Spring 2022

ELUCIDATING THE ROLE OF O-GLCNACYLATION AND GSK3B IN ALZHEIMER'S DISEASE

Emily Valencia
Southeastern University - Lakeland

ELUCIDATING THE ROLE OF *O*-GLCNACYLATION AND GSK3ß IN ALZHEIMER'S DISEASE

by

Emily Valencia

Submitted to the School of Honors Committee

in partial fulfillment

of the requirements for University Honors Scholars

Southeastern University

2022

Acknowledgement

First, I would like to thank Dr. Aimee Franklin for all of her support throughout my years at Southeastern University as my professor, advisor, and mentor. Thank you for sharing your wisdom about both academics and life. I truly appreciate you taking the time to pour into me – I always leave our conversations so filled. You are always challenging me, and this has resulted in so much growth. I cannot fully express the impact you have had on my life. Thank you for allowing me to be a part of your research team. I can honestly say that I learned so much more than just how to run a Western blot throughout this process. Thank you for your constant encouragement. I could not have completed this without you.

I would also like to thank my family and friends for their support. Thank you for listening to me when I needed to verbalize my thoughts, for comforting me during my breakdowns, and for encouraging me throughout the entire process. I would not be where I am – or who I am – without each of you. I love you all!

Finally, I would like to thank Dr. Miller, Professor Owen, and Professor Beatty for their guidance throughout the thesis process. Your support and understanding have been vital. Also, thank you for pushing me to excel in all areas of life and encouraging me along the way. I will forever cherish my memories from HLT, H3, and the many honors events.

Abstract

Alzheimer's disease (AD) is one of the most common types of neurodegenerative disease. There are currently no treatment methods for AD, but there are treatments for some of the symptoms. Aberrant levels of both *O*-GlcNAcylation and GSK3ß have been found in patients with AD. Studies have suggested that there is a relationship between the level of *O*-GlcNAcylation and the phosphorylation of GSK3ß. In order to discover new treatment methods, the phosphorylation of GSK3ß should be explored. This study uses a western blot analysis to quantify the ratio of p-GSK3ß:GSK3ß between the experimental group (higher levels of *O*-GlcNAcylation) and a control group. The preliminary results suggested that there was no significant difference in the phosphorylation of GSK3ß when the levels of *O*-GlcNAcylation are increased.

KEYWORDS: *O*-GlcNAcylation, GSK3ß, Alzheimer's disease, hippocampus, phosphorylation

Table of Contents

Introduction

Alzheimer's disease (AD) affects 11.3% of people older than 65.[1] AD primarily affects the hippocampus, the brain region that is involved in learning and memory.[2] The two hallmarks of AD are neurofibrillary tangles and neuritic plaques.[3] Although there are no effective treatment methods for AD, there are therapies to treat the symptoms.[4]

In order to develop more effective options for the treatment of AD, additional research is needed to understand the mechanism associated with the development of AD. There are aberrant levels of *O*-GlcNAcylation and GSK3β in AD. Also, recent studies have shown that *O*-GlcNAcylation and GSK3β are competitive inhibitors. Additional research should be conducted in order to elucidate the relationship between *O*-GlcNAcylation and GSK3β.

Researchers are using murine models to better understand AD and the role of *O*-GlcNAcylation. Due to the complete knockout of *O*-GlcNAcase (OGA) being embryonic lethal, OGA must be manipulated post the birth of the mouse. Therefore, researchers are using a Tet-Off system. This inhibits the transcription of OGA by the administration of doxycycline.

The purpose of this study is to evaluate the effects of the increased level of *O*-GlcNAcylation on the phosphorylation and transcription of GSK3β. The analysis of Western blots will be used to compare the levels of p-GSK3β and GSK3β between mice with differing levels of *O*-GlcNAcylation. Also, the total level of GSK3β compared to actin will be observed. This research is attempting to answer the following questions:

- Is the ratio of p-GSK3β:GSK3β significantly affected when the levels of *O*-GlcNAcylation are increased?
- Is the total level of GSK3β is significantly affected when the levels of *O*-GlcNAcylation are increased?

Understanding these answers will allow for a better explanation of the relationship between *O*-GlcNAcylation and GSK3β.

Review of Literature

Alzheimer's Disease

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases, along with Parkinson's disease and amyotrophic lateral sclerosis.[1] Neurodegeneration refers to the decline in neuronal function, which results from cell death.[5] In AD, the neurons within the hippocampus are particularly susceptible to damage. This decline in the function of neurons results in the impairment of memory, reasoning, language, and judgment.[6]

Dementia is a term used to describe the symptoms associated with a decline in cognitive ability.[7] AD is the most prevalent type of dementia, accounting for more than two-thirds of all dementia patients.[6] Dementia impedes a person's ability to perform independent, daily activities.[7]

Epidemiology

AD is prevalent in patients who are older than 65-years-old.[6] One of the most widespread risk factors for AD is an increase in age.[6] However, AD is not a normal aspect of aging.[6] Late-onset is the most common type of AD case.[8] Due to the age-dependent nature of the AD, it is becoming more prevalent partly due to the recent increase in the elderly population.[9] Other risk factors for AD include depression, higher parental age, family history of dementia, smoking, cardiovascular disease, diabetes, head trauma, and others.[6]

Hallmarks of AD are neurofibrillary tangles and neuritic plaques (Figure 1).[6] Neurofibrillary tangles are found within neurons.[6] In patients with AD, there is an increase in the phosphorylation of tau, which results in fibrillary intracytoplasmic structures.[6] They form insoluble paired helical filaments, which are referred to as neurofibrillary tangles.[6] These tangles

interfere with the normal function of the cells.[6] Neuritic plaques are found at axonal endings, and they are tiny lesions containing extracellular amyloid beta-peptides.[6] They are a result of the breakdown of amyloid precursor proteins, which is why they are also known as amyloid plaques.[10] Both neurofibrillary tangles and neuritic plaques lead to neuronal cell death.[6]

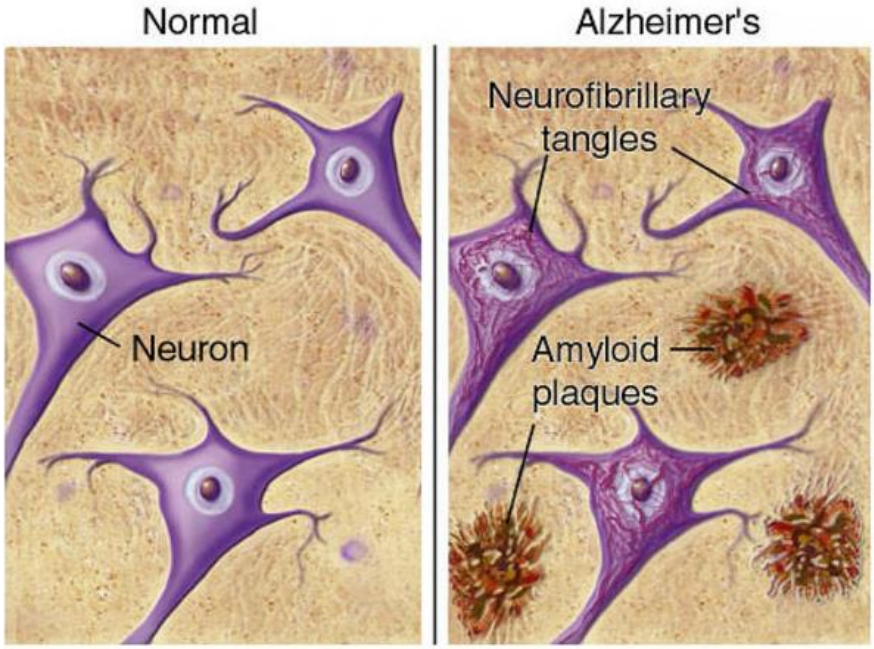


Figure 1 Neurofibrillary tangles and neuritic plaques[3]

Current Treatment Methods

In the United States, AD is the sixth leading cause of death.[6] More than 5.8 million Americans were suffering from AD in 2020, and this number is predicted to increase to 14 million Americans by 2060.[11] Although there are available treatments for some symptoms, there is currently no cure for AD.[6] The symptomatic treatments target the neurotransmitter imbalance, and they attempt to counteract it.[4] A few studies have suggested that preventative/therapeutic approaches would be beneficial for delaying the age at onset, but there have been no successful therapies discovered.[12]

Brain and Hippocampus

Central Nervous System

The nervous system is split into the central nervous system (CNS) and the peripheral nervous system (PNS).[13] The CNS is composed of the brain and spinal cord, and the other parts of the nervous system make up the PNS.[13] The CNS is a complex network that detects environmental stimuli and allows interaction between the organism and its surroundings.[13] The brain regulates the processes of the body, including movement, cognition, breathing, etc.[13] The brain is also responsible for memory, emotions, and communication.[14]

This study will focus on the brain, which is a part of the CNS. The brain is comprised of nervous tissue, which is very delicate.[14] Nervous tissue is composed of neurons and glial cells.[15] Although there are more glial cells than neurons, they take up an equal amount of space within nervous tissue. Glial cells function to support and protect neurons.[16] In the CNS, astrocytes and oligodendrocytes are the main types of glial cells.[15] Astrocytes surround the body of neurons.[16] Astrocytes assist in the migration of neurons and aid in detoxification.[16] Astrocytes also form the blood-brain barrier.[16] Additionally, astrocytes manage synaptic transmission.[16] The primary function of oligodendrocytes is to generate myelin sheath.[16] Myelin sheath surrounds nerves and acts as an insulator which allows for the fast and efficient transmission of electrical impulses.[16] Due to the importance of the brain, it is protected by the skull, meninges, and cerebrospinal fluid.[14] Also, the blood-brain barrier blocks harmful substances from entering the brain via the blood.[14]

