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The Neuroprotective Effects of the Modulation of Glycogen Synthase Kinase-3 in
Diabetic Encephalopathy Through Pharmacological and Genetic Means

A thesis submitted in partial satisfaction of the requirements for the degree Master of

Science

in

Biology

by

Matthew Richard King

Committee in charge:

Professor Nigel Calcutt, Chair
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Professor Yimin Zou
Professor Roberto Malinow

2013

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University of California, San Diego

2013

Dedication

I dedicate this thesis to my parents for their unceasing support and encouragement.

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ABSTRACT OF THE THESIS

The Neuroprotective Effects of the Modulation of Glycogen Synthase Kinase-3 in
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by

Matthew Richard King

Master of Science in Biology

University of California, San Diego, 2013

Professor Nigel Calcutt, Chair

Professor Nicholas Spitzer, Co-Chair

Diabetes is a major medical problem, with complications such as nephropathy, retinopathy and neuropathy shortening life span and impairing quality of life. One of

these complications of chronic diabetes, diabetic encephalopathy, is characterized by deficient cognition and may progress to an Alzheimer's disease-like condition over time. The primary pathogenic mechanisms underlying diabetic encephalopathy include impaired insulin signaling and hyperglycemia. Deficient insulin signaling results in an over-activation of glycogen synthase kinase-3 (GSK3) leading to an increase in amyloid-beta ($A\beta$) protein and the hyper-phosphorylation of the structural protein tau. In an effort to combat the cognitive decline seen in chronic diabetes, we treated streptozotocin (STZ)-diabetic mice with one of two drugs: AR-A014418, a GSK3 inhibitor, or TX14(A), a neuropeptide derived from prosaposin that possesses both GSK3 inhibitory and neuroprotective properties. DN-GSK3 mice, a model expressing inactive GSK3 β , were used to compare the effects of endogenous versus pharmacological inhibition. Treatment with AR-A014418 was sufficient to prevent the development of cognitive deficits while TX14(A) was able to both prevent and reverse them. No prevention of cognitive decline was seen with endogenous inhibition of GSK3. These results may open a new avenue for treatment to prevent or ameliorate some of the detrimental effects of diabetic encephalopathy and Alzheimer's disease.

Introduction

Diabetes mellitus is a metabolic disease that affects an estimated 25.8 million people in the United States alone, roughly 8.3 percent of the population (NIDDK, 2012). Diabetes stems from the body's inability to adequately regulate glucose uptake due to a dysfunction of insulin signaling. The American Diabetes Association characterizes diabetes as having a fasting blood glucose level of over 126 mg/dL and a casual blood glucose level of over 200 mg/dL. The acute symptoms of diabetes mellitus include hyperglycemia, glucosuria, polyuria, and polydipsia.

There are three main types of diabetes: type 1, type 2, and gestational. Type 1 diabetes, also known as insulin deficient or juvenile diabetes, results from the autoimmune destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. This leads to a lack of insulin secretion and signaling, dramatically reducing the translocation of the GLUT-4 glucose transporter, thus hampering the uptake of glucose from the bloodstream. Type 1 diabetes is most common in individuals under the age of 15 but can also present during adulthood. The symptoms of type 1 diabetes include polyphagia, hyperlipidemia, and potentially ketoacidosis due to the inability to transport glucose into the cells and subsequent beta-oxidation of fat to supplement the low glucose. In contrast, type 2 diabetes, the most common form of diabetes, is characterized by insulin resistance rather than lack of insulin. Typically, this results from obesity and a sedentary lifestyle. Gestational diabetes presents during pregnancy and typically reverts to normal upon termination of pregnancy. As of yet there is no cure for diabetes, so prevention is paramount. Short of that, management is the only option. Glycemic control is crucial, with insulin treatment being important for preventing hyperglycemia. Lifestyle

changes such as a regulated diet and exercise are important for patients with type 2 diabetes. A number of anti-diabetic drugs are also used to regulate blood glucose levels through a variety of mechanisms (Gries et al. 2003). Unfortunately, none of these treatment regimens is ideal and diabetes continues to be a major problem throughout the world.

Chronic diabetes leads to angiopathy and further severe systemic complications like nephropathy, retinopathy, and neuropathy (Calcutt et al. 2009). Classically, hyperglycemia has been thought of as the primary pathogenic factor for inducing the complications of diabetes (DCCT 1993), but there is an increasing awareness that deficient insulin signaling has its own pathogenic implications due either to lack of insulin or the resistance of cells to insulin (Zochodne 2008).

Diabetic neuropathy is a common complication of long-term diabetes. This complication affects anywhere from 30 to 50% of diabetic patients depending on the study (Gries et al. 2003). Among the pathogenic mechanisms suggested as leading to neuropathy, hyperglycemia is the most commonly implicated initiating lesion (Tomlinson and Gardiner 2008), while deficient insulin signaling has been gaining more and more attention (Kan et al. 2012). There are a number of potential mechanisms by which elevated plasma or cellular glucose levels can cause neurodegeneration. For example, hyperglycemia causes non-enzymatic glycation, leading to the presence of advanced glycation end products that can modify the function of proteins, lipids, and DNA. Moreover, increased glucose metabolism can promote many potentially toxic events, including the formation of reactive oxygen species and the loss of neurotrophic support (Tomlinson and Gardiner 2008).

The pathology of diabetic neuropathy includes an increase in wall thickness and a decrease in caliber of endoneurial blood vessels. There is thickening of the perineurium, demyelination, and axonal degeneration, leading to a decrease in nerve density and occupancy of both large and small fibers. This begins distally, typically in the hands and feet, and then spreads proximally through the limbs to the trunk. A few clinical hallmarks are common to patients suffering from neuropathy. Most noticeable is an increase in noxious sensation to innocuous touch stimuli, known as tactile allodynia. Patients also experience thermal hypoalgesia, a lessened response to heat stimuli. Motor nerve conduction velocity is slowed, but goes un-noticed by patients. Unfortunately, glycemic control is not always sufficiently stringent to prevent neuropathy (Gries et al. 2003).

