doxycycline, no statistically significant differences were expected in this antibody relative to actin between mutant mice on and off doxycycline, which was found to be true.

Furthermore, it was hypothesized that females may display increased OGT relative to males due to the additional X chromosome they possess, whether mutant, wild type, on doxycycline, or off doxycycline. This would in turn produce an increase in *O*-GlcNAcylation. However, no significant difference in OGT relative to actin or *O*-GlcNAc relative to actin was observed between wild type males and females or mutant males and females on doxycycline or

mutant males and females off doxycycline. Therefore, the data suggests that male and female brains express the same relative amount of OGT, and the second X chromosome found in females does not alter expression of this enzyme.

*Strengths of the study*

Several aspects of this study lent strength to the analysis and results. Each blot contained eight samples and was run two to three times with averages of the runs calculated. This was done to reduce any error that may have occurred during one particular run. Additionally, each blot contained one of each sample type. Therefore, three of each sample type were utilized, creating an n=3. This allowed for proper statistical analysis to be done and reduced error that may have resulted from one particular sample. Further, the samples were run blind to reduce bias. This meant the genotype, sex, and doxycycline status of each sample was unknown during the procedure, so the analysis of each was not skewed

*Weaknesses of the study*

Other aspects of this study weakened the analysis and results. While an n=3 allows for statistical analysis, it is the lowest level that does. Additionally, samples and antibodies utilized went through repeated freeze/thaw cycles. This may have impacted the protein content and antibody binding ability. Further, the use of actin as a loading control is a less accurate method than a BCA protein assay. As a result, the relative amounts of OGT, OGA, and *O*-GlcNAc may be skewed.

*Future directions*

This study indicated that no difference exists between OGT, OGA, and *O*-GlcNAc levels in the hippocampus of OGA knockdown and wild type male and female mice on and off doxycycline. In the future, additional samples of each type should be analyzed to increase the n

value and add credibility to the conclusions. Furthermore, differences in subregions might be present but are diluted by combining CA3, CA1, CA2 and dentate gyrus as was done in this study. Additionally, other brain regions should be analyzed to determine if OGA and *O*-GlcNAc present differently between groups in regions other than the hippocampus. Future studies may also examine if OGA and *O*-GlcNAc levels are found to be different between mice on and off doxycycline after a longer period of time off of the medication.

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## Appendix A: List of Abbreviations

*O*-GlcNAc……..……*O*-linked β-*N*-acetylglucosamine

OGA……*O*-GlcNAcase

OGT……..…….*O*-GlcNAc transferase

GABA……..……...Gamma-aminobutyric acid

LTP……..…….….Long term potentiation

LTD……..…….…..Long term depression

NMDA……..……..N-methyl-D-aspartate

AMPA……..α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

cAMP……..…….Cyclic adenosine monophosphate

PFC……..……..….Prefrontal cortex

PKA……..…….…….Protein kinase A

mGluR……..…...…….Metabotropic glutamate receptor

Aβ-42……..……..…….….Amyloid-beta 42

UDP……..……..,…….….Uridine diphosphate

ATP……..……...…….….Adenosine triphosphate

CoA……..……...…….….Coenzyme A

TET……..……..Ten-eleven translocation protein

IGF……..……..Insulin-like growth factor

HBP……..……..….Hexosamine biosynthetic pathway

CamKII…….Calcium/calmodulin-dependent protein kinase II

TetR……..……...….Tet repressor protein

TetO……..…….….Tet operator

tA……..……...…….….Transcription activator

BME……..…….….Beta-mercaptoethanol

PVDF……..……..Polyvinylidene difluoride

TBST……..……..Tris-buffered saline with 1% Tween