Neurons

Neurons are comprised of a soma (cell body), dendrites, an axon, and an axon terminal.[17] The nucleus and other essential organelles are housed in the cell body.[17] The dendrites receive afferent signals from their environment and other neurons; they are considered the "ears" of the neuron.[17] Most neurons have multiple dendrites, which allows them to receive multiple signals simultaneously.[18] Neurons only have one axon.[18] The axon sends efferent signals to other neurons, and it is considered the "mouth" of the neuron.[17]

A neuron is a cell that can be electrically excited.[18] Neurons send signals all over the body.[18] Neurons interact with other neurons through the synaptic cleft, the space between the presynaptic neuron's axon terminal and the postsynaptic neuron's dendrite.[18] The information is transmitted through both electrical and chemical components.[18]

Once a dendrite is stimulated, an electrical signal is sent to the soma.[19] The soma contains the axon hillock, which controls neuronal firing.[19] If the combined signal strength is greater than -40 mV, the threshold limit, a signal will be fired.[19] This signal is known as an action potential.[19] The electrical signal is sent down the axon by a depolarizing current.[19]

The first stage of the action potential occurs when voltage-gated sodium channels (VGNaC) open and sodium ions rush into the cell.[20] The increase of positive ions leads to the depolarization of the cell, and this leads to a positive-feedback cycle of more VGNaC opening.[20] After 1 msec, the depolarization of the neuron ceases and the VGNaC become inactive.[20] The second stage that takes place is repolarization.[20] This occurs when voltage-gated potassium channels (VGKC) open and potassium ions flow out of the cell.[20] This allows for the cell to return to its resting membrane potential (RMP), -70 mV.[20] The final stage of the action potential is termed hyperpolarization.[20] This refers to the short period when the membrane potential is

lower than the RMP, due to the VGKC being open a little longer.[20] The action potential beings at the axon hillock, and it travels down to axon to the axon terminal.[20]

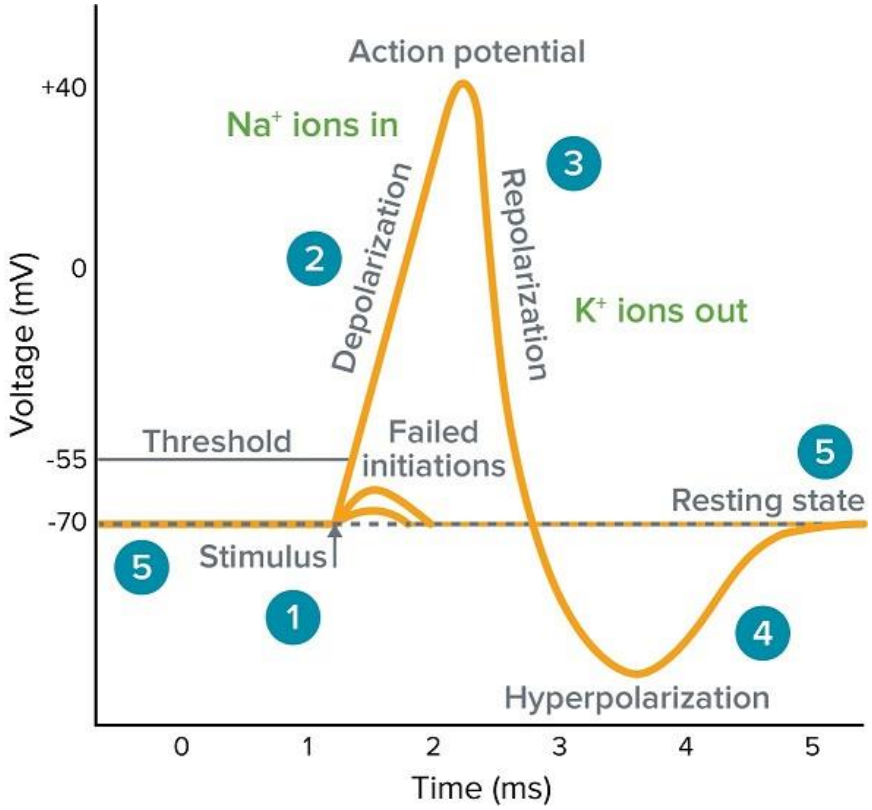


Figure 2 Representative graph of an action potential[21]

Once the action potential reaches the axon terminal, it triggers the opening of voltage-gated calcium channels.[22] This leads to an influx of the flow of calcium into the presynaptic neuron.[22] The increase of calcium causes the vesicles filled with neurotransmitters to dock on and fuse with the plasma membrane.[22] The neurotransmitter is then released into the synaptic cleft and binds to receptors on the postsynaptic neuron.[22] This produces a signal in the postsynaptic neuron.[22]

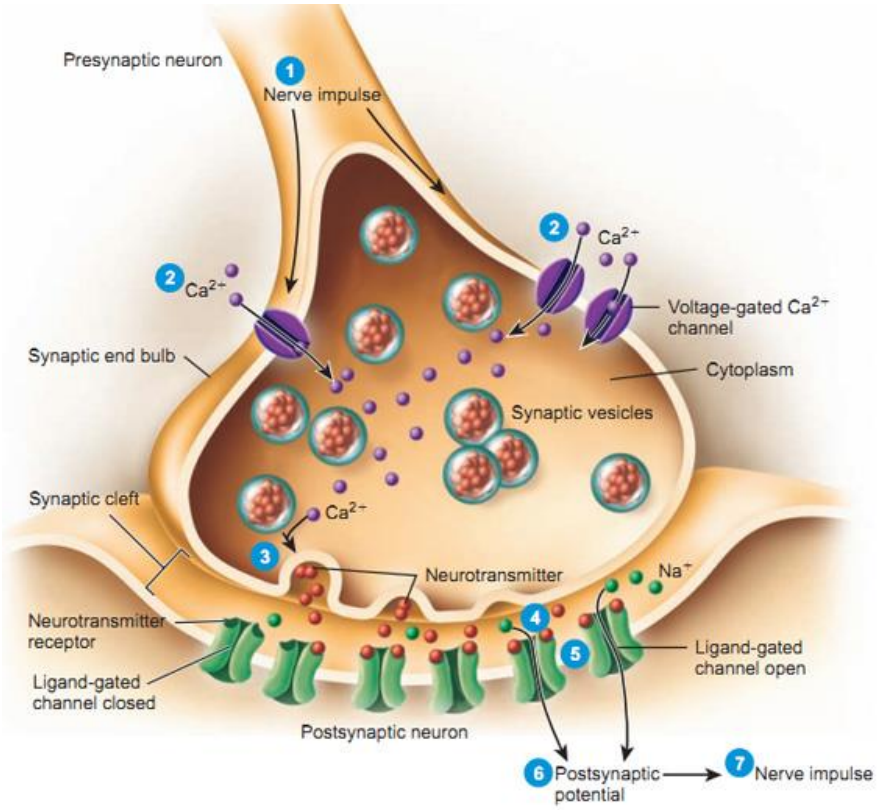


Figure 3 Communication between pre- and postsynaptic neurons[23]

Neurotransmitters can excite, inhibit, or modify the target tissue.[22] A few excitatory neurotransmitters are glutamate, acetylcholine, histamine, and norepinephrine.[22] These neurotransmitters cause the neuron to be depolarized, increasing the chance of propagating an action potential.[22] A couple of inhibitory neurotransmitters are gamma-aminobutyric acid (GABA) and serotonin.[22] These neurotransmitters cause the neuron to be hyperpolarized, decreasing the chance of firing an action potential.[22] Other types of neurotransmitters are neuromodulators and neurohormones.[22]

Synaptic Plasticity

Synaptic plasticity refers to the ability of the nervous system to adapt to stimuli.[24] Synaptic plasticity changes the way neurons communicate with other neurons, increasing or

decreasing synaptic strength.[25] This specifically relates to the hippocampus and the changes it undergoes during the process of learning.[24] Synaptic plasticity includes both the short- and long-term adaptations.[24] Short-term synaptic plasticity refers to a rapid change, occurring within milliseconds.[26] On the contrary, long-term potentiation (LTP) can persist for upwards of a couple of months.[27]

Long-term synaptic plasticity encompasses both LTP and long-term depression (LTD). LTP refers to the increase in the strength between the pre- and post-synaptic neurons.[25] Oftentimes, this is due to the activation of both the presynaptic and postsynaptic neurons within 100 ms.[28] The amount of neurotransmitter released and the number of postsynaptic receptors will increase.[29] This results in long-lasting elevated levels of communication between the two neurons, leading to changes in behavior and choices associated with memory.[25] LTD is the opposite of LTP. It results in a decrease in the communication between two neurons.[25]