Diabetic encephalopathy, defined as damage to the central nervous system resulting from the presence of diabetes, is another major chronic complication (Cukierman et al. 2005). Encephalopathy consists of electrophysiological and structural disturbances in the brain that are associated with cognitive deficits including deficits in neurotransmission, cerebral signal conduction, and synaptic plasticity (Biessels 2007). This occurs in both type 1 and type 2 diabetic patients (Ryan et al. 1993, Reaven et al. 1990). There is a strong co-incidence of diabetes and cognitive defects, so much so that diabetic patients have a 1.2 to 1.5 fold greater decrease in cognition compared to their non-diabetic counterparts (Cukierman et al. 2005). Typical symptoms of diabetic encephalopathy include deficits in both visual and verbal memory, deficits in abstract reasoning, and deficits in executive functions such as planning, reasoning, and action initiation (Desrocher and Rovet 2004, Reaven et al. 1990, Ryan and Williams 1993). The causes of diabetic encephalopathy are thought to include aberrant glucose levels, the

constant insult of advanced glycation end products, and insulin deficits (Biessels 2007, Desrocher and Rovet 2004). It has been posited that both hypo and hyperglycemia have detrimental effects on the brain (Desrocher and Rovet 2004). There is a strong co-incidence of microvascular complications and decreased cognitive ability. In patients with symptoms of diabetic encephalopathy, a decreased density of the cortical grey matter and a high number of white matter lesions have been seen (Biessels et al. 2008). Further pathological hallmarks include demyelination, neuronal damage, and ganglion degeneration (Desrocher and Rovet 2004). Currently, the only treatment for diabetic encephalopathy is prevention and the only way to achieve this is through strict glycemetic control and the avoidance of vascular risk factors, but even this is not sufficient to completely prevent cognitive decline (Biessels 2007, Biessels et al. 2008). Over time, the severity of cognitive deficits increases, with a gradual progression towards Alzheimer's disease.

Many clinical studies have shown a positive correlation between diabetes mellitus and Alzheimer's disease. Studies have shown that diabetic patients have a relative risk of approximately 1.9 to 2.3 of developing Alzheimer's disease (Ott et al. 1996, Ronnema et al. 2009). Uncontrolled type 2 diabetes was reported to lead to the development of Alzheimer's disease, while glycemetic control seemed to attenuate the development of cognitive deficits suggesting a role for glucose or insulin dysregulation in cognitive decline (Xu et al. 2009). Diabetes has also been shown to accelerate the progression from mild cognitive deficits to Alzheimer's disease (Velayudhan et al. 2010). A great deal of evidence supporting the diabetes:Alzheimer's correlation has also been found in both murine and in-vitro models. The exposure of vascular endothelial cells to high levels of

glucose and low levels of amyloid beta led to an increase in advanced glycation end products and reactive oxygen species, both of which are known to cause neuronal damage (Burdo et al. 2008). Diabetes has also been shown to worsen cognition in mice that over-express amyloid precursor protein, the precursor to amyloid beta (Burdo et al. 2008, Jolivalt et al. 2010). In STZ-treated type 1 diabetic mice, learning deficits and memory impairment were seen along with a marked increase in phosphorylated tau protein and amyloid beta levels, all hallmarks of Alzheimer's disease (Jolivalt et al. 2008).

In diabetic rats, impaired spatial learning and long term potentiation has been seen, suggesting that the hippocampus is involved in diabetes-related learning deficits (Biessels et al. 1996). The hippocampus, more specifically the entorhinal cortex, is where the pathological hallmarks of Alzheimer's disease are first seen. Interestingly, the hippocampus has high levels of insulin receptor expression (McNay and Reznagel 2011). In STZ-diabetic mice, insulin treatment ameliorated cognitive decline along with levels of amyloid beta and phosphorylated tau (Jolivalt et al. 2008). In mice, cognitive deficits are seen to appear in type 1 diabetes earlier than in type 2 diabetes (Jolivalt et al. 2008). Furthermore, in mice with type 1 diabetes, intranasal administration of insulin was shown to reduce cognitive decline as well as the appearance of pathological brain defects detected by MRI (Francis et al. 2008). All of this evidence supports the idea that insulin deficits *per se*, rather than hyperglycemia, contribute to cognitive decline. The mechanism by which insulin affects cognition is unknown. There are a number of theories as to how this is achieved ranging from metabolic and vascular effects, to synaptic modulation and insulin signaling pathways (McNay and Reznagel 2011). In diabetic mice, a decrease in phosphorylated insulin receptor and GSK3 was seen,

suggesting that the phosphatidylinositol 3-kinase (PI3K) signaling pathway downstream of the insulin receptor plays a role in the development of cognitive deficits and Alzheimer's disease (Jolivald et al. 2008, Jolivald et al. 2010, Li et al. 2007).

Insulin binding marks the start of a variety of intracellular signals including activation of the phospholipase C γ , mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways (Jolivald et al. 2008, Jope and Johnson 2004). Phospholipase C γ is an enzyme that participates in cellular signal transduction pathways. It is part of the inositol phospholipid pathway and functions to cleave phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol trisphosphate (IP₃). This in turn activates protein kinase C through the release of calcium from the endoplasmic reticulum. The activation of protein kinase C has diverse effects on cellular function. The MAP kinase pathway is a long lasting cellular signal that acts on various downstream kinases in the cell. It plays an important role in gene regulation. The PI3K pathway plays a role in the mTOR pathway in addition to activating glycogen synthase kinase-3 (GSK3). This pathway is crucial for both cell survival and growth (Alberts et al. 2008).