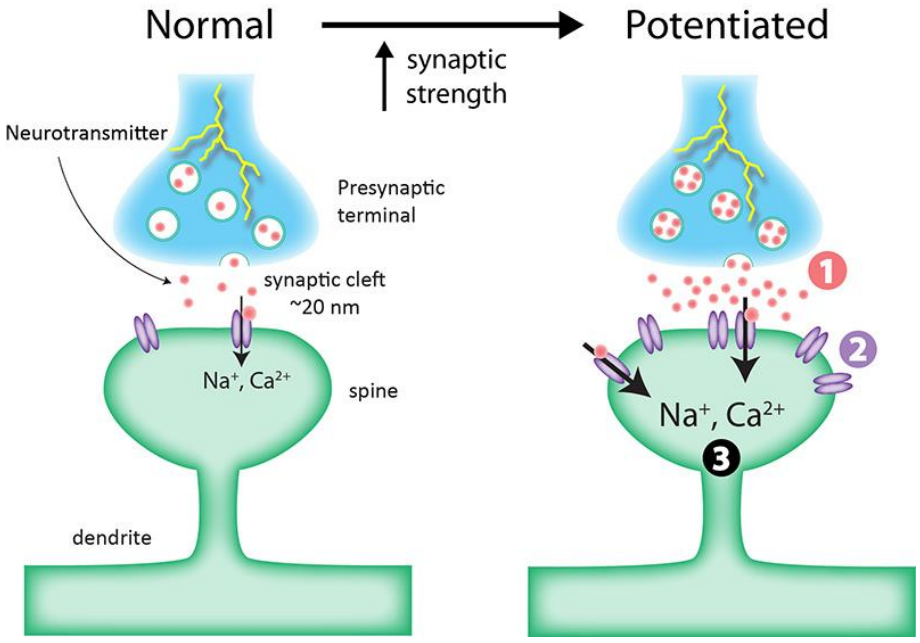


Figure 4 Synaptic plasticity (long-term potentiation)[30]

Hippocampus

The hippocampus is composed of "the dentate gyrus, the hippocampus proper, and the subiculum."[31] The hippocampus is located within the temporal lobe.[31] It is part of the limbic system, where behavior and emotion are regulated.[32] The hippocampus is also is involved with learning and memory.[13] Learning is composed of three stages: cognitive, associative, and autonomous. Memory is also comprised of three phases: registration, storage, and retrieval of information.[31] It is a plastic structure, meaning that it is affected by stimuli.[32]

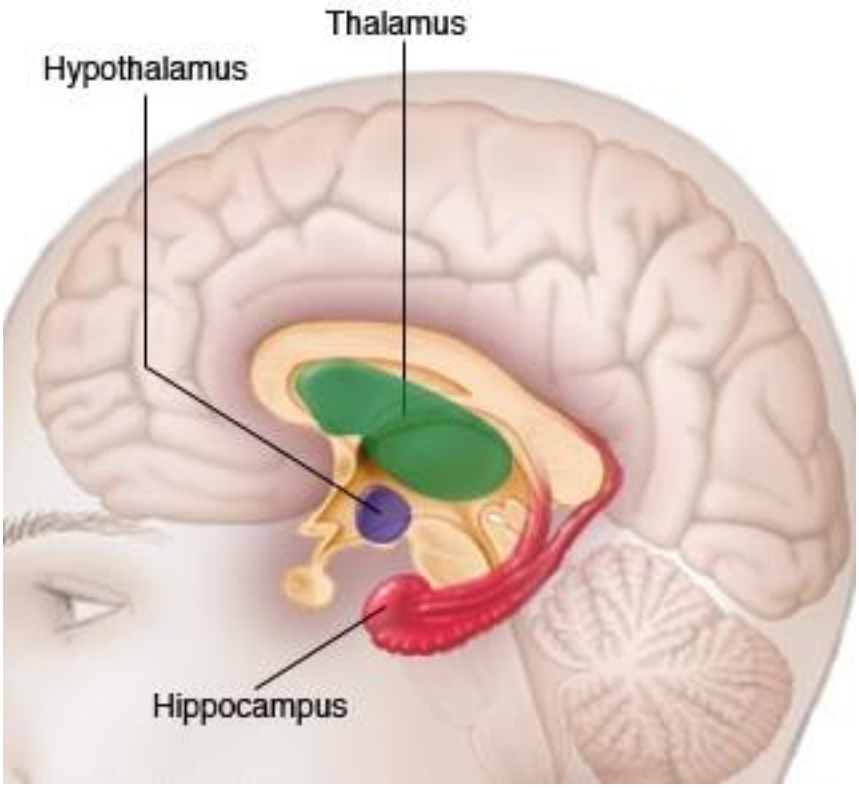


Figure 5 The hippocampus[33]

O-GlcNAcylation

Posttranslational Modifications

Proteins are essential to all biological processes.[34] Proteins are composed of a string of amino acids connected through peptide bonds.[34] This linear strand of monomer units makes up the primary structure of the protein.[34] The spontaneous folding of proteins into α-helices and β-sheets forms the secondary structure of the protein.[34] The interactions of the protein side chains promote further folding to form a 3-D structure, known as the tertiary structure.[34] The specific function of each protein is dictated by the functional groups – such as alcohols, esters, carboxylic acids – it contains.[34] The quaternary structure of the protein consists of multiple amino acid chains connected to each other.[34]

The central dogma of biology states that DNA is transcribed into mRNA which then is translated into a protein.[35] After the protein is formed, it may undergo post-translational modifications (PTM).[36] Over 200 different PTMs have been identified, and these changes allow for more variation in protein function.[36] Among these, phosphorylation, acetylation, carboxylation, methylation, and *O*-GlcNAcylation are just a few.[36] PTMs regulate enzyme activity, protein-protein interactions, and other processes.[36]

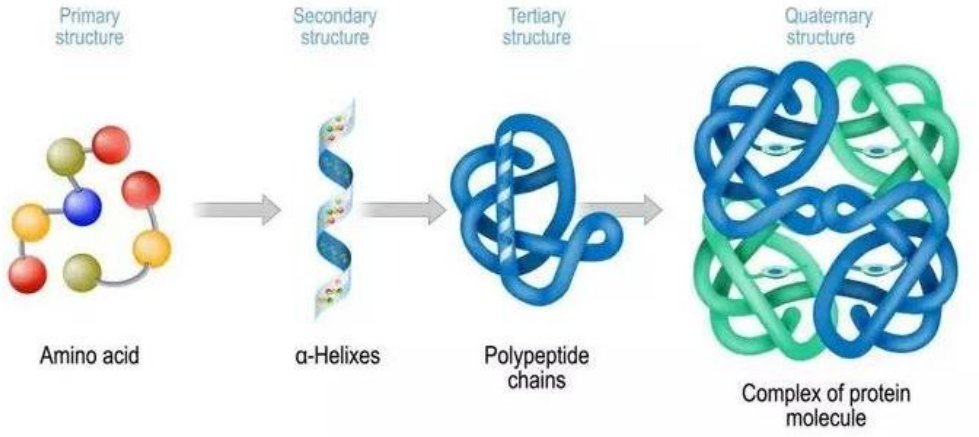


Figure 6 Levels of protein structure[37]

O-GlcNAcylation as a Posttranslational Modification

O-linked N-acetylglucosaminylation (*O*-GlcNAcylation) is a post-translational modification.[38] An N-acetylglucosamine (GlcNAc) is added to a Serine or Threonine residue on the protein of choice.[38] *O*-GlcNAcylation is regulated by two enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA).[38] OGT transfers a single GlcNAc to the protein of choice, while OGA reversibly removes GlcNAc.[38] *O*-GlcNAcylation regulates some essential processes in the cell.[38]

O-GlcNAcylation is key in epigenetics, regulating transcription, cell signaling, and enhancing cell survival.[38] Crucial transcription factors are *O*-GlcNAcylated to turn on the nuclear factors of T and B lymphocytes to become active.[38] *O*-GlcNAc is involved in gene expression due to the interactions with transcription regulators.[39] *O*-GlcNAcylation also plays a role in the binding of DNA, translocation, transactivation, and the regulation of transcription factors.[38]

The regulation of *O*-GlcNAcylation is due to a response to the availability of nutrients and the level of cellular stress.[38] A key nutrient in regulation is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the substrate that is donated in *O*-GlcNAcylation.[38] This molecule is produced by the hexosamine biosynthetic pathway.[38] When there is a sufficient supply of this substrate, *O*-GlcNAcylation proceeds.[38]

Enzymes Involved in O-GlcNAcylation

The regulation of *O*-GlcNAcylation is managed by one pair of enzymes: OGT and OGA.[38] OGT transfers a single GlcNAc, from UDP-GlcNAc, onto the target protein.[38] OGA works in opposition to OGT, removing a single GlcNAc from the protein.[38] These two enzymes work together to maintain homeostasis.[38]

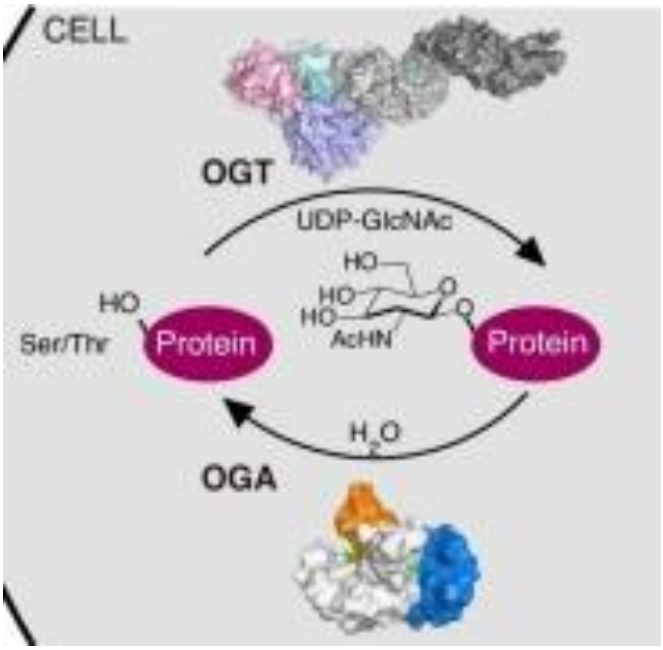


Figure 7 OGA/OGT-mediated *O*-GlcNAcylation[40]

OGT has three isoforms: nucleocytoplasmic (ncOGT), mitochondrial (mOGT), and short (sOGT).[38] In the cytoplasm, both ncOGT and sOGT are present.[38] In the mitochondria, mOGT is present.[38] Although they all contain a common catalytic carboxy-terminal (C-terminal) and intervening domain (ID), they vary in length.[41] The amino-terminal lobe of the catalytic domain plays a role in the binding cleft.[41] The function of the ID remains unknown.[41] The difference of the three isoforms is due to the fluctuation of the length of the amino-terminal (N-terminal) tetratricopeptide repeats (TPRs).[41] The recipient substrate is bound to OGT through interactions with the TPR domain.[41] The TPR domain mediates protein-protein interaction, and it is required for substrate recognition.[41]

OGA has two isoforms: nucleocytoplasmic (ncOGA) and short (sOGA).[38] ncOGA contains an *O*-GlcNAc hydrolase at its N-terminal and a histone acetyltransferase-like domain at its C-terminal.[38] sOGA only contains an *O*-GlcNAc hydrolase as its N-terminal, and it is present in the endoplasmic reticulum and lipid droplets.[38]

O-GlcNAcylation and Diseases

The dysregulation of *O*-GlcNAcylation is prevalent in numerous human diseases, including neurodegeneration, cardiovascular disease, cancer, and others.[42] Due to *O*-GlcNAcylation's influence on neuronal development and synaptic plasticity, Alzheimer's disease is associated with aberrant levels of *O*-GlcNAcylation.[42] *O*-GlcNAcylation acts as a protector by inhibiting necroptosis.[43] Therefore, a decrease in *O*-GlcNAcylation has been found in patients with AD.[43] Additionally, *O*-GlcNAc is present in both the protein Tau, which cause neurofibrillary tangles when hyperphosphorylated, and amyloid precursor proteins, which are found in neuritic plaques.[44]

The activity of OGT is upregulated in the heart versus other tissue.[45] *O*-GlcNAcylation contributes to "ischemic cardioprotection, hypertrophy, diabetic complications, hypertension, and heart failure."[45] As a result, improper levels of *O*-GlcNAc can have notable implications. Both an increase and a decrease in the levels of *O*-GlcNAc were shown to be associated with heart failure.[45]

Cell division and metabolism are regulated by *O*-GlcNAcylation.[42] Therefore, abnormal levels of *O*-GlcNAcylation are prevalent in tumors and cancer cell lines due to its effects on tumorigenesis and tumor metastasis.[42] This is due to a decrease in the levels of *O*-GlcNAc as a result of an increase in the enzymatic activity of OGA.[42]

GSK3

Kinases

Protein kinases add a phosphate group to specific amino acids within a substrate.[46] These enzymes play a regulatory role in cells and modify protein function.[46] The addition of the phosphate changes the protein's conformation by modifying the protein to a hydrophilic polar state, rather than the original hydrophobic apolar state.[47] This can either increase or decrease the activity of the target enzyme, and it changes the protein's interaction with other molecules.[46] Adenosine triphosphate (ATP) is used by the kinases to phosphorylate other proteins through covalent bonding.[48] This process results in a phosphorylated substrate and an ADP molecule.[48]

Phosphorylation is involved in the regulation of cell division and growth, protein synthesis, signal transduction, and other important cellular processes.[48] Phosphorylation is a reversible process with the protein phosphatase conducting dephosphorylation.[47] The dysregulation of phosphorylation leads to several diseases, including tumors.[47] Therefore, this process is highly regulated within the body. Most kinases are activated through phosphorylation.[48] Phosphorylation is site-specific, and it often occurs on serine and threonine residues.[48] Also, the kinase must be localized to the target substrate for phosphorylation to proceed.[48]

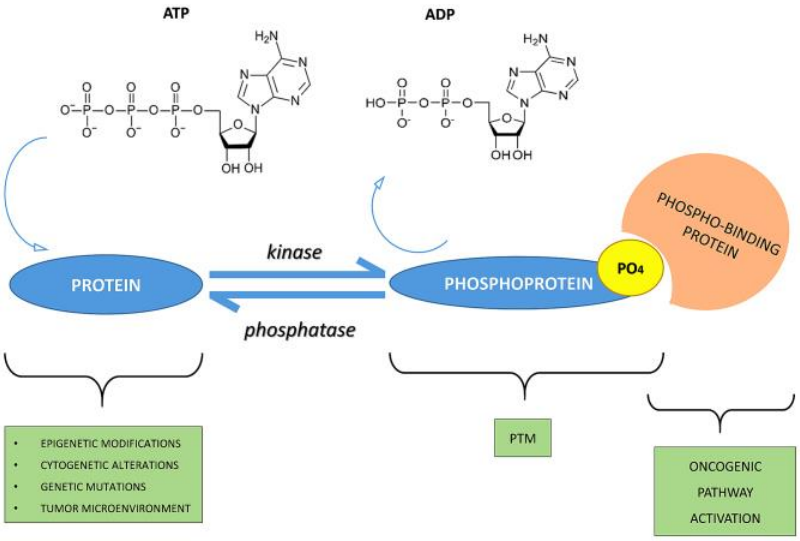


Figure 8 Mechanism of phosphorylation and dephosphorylation

GSK3 as a Kinase

Glycogen synthase kinase-3 (GSK3) phosphorylates over 100 substrates.[49] GSK3 contains two isoforms: GSK3α and GSK3β.[49] GSK3 contains two main domains: "a primed-substrate binding domain" and "a kinase domain" to phosphorylate the bound substrate.[49] Unlike most kinases, GSK3 is innately active, and it is inhibited most frequently by serine-phosphorylation.[49]

GSK3 is controlled through the addition of phosphate and by substrate availability, and it is also regulated by substrate recognition. [49] The majority of GSK3 substrates must be primed through pre-phosphorylation.[49] Along with being primed, GSK3 substrates must be co-localized with GSK3.[49] The coordination of these processes and the signaling determines if and when the substrate will be phosphorylated by GSK.[49] This specificity limits the activity of GSK3.

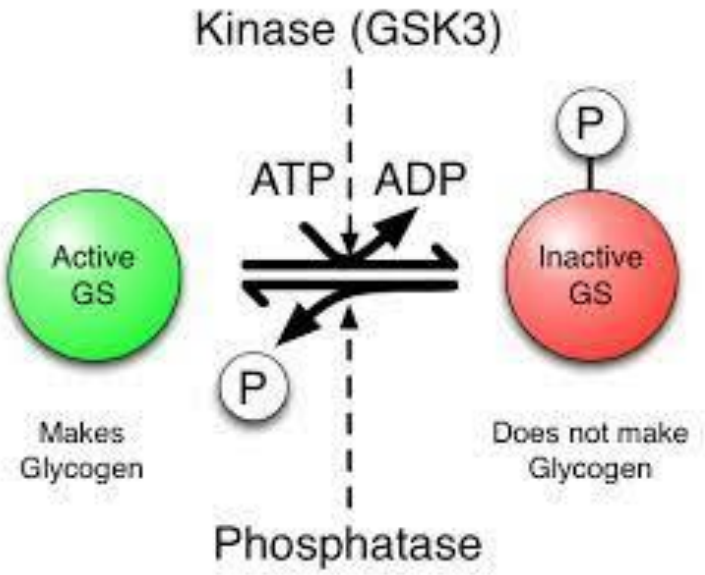


Figure 9 GSK3 activity[50]

GSK3 and Diseases

GSK3 is correlated with neurological diseases, including psychiatric illnesses.[49] GSK3 is also associated with neurodegenerative diseases, including Alzheimer's disease.[49] It causes neurofibrillary tangles, a hallmark of Alzheimer's disease.[49] GSK3 contributes to the development of the neurofibrillary tangles because it is one of the kinases that is involved in the hyperphosphorylation of the protein tau.[51]

The Relationship between *O*-GlcNAcylation and GSK3

O-GlcNAc and kinases are shown to be competitive inhibitors for residues with serine and threonine acceptor regions.[39] This is because a few of the *O*-GlcNAc sites are also the sites of phosphorylation.[52] This was suggested by the increase in the level of *O*-GlcNAcylation due to the inhibition of GSK3.[52] Furthermore, OGT is regulated by GSK3β.[53] *O*-phosphorylation is regulated in a site-specific manner by *O*-GlcNAcylation.[52] Also, it is hypothesized that if a GlcNAc group is added to GSK3, it may change the protein's conformation, leading to a decrease in the phosphorylation of GSK3.[54]

Due to the competitive inhibition findings, there may be a link between an increase of *O*-GlcNAcylation and the decrease of p-GSK3. Due to GSK3 being inherently on, a decrease in p-GSK3 may lead to an increase in the activity of GSK3. This may allow for more phosphorylation of the downstream targets of GSK3.