GSK3 is an enzyme, located downstream of PI3K/AKT, which regulates cellular structure, function, and survival (**Figure A**). It also plays important roles in glucose homeostasis, immune function, cellular migration, proliferation, and apoptosis (Alberts et al. 2008). GSK3 also is part of the Wnt pathway, which controls cell-cell communication (Martinez 2008). GSK3 has two separate isoforms: GSK3 α and GSK3 β . The activity of GSK3 is downregulated by its phosphorylation on serine 21 and 9 of GSK3 α and GSK3 β , respectively (Sutherland et al. 1993). GSK3 α activity promotes more particularly the

formation of amyloid beta ($A\beta$), a neurotoxic protein that aggregates into plaques and inhibits axonal transport, via modulating the processing of amyloid precursor protein (APP) (Kasa et al. 2000), while active GSK3 β , more particularly, phosphorylates tau, among a myriad of other proteins (Jope and Johnson, 2004). Tau is a protein that binds and stabilizes microtubules. When tau is phosphorylated, its ability to bind microtubules is decreased, consequently disturbing the neuronal cytoskeleton, impeding axonal transport, and facilitating the formation of neurofibrillary tangles (reviewed in Grundke-Iqbal and Iqbal 1989). A wide variety of drugs exist that have the ability to inhibit the activity of GSK3. These drugs are small molecules that are capable of passing through the blood-brain barrier, making them particularly useful in treating pathologies of the central nervous system. Both ATP competitive and non-competitive examples exist. GSK3 inhibitors have shown promise in animal models in combating a wide variety of diseases and disorders including cancer, Alzheimer's disease, diabetes, ALS, Parkinson's disease, chronic inflammation, and more (Martinez 2008).

A number of animal models exist for both diabetes and Alzheimer's disease (Rees and Alcolado 2004, Spires and Hyman 2005). For our purposes, we chose to use two different mouse models: streptozotocin (STZ)-treated mice and dominant negative GSK3 mice, to assess the role of GSK3 in the development of AD-like features in the diabetic brain

Streptozotocin is a chemical isolated from *streptomyces achromogenes* that possesses antibiotic properties (Rees and Alcolado 2004). Upon administration, it is taken up into the beta cells of the pancreatic islets of Langerhans by GLUT-2 transporters. STZ is an alkylating agent, so it damages the DNA of the beta cells leading to their death

(Bolzan and Bianchi 2002). As the beta cells are the site of insulin production and secretion, their destruction leads to insulinopenia, which in turn leads to hyperglycemia. This makes STZ-treated rodents a widely used animal model of type 1 diabetes (Rees and Alcolado 2004).

Dominant negative GSK3 (DN-GSK3) mice are a transgenic mouse model in which the activity of GSK3 β is endogenously inhibited (Rockenstein et al. 2007). These mice overexpress wild-type GSK3 β under the control of the mThy1 promoter. This overexpression activates AKT, the enzyme that phosphorylates GSK3 β at serine 9, increasing the phosphorylation of GSK3 β . The aforementioned feedback loop inactivates GSK3 β , resulting in a much lower level of active GSK3 β . For this reason, they are called dominant negative. When the DN-GSK3 mice were crossed with hAPP mice (a transgenic mouse model for Alzheimer's disease that overexpresses amyloid precursor protein) a decrease in phosphorylated tau protein was seen with a corresponding amelioration of cognitive decline (Rockenstein et al. 2007), demonstrating a role of GSK3 in the phosphorylation of tau and memory behavior.

In addition to the genetic modulation of GSK3 protein and activity in the DN-GSK3 mice, we employed pharmacological approaches to GSK3 inhibition. AR-A014418 is one of the many GSK3 inhibitors currently available (Martinez 2008). This drug blocks the binding of ATP to the ATP binding pocket on GSK3, preventing GSK3 from phosphorylating its downstream targets. AR-A014418 is one of the better GSK3 inhibitors due to its high specificity and lack of interference with the related cyclin dependent kinases 2 and 5 (Bhat et al. 2003). It has been shown to prevent tau

phosphorylation in JNPL3 mice, suggesting a beneficial role in preventing Alzheimer's disease (Noble et al. 2005).

TX14(A) is a 14 amino acid peptide derived from prosaposin that possesses neuroprotective properties, and prevents small and large fiber deficits from developing in diabetic rats (Calcutt et al. 1999). Furthermore, TX14(A) has been shown to reverse established nerve conduction deficits and to have anti-allodynic properties (Mizisin et al. 2001, Jolivald et al. 2005). Preliminary data from our group has shown TX14(A) to also possess GSK3 inhibitory properties.

In order to determine the effect of GSK3 inhibition and/or the loss of neurotrophic support in diabetic encephalopathy in a model of type 1 diabetes, three studies were performed. To test the preventive potential of GSK3 inhibition and maintained neurotrophic support, mice were treated with the drugs AR-A014418 or TX14(A) for 10 weeks from the onset of diabetes. To more closely reflect the clinical setting, we tested the potential of these two drugs to reverse established disorders by treating mice for 8 weeks following 10 weeks of uncontrolled diabetes. Finally, in order to further test the effect of GSK3 inhibition on the development of diabetic encephalopathy, DN-GSK3 mice were made diabetic and followed for 12 weeks.