Methodology

Genetic Model

Purpose

Gene function and gene therapy can be studied through inducible transgene expression systems.[55] The Tet-On and Tet-Off systems use tetracycline or doxycycline, its derivative, to control gene expression.[55] A few, amongst several, advantages of the tetracycline-controllable expression system are accurate on/off regulation, specificity, and lack of cellular pathway interference.[55] The Tet-Off system uses a negative control of transgene expression while the Tet-On system uses a positive control.[55]

This experiment used the Tet-Off system to regulate the transcription of OGA. OGA was turned off by silencing its original gene and inserting a gene that can be externally manipulated. The Tet-Off system used in this model resulted in the silencing of the gene used to transcribe OGA through the administration of doxycycline. Therefore, the synthesis of OGA did not occur. Due to OGA's function of removing GlcNAc from proteins, the lack of performance should lead to an increase in GlcNAc.

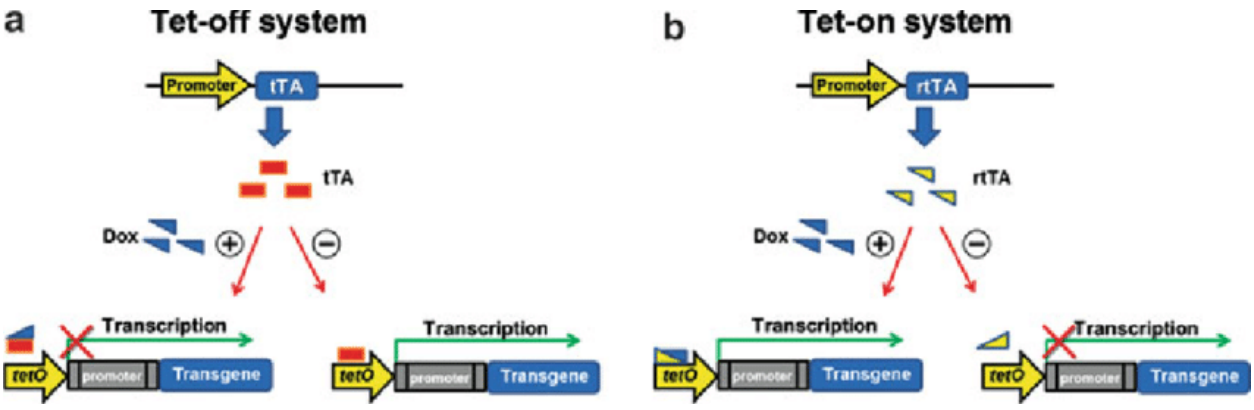


Figure 10 Tet-Off/Tet-On doxycycline system and gene transcription [56]

Table 1 Male Mice On/Off Doxycycline

Mouse ID	On/Off Doxycycline
971	ON
973	ON
974	OFF
977	OFF
989	OFF
1008	ON
1011	ON
1022	ON
1024	OFF
1026	OFF
1027	OFF
1038	ON
1044	OFF
1056	ON
1009	ON
1010	OFF
1023	ON
1025	OFF
1030	ON
1043	OFF
1045	OFF
1058	ON
1063	OFF

Table 2 Female Mice On/Off Doxycycline

Mouse ID	On/Off Doxycycline
564	ON
969	ON
996	ON
1002	OFF
1003	OFF
1004	ON
1017	OFF
1018	OFF
1021	ON
1032	OFF
1033	ON
1034	OFF
1036	ON
1040	ON
1041	ON
1042	OFF
1048	OFF
1051	OFF
1062	ON
1069	OFF

Obtaining the Samples

The mice used in this study were identified in multiple ways. First, they were given a number that correlated to its cage. Due to there being multiple mice in each cage, the ears of the mice were also tagged in order to identify the mouse of interest from the other mice. An isoflurane chamber was used to make the mouse unconscious. After this occurred, a guillotine was used to sacrifice the mouse, and then the brain of the mouse underwent dissection over dry ice. The cerebellum, frontal cortex, hippocampus, and excess tissue were placed in their corresponding labeled test tubes. The test tubes were placed in liquid nitrogen for several hours until they were transported for storage in the -80°C freezer.

Homogenizing the Samples

The samples were homogenized, and then they were stored at -20°C. Briefly, a mixture of protease inhibitor and phosphatase inhibitor at a 1:100 ratio to tissue protein extraction reagent was used to make the lysis buffer. A homogenizer was used to homogenize the samples. The samples were then put on ice for 30 minutes, and then they were centrifuged at 4°C for 15 minutes at 3,200 revolutions per minute. The supernatant was stored at -20°C after the pellets were discarded.

Western Blot

Purpose

The Western Blot technique is used to identify particular proteins within a multitude of proteins derived from cells.[57] Once identified, the concentration of the proteins within a particular tissue sample can be determined.[57] There are three steps in the Western Blot process. First, gel

electrophoresis is used to separate the proteins by size.[57] Second, the proteins are transferred onto a polyvinylidene fluoride (PDVF) paper.[57] Lastly, the PDVF paper, which now contains the proteins, goes through a series of washes.[57] A primary and secondary antibody are used to bind to the target protein.[57] The primary antibody first binds to the epitope of the protein.[58] The proteins of interest for this experiment are p-GSK3β, GSK3β, and *O*-GlcNAc. The secondary antibody is designed to bind to the primary antibody.[58] The secondary antibody contains an enzyme that produces light.[58] The amount of enzyme present causes for differing amounts of light, and this production of light can be quantified to determine the relative concentrations of the target proteins in a given sample.[58]

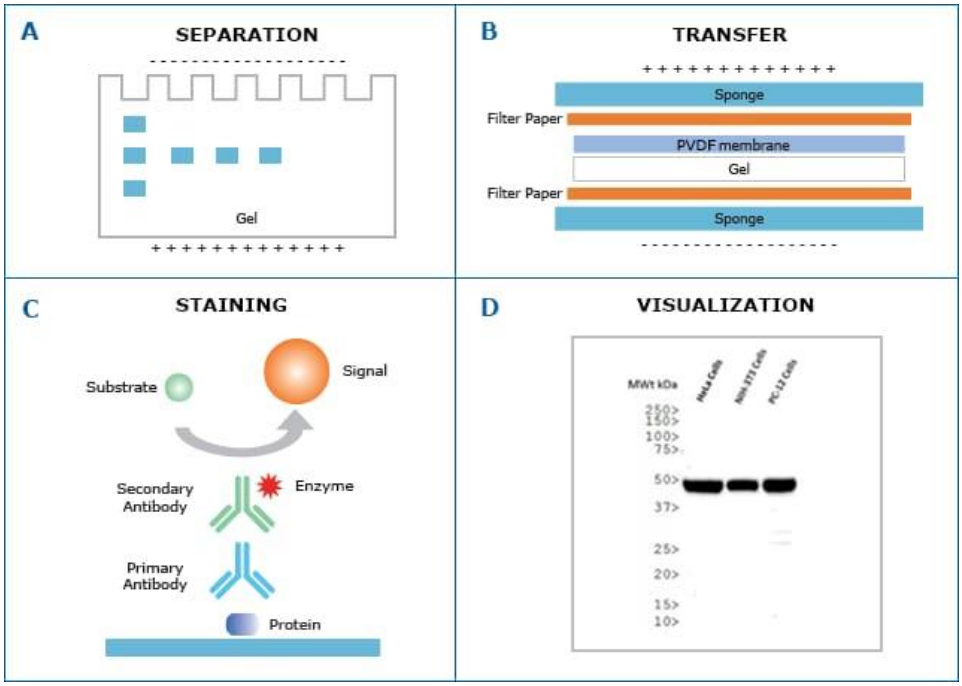


Figure 11 Visualization of the Western Blot protocol[59]

Procedure

Specific Aim I

To determine whether the ratio of p-GSK3β:GSK3β is significantly different between the control group compared to the experimental group.

Tissue Samples Used

Mouse ID	On/Off Doxycycline	Genotype
971	ON	CAMK tTA+TREOGNgk +DOX
973	ON	WT+ DOX
974	OFF	WT
977	OFF	WT
989	OFF	CAMK tTA+TREOGNgk
1008	ON	WT+ DOX
1011	ON	CAMK tTA+TREOGNgk +DOX
1022	ON	CAMK tTA+TREOGNgk +DOX
1024	OFF	CAMK tTA+TREOGNgk__
1026	OFF	CAMK tTA+TREOGNgk__
1027	OFF	WT__
1038	ON	WT +DOX
1044	OFF	CAMK tTA+TREOGNgk__
1056	ON	CAMK tTA+TREOGNgk +DOX

Table 3 Tissue samples utilized with genotype listed

Western Blot Protocol (p-GSK3β)

Preparing Samples: 2 µL of homogenate, 3.3 µL of sample buffer, and 14.7 µL of deionized water were added to each microcentrifuge tube per tissue sample. The tube was then vortexed and heated for 10 minutes at 70°C.

Running the Gel: A gel of 7.5% acrylamide was made. 2 µL of standard ladder was added to the leftmost well, and 20 µL of samples were added to the remaining wells. The gel was run in running buffer for 40 minutes at 200 V.

Transfer: PVDF paper was washed in methanol for 10 seconds, and then it was soaked in transfer buffer. Then, a "sandwich" was made in the order: sponge, filter paper, PVDF paper, gel, filter paper, sponge. This was put into a chamber containing a stir bar and ice pack. The transfer ran for one hour at 100 V in the cold room (4°C).

Wash 1: The blot was washed for 5 minutes in TBS-T.

Milk Block: The blot was blocked for 60 minutes in 5% milk with agitation while at room temperature.

Wash 2: The blot was washed for 15 minutes in TBS-T three times.

Primary Antibody: The primary antibody solution was prepared [1:5000] using 1 µL of anti-p-GSK3β and 5 mL of 5% milk. The solution was added to the blot, and it was blocked overnight with agitation in the cold room (4°C).

Wash 3: The blot was washed for 15 minutes in TBS-T four times.

Secondary Antibody: The secondary antibody solution was prepared [1:5000] using 1 µL of anti-rabbit and 5 mL of 5% milk. The solution was added to the blot, and it was blocked for one hour with agitation at room temperature.

Wash 4: The blot was washed for 15 minutes in TBS-T three times.

Developing Blot: The blot was developed for 5 minutes in a 1:1 ECL solution. Bio-Rad Chemiluminescence Doc Imager was used to view the blot. The exposure was adjusted as needed.

Western Blot Protocol (GSK3β)

After p-GSK3β was detected, the PVDF blot was stripped with stripping buffer for 5 minutes. The Western Blot Protocol was then followed starting at Wash 1. For primary antibody, the solution was prepared [1:8000] using 1 µL of anti-GSK3β and 8 mL of 5% milk. For secondary antibody, the solution was prepared [1:5000] using 1 µL of anti-rabbit and 5 mL of 5% milk.

Image Lab

Image Lab was used to determine the relative amount of p-GSK3β between the experimental and the control group.

Specific Aim II

To determine whether the total level of GSK3β is significantly different between the control group compared to the experimental group.

Western Blot Protocol (O-GlcNAc)

After GSK3β was detected, the PVDF blot was stripped with stripping buffer for 10 minutes. The Western Blot Protocol was then followed starting at Wash 1. For primary antibody, the solution was prepared [1:2000] using 1.5 µL of anti-*O*-GlcNAc and 3 mL of 5% milk. For secondary antibody, the solution was prepared [1:5000] using 1 µL of anti-mouse and 5 mL of 5% milk.

Western Blot Protocol (Actin)

After GSK3β was detected, the PVDF blot was stripped with stripping buffer for 10 minutes. The Western Blot Protocol was then followed starting at Wash 1. For primary antibody, the solution was prepared [1:5000] using 1 µL of anti-*O*-GlcNAc and 5 mL of 5% milk. For secondary antibody, the solution was prepared [1:5000] using 1 µL of anti-mouse and 5 mL of 5% milk.

Image Lab

Image Lab was used to determine the relative amount of GSK3β between the experimental group and the control group.

Data Analysis

Specific Aim I

For the first specific aim, I compared the ratio of p-GSK3β:GSK3β between the control group, off Doxy, and the experimental group, on Doxy. Figure 12 displays the levels of p-GSK3β and GSK3β for Western Blot 1. Table 3 explains the genetic makeup of the mice that were used in this study. The top band is composed of p-GSK3β (molecular weight 47 kD), and the bottom band is composed of GSK3β (molecular weight of 47 kD).

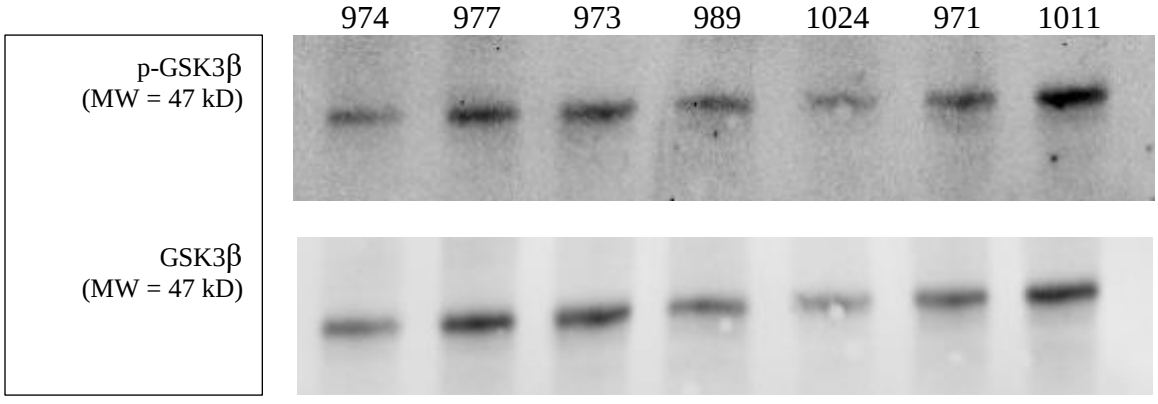
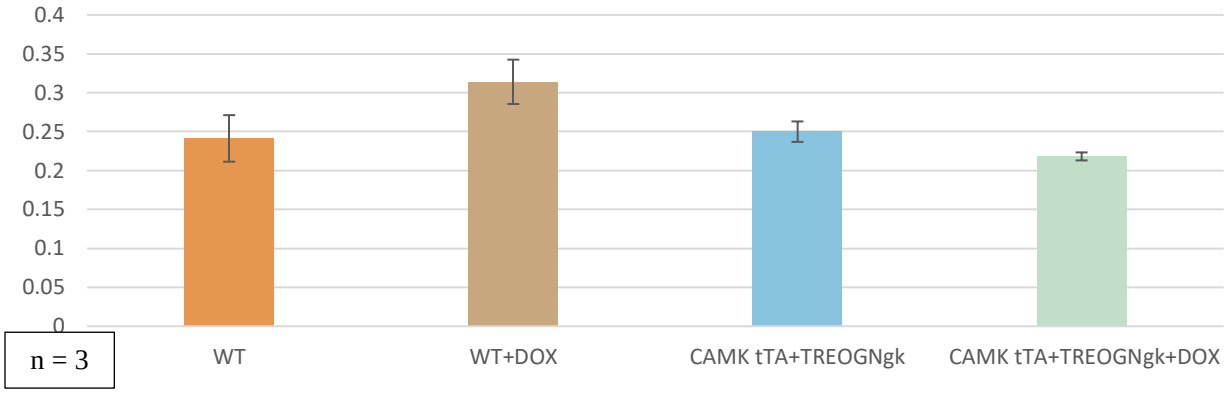


Figure 12 Western Blot analysis of Blot 1 results

Image Lab was used to calculate the amount of protein in each band. Once this information was collected, the amount of p-GSK3β in each band was compared to the amount of GSK3β to determine the ratio of p-GSK3β:GSK3β. The genetic makeup of each mouse was taken into consideration when the comparison was being conducted. The four groups of genetic makeup are: wild type (WT), wild type on doxycycline (WT + DOX), CAMK tTA+TREOGNgk +DOX (OGA KD + DOX), and CAMK tTA+TREOGNgk (OGA KD).



Graph 1 Ratio of p-GSK3β:GSK3β

Due to the evaluation of only three successful Western Blots in this study (n=3), this research is only considered preliminary data. This was not enough data to provide statistical analysis.

It was expected that the ratio of p-GSK3β:GSK3β would be significantly lower in the experimental group compared to the control group. However, the data collected for specific aim I showed that there was no significant difference for the levels for p-GSK3β compared to GSK3β.

Specific Aim 2

For the second specific aim, I compared the amount of GSK3β relative to actin between the control group, off Doxy, and the experimental group, on Doxy. Figure 13 displays the levels of GSK3β and actin for Western Blot 10. As suggested before, one should refer to Table 3 to

understand the genetic makeup of the mice that were used in this study. The top band is composed of GSK3β (molecular weight 47 kD), and the bottom band is composed of actin (molecular weight of 42 kD).