Insulin Signaling Pathway

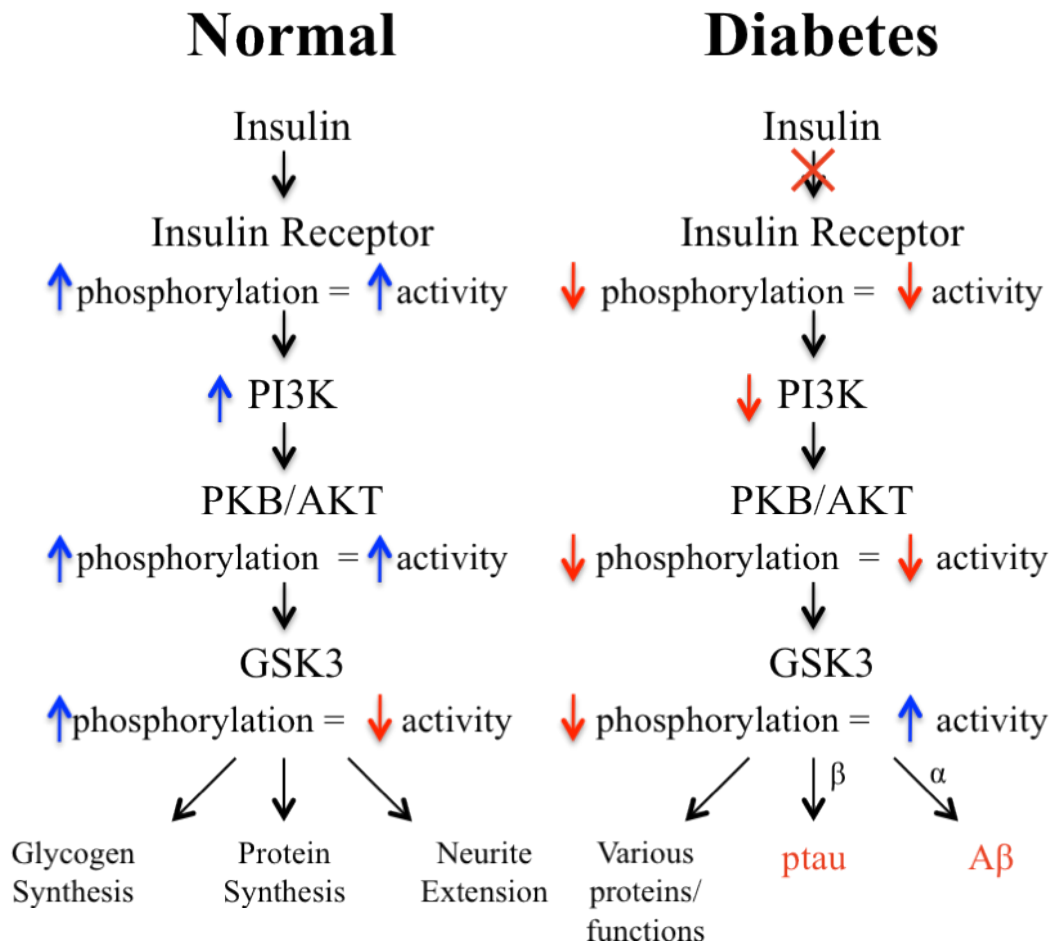


Figure A: Insulin signaling through the PI3K/AKT pathway under normal conditions and type 1 diabetes.

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Materials and Methods

2.1 Animals:

Prevention and reversal studies: Adult female Swiss Webster mice were used in the prevention and reversal studies. Eight to twelve animals were used per group. These animals were housed four to a cage with free access to food and water. All cages were maintained in a vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. All study protocols were approved by the Institutional Animal Care and Use Committee of the University of California San Diego.

DN-GSK3 study: Adult male DN-GSK3 mice were used in the DN-GSK3 study. These mice were obtained from Dr. Eliezer Masliah at UCSD. DN-GSK3 mice overexpress GSK3 β causing a feedback loop that results in the inactivation of GSK3 β (Rockenstein et al 2007). Five to seven animals were used per group. These animals were housed four to a cage with free access to food and water. All cages were maintained in a vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. All study protocols were approved by the Institutional Animal Care and Use Committee of the University of California San Diego.

2.2 Induction of Diabetes:

Studies were performed in a mouse model of insulin deficient type 1 diabetes. Diabetes was induced using streptozotoin (STZ: 2-deoxy-2-({[methyl(nitroso)amino]carbonyl}amino)- β -D-glucopyranose). Diabetes was induced at six to eight weeks of age. Intraperitoneal injection of STZ (Sigma, St. Louis, MO) at 90mg/kg dissolved in 0.9% sterile saline solution was performed after an overnight fast on two consecutive days. STZ competes directly with glucose for entrance to the pancreatic beta

cells so animals are fasted to ensure maximal effectiveness of the STZ. Hyperglycemia was confirmed 4 days post STZ administration using a strip-operated glucose meter (One Touch Ultra, Lifescan, Milpitas, CA, USA) on a blood sample obtained via tail prick as well as on a blood sample obtained at the conclusion of the study.

2.3 Drugs:

TX14(A) (Prosaptide) was supplied by Myelos Corporation in solution at 4mg/ml. TX14(A) is a 14 amino acid peptide derived from the N-terminal neurotrophic region of prosaposin, the glycoprotein precursor of the saposin family (Calcutt et al. 1999). TX14(A) is known to prevent both large and small fiber deficits associated with diabetes. It was administered at 1mg/kg 3 days a week. The ATP-competitive GSK3 inhibitor AR-A014418 (Astra-Zeneca) was purchased from Sigma. AR-A014418 was dissolved in DMSO and then diluted with PBS, giving a final DMSO concentration of 3.7%. It was administered as an intraperitoneal injection 6 days a week at 30 μ mol/kg. Vehicle consisting of 4% DMSO in PBS was administered to STZ and control mice as a control. All drugs and vehicle were administered as an intraperitoneal injection at 10ml/kg over the course of 10 weeks for the prevention study and over the course of 8 weeks for the reversal study.

2.4 Rotarod:

Mice were placed on a rotarod (Stoelting Co., Wood Dale, IL, USA) to assess motor coordination and physical condition. The device accelerated from 4 to 40 RPM over 5 minutes. The amount of time spent on the rod before loss of balance was recorded over three trials. All trials were performed on the same day.