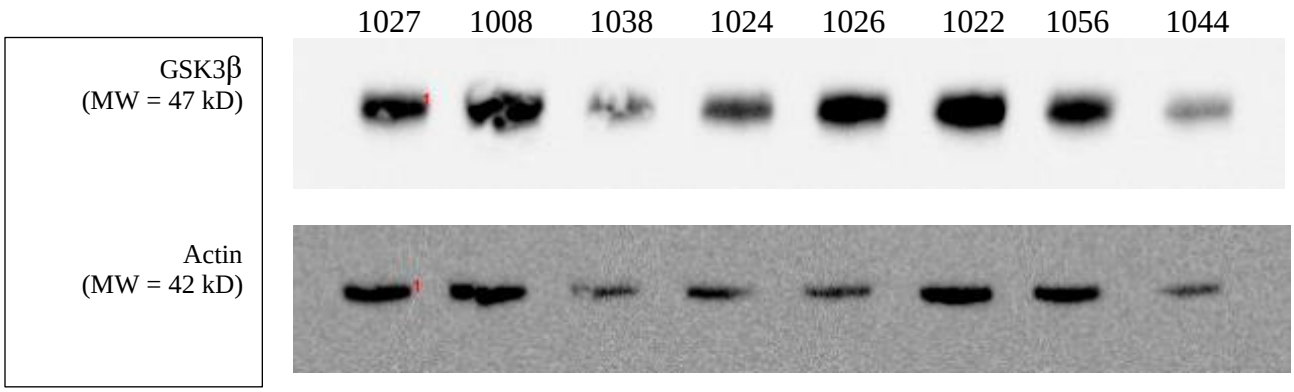
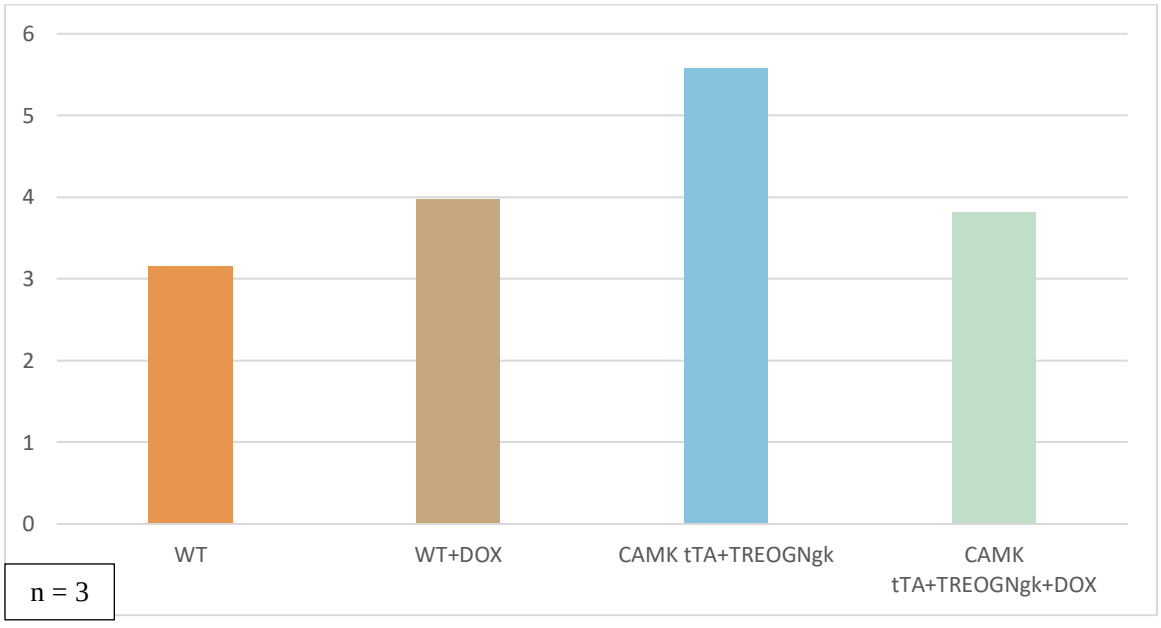


Figure 13 Western Blot analysis of Blot 10 results

Image Lab was used to calculate the amount of protein in each band. Once this information was collected, the amount of GSK3β in each band was compared to the amount of actin to determine the relative amount of GSK3β. Again, the genetic makeup of each mouse was taken into consideration when the comparison was being conducted.



Graph 2 Ratio of GSK3β:Actin

This research is only considered preliminary data due to the evaluation of only three successful Western Blots in this study (n=3). Additionally, this was not enough data to provide a statistical analysis.

It was expected that there is no significant difference of the total level of GSK3β between the control group compared to the experimental group. The data I collected suggested that my hypothesis was correct. Therefore, it would indicate that the level of *O*-GlcNAc does not affect the synthesis of GSK3β.

Conclusion

Based on the research I gathered, the trends suggest that the levels of *O*-GlcNAcylation do not affect the phosphorylation of GSK3β. Additionally, it also appeared that the synthesis of GSK3β was not affected by the levels of *O*-GlcNAcylation. In order to provide a statistical analysis, additional data would need to be collected. Although this preliminary data is significant because indicates that *O*-GlcNAcylation does not have a direct effect on GSK3β in the hippocampus, it would be beneficial for future studies to observe the effects on GSK3β's activity assay.

Research Limitations

My research had several limitations. The most critical drawback was the insufficient number of Western blots performed. I was only able to conduct three successful pairs of Western blots (in order to collect data for all of my samples, I needed to run two Western blots). This resulted from the time it took for me to discover the correct concentrations of primary and secondary antibodies that would result in a successful Western blot. I had many failed attempts

due to incorrect concentrations. Statistical analysis was not able to be conducted due to the sample size of three. Also, this study only utilized fourteen different tissues samples. The inadequate sample size inhibits the results to be generalized.

Future Studies

Future studies should use a greater number of samples when conducting this same experiment. This would allow for the findings to be more precise and the conclusions made from this study would be supported or disproved by the supplementary data. Additionally, adding female samples to the study to establish whether there are significant differences between males and females in this study, specifically in respect to the Tet-Off system. The level of phosphorylation of GSK3β substrates between the experimental group and the control group should be studied. Although the potential activity of GSK3β within the hippocampus was not affected, it would be beneficial to study the competitive inhibition between GSK3β and *O*-GlcNAc within the hippocampus to determine if the activity assay of GSK3β is affected by the levels of *O*-GlcNAc. Also, future studies should observe the relationship between *O*-GlcNAcylation and the phosphorylation of GSK3β, as well as the activity assay of GSK3β, in other regions of the brain.

References

1. Facts and Figures. Alzheimer's Disease and Dementia. Accessed January 9, 2022. https://www.alz.org/alzheimers-dementia/facts-figures

2. Mu Y, Gage FH. Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Mol Neurodegener*. 2011;6:85. doi:10.1186/1750-1326-6-85

3. Amyloid Plaques and Neurofibrillary Tangles | BrightFocus Foundation. Accessed October 29, 2021. https://www.brightfocus.org/alzheimers-disease/infographic/amyloid-plaques-and-neurofibrillary-tangles

4. Yiannopoulou KG, Papageorgiou SG. Current and Future Treatments in Alzheimer Disease: An Update. *J Cent Nerv Syst Dis*. 2020;12:1179573520907397. doi:10.1177/1179573520907397

5. Checkoway H, Lundin JI, Kelada SN. Neurodegenerative diseases. *IARC Sci Publ*. 2011;(163):407-419.

6. Kumar A, Sidhu J, Goyal A, Tsao JW. Alzheimer Disease. In: *StatPearls*. StatPearls Publishing; 2021. Accessed October 29, 2021. http://www.ncbi.nlm.nih.gov/books/NBK499922/

7. Gale SA, Acar D, Daffner KR. Dementia. *Am J Med*. 2018;131(10):1161-1169. doi:10.1016/j.amjmed.2018.01.022

8. Guerreiro R, Bras J. The age factor in Alzheimer's disease. *Genome Med*. 2015;7:106. doi:10.1186/s13073-015-0232-5

9. Gitler AD, Dhillon P, Shorter J. Neurodegenerative disease: models, mechanisms, and a new hope. *Dis Model Mech*. 2017;10(5):499-502. doi:10.1242/dmm.030205

10. The neuritic plaque in Alzheimer's disease: perivascular degeneration of neuronal processes - PubMed. Accessed December 7, 2021. https://pubmed.ncbi.nlm.nih.gov/31437721/

11. What is Alzheimer's Disease? | CDC. Published April 7, 2021. Accessed January 1, 2022. https://www.cdc.gov/aging/aginginfo/alzheimers.htm

12. Association A. 2015 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2015;11(3):332-384. doi:10.1016/j.jalz.2015.02.003

13. Ludwig PE, Reddy V, Varacallo M. Neuroanatomy, Central Nervous System (CNS). In: *StatPearls*. StatPearls Publishing; 2021. Accessed April 30, 2021. http://www.ncbi.nlm.nih.gov/books/NBK442010/

14. Thau L, Reddy V, Singh P. Anatomy, Central Nervous System. In: *StatPearls*. StatPearls Publishing; 2021. Accessed September 20, 2021. http://www.ncbi.nlm.nih.gov/books/NBK542179/

15. Jessen KR. Glial cells. *Int J Biochem Cell Biol*. 2004;36(10):1861-1867. doi:10.1016/j.biocel.2004.02.023

16. Jäkel S, Dimou L. Glial cells and their function in the adult brain: a journey through the history of their ablation. *Front Cell Neurosci*. 2017;11:24.

17. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Overview of Neuron Structure and Function. *Mol Cell Biol 4th Ed*. Published online 2000. Accessed April 30, 2021. https://www.ncbi.nlm.nih.gov/books/NBK21535/

18. Ludwig PE, Reddy V, Varacallo M. *Neuroanatomy, Neurons*. StatPearls Publishing; 2021. Accessed January 4, 2022. https://www.ncbi.nlm.nih.gov/books/NBK441977/

19. Neuronal communication. *Nat Struct Mol Biol*. 2019;26(7):527-527. doi:10.1038/s41594-019-0265-3

20. Grider MH, Jessu R, Kabir R. *Physiology, Action Potential*. StatPearls Publishing; 2021. Accessed January 4, 2022. https://www.ncbi.nlm.nih.gov/books/NBK538143/

21. What is an action potential? Molecular Devices. Accessed January 4, 2022. https://www.moleculardevices.com/applications/patch-clamp-electrophysiology/what-action-potential

22. Sheffler ZM, Reddy V, Pillarisetty LS. Physiology, Neurotransmitters. In: *StatPearls*. StatPearls Publishing; 2021. Accessed January 4, 2022. http://www.ncbi.nlm.nih.gov/books/NBK539894/

23. Upload 9 06 Nerve Impulse Notes to e. Accessed January 4, 2022. https://slidetodoc.com/upload-9-06-nerve-impulse-notes-to-e/

24. Mateos-Aparicio P, Rodríguez-Moreno A. The Impact of Studying Brain Plasticity. *Front Cell Neurosci*. 2019;13:66. doi:10.3389/fncel.2019.00066

25. Stuchlik A. Dynamic learning and memory, synaptic plasticity and neurogenesis: an update. *Front Behav Neurosci*. 2014;8:106. doi:10.3389/fnbeh.2014.00106

26. Ohno T, Hasegawa T, Tsuruoka T, Terabe K, Gimzewski JK, Aono M. Short-term plasticity and long-term potentiation mimicked in single inorganic synapses. *Nat Mater*. 2011;10(8):591-595. doi:10.1038/nmat3054

27. Nicoll RA. A Brief History of Long-Term Potentiation. *Neuron*. 2017;93(2):281-290. doi:10.1016/j.neuron.2016.12.015

28. Purves D, Augustine GJ, Fitzpatrick D, et al. Long-Term Synaptic Potentiation. *Neurosci 2nd Ed*. Published online 2001. Accessed January 10, 2022. https://www.ncbi.nlm.nih.gov/books/NBK10878/

29. Yang Y, Calakos N. Presynaptic long-term plasticity. *Front Synaptic Neurosci*. 2013;5:8. doi:10.3389/fnsyn.2013.00008

30. Long-term synaptic plasticity. Published November 22, 2016. Accessed December 7, 2021. https://qbi.uq.edu.au/brain-basics/brain/brain-physiology/long-term-synaptic-plasticity

31. Fogwe LA, Reddy V, Mesfin FB. Neuroanatomy, Hippocampus. In: *StatPearls*. StatPearls Publishing; 2021. Accessed April 30, 2021. http://www.ncbi.nlm.nih.gov/books/NBK482171/

32. Anand KS, Dhikav V. Hippocampus in health and disease: An overview. *Ann Indian Acad Neurol*. 2012;15(4):239-246. doi:10.4103/0972-2327.104323

33. Slide show: How your brain works. Mayo Clinic. Accessed January 12, 2022. https://www.mayoclinic.org/brain/sls-20077047

34. Berg JM, Tymoczko JL, Stryer L. Protein Structure and Function. *Biochem 5th Ed*. Published online 2002. Accessed April 29, 2021. https://www.ncbi.nlm.nih.gov/books/NBK21177/

35. Hoerter JE, Ellis SR. Biochemistry, Protein Synthesis. In: *StatPearls*. StatPearls Publishing; 2021. Accessed April 29, 2021. http://www.ncbi.nlm.nih.gov/books/NBK545161/

36. Duan G, Walther D. The Roles of Post-translational Modifications in the Context of Protein Interaction Networks. Radivojac P, ed. *PLOS Comput Biol*. 2015;11(2):e1004049. doi:10.1371/journal.pcbi.1004049

37. Figure 4: Primary, Secondary, Tertiary and Quaternary structure of protein. ResearchGate. Accessed January 4, 2022. https://www.researchgate.net/figure/Primary-Secondary-Tertiary-and-Quaternary-structure-of-protein_fig2_340436783

38. Yang X, Qian K. Protein O-GlcNAcylation: emerging mechanisms and functions. *Nat Rev Mol Cell Biol*. 2017;18(7):452-465. doi:10.1038/nrm.2017.22

39. Lewis BA, Hanover JA. O-GlcNAc and the Epigenetic Regulation of Gene Expression. *J Biol Chem*. 2014;289(50):34440-34448. doi:10.1074/jbc.R114.595439

40. Joiner CM, Li H, Jiang J, Walker S. Structural characterization of the O-GlcNAc cycling enzymes: insights into substrate recognition and catalytic mechanisms. *Curr Opin Struct Biol*. 2019;56:97-106. doi:10.1016/j.sbi.2018.12.003

41. Pathak S, Alonso J, Schimpl M, et al. The active site of O-GlcNAc transferase imposes constraints on substrate sequence. *Nat Struct Mol Biol*. 2015;22(9):744-750. doi:10.1038/nsmb.3063

42. Yang YR, Suh PG. O-GlcNAcylation in cellular functions and human diseases. *Adv Biol Regul*. 2014;54:68-73. doi:10.1016/j.jbior.2013.09.007

43. Park J, Ha HJ, Chung ES, et al. O-GlcNAcylation ameliorates the pathological manifestations of Alzheimer's disease by inhibiting necroptosis. *Sci Adv*. 7(3):eabd3207. doi:10.1126/sciadv.abd3207

44. Zhu Y, Shan X, Yuzwa SA, Vocadlo DJ. The Emerging Link between O-GlcNAc and Alzheimer Disease. *J Biol Chem*. 2014;289(50):34472-34481. doi:10.1074/jbc.R114.601351

45. Wright JN, Collins HE, Wende AR, Chatham JC. O-GlcNAcylation and cardiovascular disease. *Biochem Soc Trans*. 2017;45(2):545-553. doi:10.1042/BST20160164

46. Roskoski R. A historical overview of protein kinases and their targeted small molecule inhibitors. *Pharmacol Res*. 2015;100:1-23. doi:10.1016/j.phrs.2015.07.010

47. Ardito F, Giuliani M, Perrone D, Troiano G, Muzio LL. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). *Int J Mol Med*. 2017;40(2):271-280. doi:10.3892/ijmm.2017.3036

48. Li M, Rehman AU, Liu Y, Chen K, Lu S. Chapter Four - Dual roles of ATP-binding site in protein kinases: Orthosteric inhibition and allosteric regulation. In: Donev R, ed. *Advances in Protein Chemistry and Structural Biology*. Vol 124. Protein Kinases in Drug Discovery. Academic Press; 2021:87-119. doi:10.1016/bs.apcsb.2020.09.005

49. Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol Ther*. 2015;148:114-131. doi:10.1016/j.pharmthera.2014.11.016

50. Nick. Glycogen Synthase Kinase 3 (GSK3) and Glycogen Synthase (GS) - the phosphorylation equilibrium. Accessed December 7, 2021. https://teaching.drnickmorris.com/2012/11/glycogen-synthase-kinase-3-gsk3-and.html

51. Griebel G, Stemmelin J, Lopez-Grancha M, et al. The selective GSK3 inhibitor, SAR502250, displays neuroprotective activity and attenuates behavioral impairments in models of neuropsychiatric symptoms of Alzheimer's disease in rodents. *Sci Rep*. 2019;9(1):18045. doi:10.1038/s41598-019-54557-5

52. Wang Z, Pandey A, Hart GW. Dynamic Interplay between O-Linked N-Acetylglucosaminylation and Glycogen Synthase Kinase-3-dependent Phosphorylation*. *Mol Cell Proteomics*. 2007;6(8):1365-1379. doi:10.1074/mcp.M600453-MCP200

53. Kaasik K, Kivimäe S, Allen JJ, et al. Glucose Sensor O-GlcNAcylation Coordinates with Phosphorylation to Regulate Circadian Clock. *Cell Metab*. 2013;17(2):291-302. doi:10.1016/j.cmet.2012.12.017

54. Chang YH, Weng CL, Lin KI. O-GlcNAcylation and its role in the immune system. *J Biomed Sci*. 2020;27(1):57. doi:10.1186/s12929-020-00648-9

55. Mizuguchi H, Hayakawa T. The tet-off system is more effective than the tet-on system for regulating transgene expression in a single adenovirus vector. *J Gene Med*. 2002;4(3):240-247. doi:10.1002/jgm.261

56. Fig. 11.2 Tetracycline (tet)-controlled transcriptional regulation... ResearchGate. Accessed October 25, 2021. https://www.researchgate.net/figure/Tetracycline-tet-controlled-transcriptional-regulation-systems-a-tet-off-system_fig2_311556058

57. Mahmood T, Yang PC. Western Blot: Technique, Theory, and Trouble Shooting. *North Am J Med Sci*. 2012;4(9):429-434. doi:10.4103/1947-2714.100998

58. Ghosh R, Gilda JE, Gomes AV. The necessity of and strategies for improving confidence in the accuracy of western blots. *Expert Rev Proteomics*. 2014;11(5):549-560. doi:10.1586/14789450.2014.939635

59. Western Blotting. Novus Biologicals. Accessed October 25, 2021. https://www.novusbio.com/application/western-blotting

Appendix A: List of Abbreviations

AD – Alzheimer's disease

ATP – adenosine trisphosphate

C-terminal – carboxy-terminal

CNS – central nervous system

GABA – gamma aminobutyric acid

GlcNAc – *N*-acetylglucosamine

GSK3 – glycogen synthase kinase

ID – intervening domain

LTD – long-term depression

LTP – long-term potentiation

mOGT – mitochondrial OGT

N-terminal – amino-terminal

ncOGA(T) – nucleocytoplasmic OGA(T)

OGA – *O*-GlcNAcase

O-GlcNAc – *O*-linked GlcNAc

O-GlcNAcylation – *O*-linked N-acetylglucosaminylation

OGA – *O*-GlcNAcase

OGT – *O*-GlcNAc transferase

p-GSK3 – phosphorylated glycogen synthase kinase

PNS – peripheral nervous system

PTM – post-translational modification

PDVF – polyvinylidene fluoride

RMP – resting membrane potential

sOGA(T) – short OGA(T)

TPR – tetratricopeptide repeat

UDP-GlcNAc – uridine diphosphate N-acetylglucosamine

VGCaC – voltage-gated calcium channels

VGKC – voltage-gated potassium channels

VGNaC – voltage-gated sodium channels