2.5 Thermal Response Latency:

In order to assess possible changes in thermal nociception, a sign of peripheral neuropathy, the thermal response latency test was performed. The thermal testing apparatus consists of a large cabinet with a glass top. Inside is a light source used to apply heat to the underside of the paw. Sensors and visual observation are used to determine when the mouse responds to the stimulus, stopping a timer. A response consists of the animal withdrawing the paw from the stimulus and is generally accompanied by licking of the affected paw. Mice were placed in an observation chamber on top of the thermal testing apparatus (UARD, San Diego, CA) at 30 °C and allowed to acclimate for 15 minutes. A heat stimulus was applied to the center of the hind paw. The temperature increases by approximately 1°C per minute in order to ensure the activation of C-fiber nociceptive neurons. A heating rate higher than 2°C per minute preferentially activates A δ nociceptive neurons rather than C-fibers (Yeomans et al. 1996). The time elapsed before withdrawal was recorded and converted to a temperature measurement using a standard curve. The standard curve is created by taking temperature measurements at 5-second intervals from 0 to 20 seconds. A regression line is then applied to this data to generate the standard curve. This curve is compiled once during each day of thermal testing.

2.6 Tactile Allodynia:

The tactile allodynia test was performed to assess the presence or absence of allodynia, a pain response to a normally non-painful stimulus indicative of peripheral neuropathy. Mice were placed in an inverted beaker on top of a wire mesh platform and allowed to acclimate for 15 minutes. Weighted von Frey filaments (Stoelting, Wood Dale, IL) whose log values range from 3.22 to 4.74 were used to apply pressure to the

plantar surface of the hind paw. Beginning with filament 3.84, mice were poked with enough pressure to bend the filament 5 times, each lasting approximately 1 second. A positive response was characterized as a flinch of the hind leg away from the stimulus. In the event of a positive response, the next lighter filament was used. If no response was seen, the next heavier filament was used. This process was continued until 4 measurements were obtained after the first change in response, the lightest filament was used, or until 5 consecutive negative responses were seen. The 50% paw withdrawal threshold was then calculated using a program based on the equation: 50% gram threshold = $(10^{[X_f+k\delta]})/10000$, where X_f is the log value of the final von Frey filament, k is the value for the response pattern, and δ is the mean difference between the filament log values (Chaplan et al. 1994).

2.7 Motor Nerve Conduction Velocity:

To assess conduction slowing, sciatic nerve conduction velocity was measured. Mice were placed on a circulating water heating pad in order to maintain a constant 37° C body temperature. Two recording electrodes were placed in the hind paw. Another electrode was placed in the scruff of the neck to serve as a ground. A fourth electrode was placed alternately in the sciatic notch and ankle to deliver a stimulus. Using AD Instruments Powerlab 4/30 (AD Instruments, Colorado Springs, CO), a 50 μ s stimulus of 6.75 volts was delivered and the duration of conduction from either the sciatic notch or the ankle to the hind paw was recorded using the Scope 4 program. Each measurement was performed 3 times and the difference in conduction times between the two stimulation sites was calculated. By dividing the distance between the ankle and sciatic

notch by the median difference in conduction time, the conduction velocity in meters per second was determined.

2.8 Barnes Maze:

The Barnes Maze is a cognitive test used to assess learning in mice (Barnes 1979). The maze is composed of a brightly lit white circular platform with 20 holes spaced equidistant around the periphery (MayallsMazes, Encinitas, CA). A box is placed beneath one of the holes which has a visual cue placed above it, in this case a large red arrow. Prior to testing on the first day, the mouse being assessed was placed in the box for 1 minute in order to acclimate to the box. The mouse was then placed in the center of the table and allowed to explore until it either found the box or 5 minutes elapsed. The amount of time it took the mouse to locate the box, the amount of time it took the mouse to enter the box, and the number of erroneous visits to the other 19 holes was recorded. Afterward, the mouse was placed in the box for an additional minute in order to reinforce the mouse's desire to enter the box. This test was performed at the end of the studies every day for a week. Mice with impaired cognitive function show a slower improvement than control mice in the amount of time it takes them to locate and enter the box over the course of testing.

2.9 Object Recognition:

The object recognition test is used to assess memory. Object recognition is performed using an 18x9 inch transparent box in a dimly lit room isolated from extraneous noise. Two identical objects are placed equidistant from each other and from the sides of the box. Mice are given 10 minutes to achieve 20 seconds of exploration time of the two objects, after which they are removed back to their cage. Exploration is

defined as the mice facing and being within approximately 1 inch of the object. An hour later, one of the objects is replaced with a novel object and the mice are given 5 minutes to explore. The time the mice spend exploring the familiar and novel objects is recorded. The percentage of time that mice spend with the novel object is then calculated. Mice with normal cognitive function are expected to spend more time with the novel object.

2.10 Tissue Preparation:

Hemi-brains lacking cerebellum were homogenized in buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X, 1 mM EDTA, protease inhibitor cocktail). The homogenates were then centrifuged at 13,000g for 30 minutes. Clear extracts were stored at -80°C. A portion of the homogenate extract was boiled for 5 minutes in detergent free solution, after which the samples were centrifuged for 30 minutes to remove insoluble material. These samples, containing approximately 7µg of protein, were prepared with equal volumes of Laemmli SDS sample buffer for tau Western-blot analysis. The bicinchoninic acid method (BCA protein assay kit, Pierce, Rockford, IL, USA) was utilized to assess protein concentration prior to blotting.

2.11 Western Blotting:

Homogenates of brain tissue were centrifuged at 13,000g. Extract aliquots were boiled in Laemmli LDS sample buffer (Invitrogen, Carlsbad, CA, USA). 15µg of extract proteins were separated on 4-12% Bis-Tris SDS-PAGE gels (Novex, Invitrogen). Separated proteins were then blotted on nitrocellulose. Blots were incubated with antibodies against phospho-GSK3α/β, GSK3α/β, phospho-tau and Tau-5, Amyloid β, and synaptophysin. Blots were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Densitometric

scanning using Quantity One (BioRad, San Diego, CA, USA) was used for immunoreactivity quantification. Band intensities for each protein were normalized by calculating the ratio of band intensity of interest to the band intensity of actin, cyclophilin, and total (non-phosphorylated) protein where applicable.

2.12 Statistical Analyses:

Data was analyzed by one-way ANOVA followed by either Dunnett's Multiple Comparison test or Tukey's *post-hoc* test.

Results

3.1.0 Prevention Study

Diabetic Swiss-Webster mice were treated with AR-A014418 at 30 μ mol/kg daily or TX14(A) at 1 mg/kg 3 days a week for 10 weeks from the onset of diabetes.

3.1.1 Diabetes

During the course of the study, the control mice treated with vehicle maintained a healthy weight with a gradual increase (**Figure 1**). Diabetic mice treated with vehicle maintained a consistent weight, which was lower than that of the control mice. Treatment of diabetic mice with either drug had no effect on body weight as these mice had similar weight values to the vehicle-treated diabetic mice. All STZ-treated mice exhibited hyperglycemia while control mice maintained a normal blood glucose level (**Figure 2A**). Furthermore, all STZ-treated mice had elevated HbA1c levels (**Figure 2B**). Treatment of diabetic mice with either drug had no effect on blood glucose or HbA1c levels.

3.1.2 Learning and memory behavior

In the Barnes maze test, vehicle-treated STZ-diabetic mice took more time to find the escape box than control mice on days 2, 3, and 4 of testing after 9 weeks of diabetes (**Figure 3A**), resulting in a significantly ($p < 0.01$) increased area under the curve (**Figure 3B**). Treatment with AR-A014418 significantly ($p < 0.05$) decreased the amount of time required by STZ-diabetic mice to locate the escape box when compared to vehicle-treated STZ-diabetic mice. Mice treated with TX14(A) also showed improvement compared to untreated diabetic mice, but this was not statistically significant. All diabetic mice exhibited similar error rates (**Figure 3C**). In the locomotor test using the rotarod, the time required for mice to fall off the rotating rod was similar for all groups (**Figure 4**). In the

object recognition test, vehicle-treated diabetic mice spent significantly ($p < 0.05$) less time with the novel object as compared to control mice (**Figure 5**). AR-A014418 treatment prevented this deficit ($p < 0.05$) while treatment with TX14(A) had no effect.

3.1.3 Peripheral neuropathy

Vehicle-treated diabetic mice exhibited a significant ($p < 0.01$) thermal hypoalgesia after 11 weeks of diabetes compared to control mice (**Figure 6A**). Both drugs decreased paw withdrawal latency compared to vehicle-treated diabetic mice, but only the TX14(A) treatment effect was statistically significant ($p < 0.05$). Similar patterns were noted when values were converted from withdrawal latency to the temperature of withdrawal, as derived from the time:temperature standard curve (**Figure 6B**). After 11 weeks of diabetes, when tactile response was assessed, no significant difference in the paw withdrawal threshold was detected between any of the groups (**Figure 7**). There was no significant difference in sciatic motor nerve conduction velocity between any of the groups (**Figure 8**).

3.1.4 Western blot analysis

Phosphorylation of GSK3 β was significantly ($p < 0.05$) decreased in vehicle-treated diabetic mice compared to control mice (**Figure 9**). Treatment with either drug returned GSK3 β phosphorylation to a level similar to that of control mice. Levels of phosphorylated tau were increased in vehicle-treated diabetic mice ($p < 0.001$) compared to control mice (**Figure 10A**). Diabetic mice treated with AR-A014418 also had values significantly ($p < 0.05$) greater than control mice whereas values in diabetic mice treated with TX14(A) were elevated but not significantly different from any other group. The level of amyloid beta protein was significantly ($p < 0.01$) increased in all diabetic mice,

regardless of treatment (**Figure 10B**). Synaptophysin protein levels were significantly ($p < 0.05$) reduced in vehicle-treated diabetic mice compared to control mice (**Figure 11**). Both drugs maintained synaptophysin protein at levels that were not significantly different from control mice.

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3.2.0 Reversal Study

Diabetic Swiss-Webster mice were treated with AR-A014418 at 30 $\mu\text{mol/kg}$ daily or TX14(A) at 1 mg/kg 3 days a week for 8 weeks after 10 weeks of uncontrolled diabetes.

3.2.1 Diabetes

During the course of the study, the control mice treated with vehicle maintained a healthy weight with a gradual increase over time (**Figure 12**). Diabetic mice treated with vehicle initially lost weight then maintained a consistent weight, which was lower than that of the control mice. Treatment of diabetic mice with either drug had no effect on body weight. All STZ-treated mice exhibited hyperglycemia compared to control mice (**Figure 13**). Over the course of the study a total of 13 mice died: 1 control, 1 STZ, 5 TX14(A), and 6 AR-A014418.

3.2.2 Learning and memory behavior

In the Barnes maze test, vehicle-treated diabetic mice took more time to find the escape box than control mice on days 1, 3, and 4 after 16 weeks of diabetes (**Figure**

14A), resulting in a significantly ($p < 0.05$) increased area under the curve (**Figure 14B**). Treatment of diabetic mice with either drug had no effect on the time required to find the escape box. The time required for the mice to fall from the rotating rod was similar for all groups (**Figure 15**). In the object recognition test, there was no significant difference in time spent with the novel object amongst the groups (**Figure 16**). Diabetic mice treated with AR-A014418 show zero percent time spent with the novel object due to a lack of exploration.

3.2.3 Peripheral neuropathy

Vehicle-treated diabetic mice exhibited significant ($p < 0.01$) thermal hypoalgesia after 15 weeks of diabetes compared to control mice (**Figure 17**). Treatment with either drug had no effect on the thermal withdrawal latency. There was no significant difference in sciatic motor nerve conduction velocity between any of the groups (**Figure 18**).

3.3.0 DN-GSK3 Study

STZ-diabetic, non-diabetic wild type, and DN-GSK3 mice were monitored for 12 weeks.

3.3.1 Basal physiology and diabetes

During the course of the study, the wild type (WT) mice maintained a healthy weight with a gradual increase followed by a plateau (**Figure 19**). The DN-GSK3 mice maintained a consistent weight slightly lower than that of the WT mice. All diabetic mice maintained a consistent weight lower than that of their respective counterparts. All STZ-treated mice exhibited hyperglycemia while WT and DN-GSK3 mice maintained a normal blood glucose level (**Figure 20**). The plasma insulin level of WT mice was significantly ($p < 0.001$) higher than that of the other three groups (**Figure 21**). Non-

diabetic DN-GSK3 mice had a significantly ($p < 0.001$) lower insulin levels than non-diabetic WT mice, while both groups of diabetic mice had similar plasma insulin levels.

3.3.2 Learning and memory behavior

In the Barnes maze test, both diabetic and DN-GSK3 mice took more time to find the escape box than WT mice on days 2, 3, 4, and 5 (**Figure 22A**), resulting in a significant ($p < 0.01$) increase in the area under the curve (**Figure 22B**). Diabetic DN-GSK3 mice also took significantly ($p < 0.001$) more time to locate the escape box than the WT mice, with a time similar to that of non-diabetic DN-GSK3 mice (**Figure 22**). The time required for the mice to fall from the rotating rod was similar for the WT and diabetic mice (**Figure 23**). Both DN-GSK3 and diabetic DN-GSK3 mice spent a similar amount of time on the rotarod and this was significantly ($p < 0.001$) less than STZ-diabetic mice (**Figure 23**). In the object recognition test, there was no significant difference in the amount of time spent with the novel object amongst any of the groups (**Figure 24**).

3.3.3 Peripheral neuropathy

WT mice had a significantly ($p < 0.05$) higher paw thermal withdrawal latency than DN-GSK3 mice at 10 weeks (**Figure 25**). Both the diabetic and diabetic DN-GSK3 mice had similar paw withdrawal latency, which was higher than that of the DN-GSK3 mice but similar to that of the WT mice. No significant difference was noted for the other groups. There were no significant differences in sciatic motor nerve conduction velocity amongst any of the groups of mice (**Figure 26**), although the motor nerve conduction velocity of the diabetic DN-GSK3 mice was slightly lower than that of the other groups.

Discussion

In order to assess the role of GSK3 in the development of Alzheimer's disease-like features in type 1 diabetes, three studies utilizing the inhibition of GSK3 were performed, each testing a different facet of the effects of GSK3 inhibition. Prevention and reversal studies were performed to assess the effects of pharmacological GSK3 inhibition on the prevention and reversal of the cognitive deficits seen in the STZ mouse model of diabetes. The DN-GSK3 study used the DN-GSK3 mouse model, which has endogenous inhibition of GSK3 β , and was performed as a proof of concept.

4.1 Diabetes

In all three studies, control, wild-type, or diabetic mice all maintained a healthy weight throughout the study duration. Additionally, in each study, diabetic mice had a lower weight than their non-diabetic counterparts due to their inability to utilize glucose. In both the prevention and reversal studies, all of the diabetic mice, despite receiving different treatments, had very similar weights with no significant differences between the groups. This same pattern held true for diabetic mice in the DN-GSK3 study despite their differing genetic profiles. Together, these data suggest that the GSK3 inhibitors did not induce a general toxicity in control or diabetic mice.

At the terminus of each study, the blood glucose of each mouse was measured to both confirm the presence of, and assess the severity of diabetes. For each of the studies, all of the non-diabetic groups had blood glucose measurements well within normal physiological parameters. All of the STZ-injected animals had measurements indicating that they were clearly diabetic. There were no significant differences in blood glucose

levels between any of the groups receiving different treatments. Plasma insulin levels were also measured in the mice of the DN-GSK3 study. As expected, the wild-type mice had normal plasma insulin levels while the diabetic mice had significantly reduced amounts of insulin, as would be expected in type 1 diabetes. Interestingly, the non-diabetic DN-GSK3 mice had a plasma insulin level near that of the diabetic groups, although the reduced insulin was not accompanied by hyperglycemia. This is a novel observation for this mouse strain and the reason for this is not known.

Overall, neither the drug treatments nor the genetic manipulations had any effect on the presence of diabetes in any of the mice. Furthermore, there were no significant differences in the severity of the diabetes between any of the groups. Therefore, any effects of treatments on PNS and CNS function cannot be attributed to an amelioration of diabetes and likely result from actions downstream of hyperglycemia and/or insulin deficiency in the mice.

4.2 Learning and memory behavior

Diabetes caused the development of learning deficits as seen through an increase in Barnes maze latency, suggesting that visuospatial cognition is hampered. Altering the activation of GSK3 had various effects on the central nervous system. Treatment with either AR-A014418 or TX14(A) was sufficient to prevent some of the learning deficits typical of Alzheimer's disease that are revealed using the Barnes maze. It appears that AR-A014418 is more effective than TX14(A) at the doses used. Dosages were chosen based upon previous studies (Calcutt et al. 1999, Noble et al. 2005). Higher dosages may prove to be more effective, but excessive dosing may also prove to be toxic or cause

detrimental over-inhibition of GSK3 (Calcutt, unpublished observations). In contrast, treatment with AR-A014418 was unsuccessful at reversing learning deficits while TX14(A) was seen to have a marginal effect. Since inhibition of GSK3 β was seen to have prevented learning deficits, it was expected that the DN-GSK3 mice would not be subject to the diabetes-induced learning deficits due to their endogenous GSK3 β inhibition. Interestingly, the non-diabetic DN-GSK3 mice performed as poorly as their diabetic control counterparts and diabetes in the DN-GSK3 mice did not affect their learning ability. These results are different from the original study using the DN-GSK3 mice, in which DN-GSK3 mice performed as well as their wild-type counterparts in the Morris water maze which tests similar parameters to the Barnes maze (Rockenstein et al. 2007). This might be explained by varying levels of GSK3 β inhibition between the mice in our study and the original study, or by the low level of plasma insulin that was measured in our mice, resulting in decreased activation of the insulin receptor pathway and consequent increased activation of GSK3. Western blot analysis of brain tissues collected during our study will help clarify the difference between the studies.

Memory ability was also assessed, through the use of the object recognition test. This test is an excellent way to assess cognition as it tests both memory and comprehension of a novel object and has been extensively used in other studies to measure the effect of pharmacological treatment on cognition with great success (Fonseca et al. 2013, Rushaidhi et al. 2013, Zhao et al. 2012). Diabetes induces memory deficits which result in hampered novel object recognition. Treatment with AR-A014418 completely prevented the development of memory deficits while TX14(A) had no effect. However these drugs have limited ability to reverse established memory deficits.

Unfortunately, no statistical significance can be derived from the data of the reversal study because of the small sample size. Similarly, there were no differences seen between the groups in the DN-GSK3 study, but once again the sample sizes were small due to an unwillingness of many mice to explore during the test.

In all three of the studies, there was no significant difference between the groups in terms of rotarod performance, indicating that none of the mice had any motor or coordination deficits. Therefore, the effects seen in the Barnes maze and object recognition tests were not the result of impaired movement. This was confirmed by calculating the rate of errors made by the diabetic mice during the Barnes maze, which was indicative of ample movement throughout the test.

In terms of the central nervous system, it appears that pharmacological inhibition of GSK3 β has significant preventive effects but limited reversal effects. The results from the DN-GSK3 study would seem to refute this conclusion. However, this might be explained by varying degrees of inhibition of GSK3 β . AR-A014418 and TX14(A) only partially inhibit GSK3 β while the endogenous inhibition might be pronounced. In the original DN-GSK3 study, the activity of GSK3 β was reduced by approximately 33% (Rockenstein et al. 2007). Since these DN-GSK3 mice performed similarly to diabetic mice in which there is over-activation of GSK3 due to the lack of insulin signaling (Jolivald et al. 2008), we propose that over-inhibition of GSK3 β is as detrimental as over-activation. To test this hypothesis, future studies will measure the activity of GSK3 β in our mice in order to verify whether or not we had a similar degree of inhibition. Furthermore, it may be the case that AR-A014418 and TX14(A) inhibit both GSK3 α and GSK3 β , while the DN-GSK3 mice only have inactivation of GSK3 β , and that this is what

accounts for the difference in behavioral data seen in the DN-GSK3 study as compared to the prevention and reversal studies. This possibility will be explored.

4.3 Peripheral neuropathy

Peripheral neuropathy is a common complication of diabetes in humans and rodents. It typically can be detected as thermal hypoalgesia, tactile allodynia, and motor nerve conduction velocity slowing. Previous studies from our laboratory have shown TX14(A) to prevent the development of both small and large fiber deficits in rats (Calcutt et al. 1999). Treatment with TX14(A) preserves axonal caliber and prevents the development of thermal hypoalgesia (Calcutt et al. 1999). It has also been shown to acutely alleviate tactile allodynia induced by a wide variety of neurological insults (Jolivalt et al. 2005). Furthermore, TX14(A) has the capacity to reverse both sensory and motor nerve conduction deficits in rats, as well as to reduce axonal loss (Mizisin et al. 2001). The mechanisms by which TX14(A) ameliorates diabetic neuropathy are not known, and the ability of TX14(A) to reduce GSK3 activity in diabetes is a recent finding. We are not aware of any studies reporting the efficacy of GSK3 inhibitors on diabetic peripheral neuropathy.

Thermal hypoalgesia was seen in the non-treated STZ-diabetic mice of all studies and is a well-documented early symptom of uncontrolled diabetes (Jolivalt et al. 2011, Jolivalt et al. 2012). Treatment with AR-A014418 or TX14(A) partially ameliorated the thermal hypoalgesia when administered in a preventive manner. TX14(A) has previously been shown to prevent thermal hypoalgesia in STZ-diabetic rats (Calcutt et al. 1999), whereas the efficacy of AR-A014418 is a novel finding. Neither of the drugs had any

restorative effects when administered after the onset of hypoalgesia. The endogenous inhibition of GSK3 β also had no effect on hypoalgesia. For the prevention study, there was no significant difference between the groups for the tactile allodynia test. Due to the inconclusive nature of the test, it was not repeated for the subsequent studies.

Additionally, there were no significant differences in motor nerve conduction velocity amongst any of the groups for any of the studies. This data suggests that both AR-A014418 and TX14(A) have peripheral neuroprotective properties on small fiber dysfunction as detected as paw thermal hypoalgesia, but are less effective at reversing established small fiber neuropathy.

4.4 Western blot analysis

Western blotting was performed to assess the degree of GSK3 β inhibition as well as to determine potential effects on other important proteins as a result of the preventive administration of AR-A014418 and TX14(A). The inactivation of GSK3 β is achieved by its phosphorylation at serine 9. GSK3 β 's phosphorylation was significantly decreased in non-treated STZ-diabetic mice. This agrees with prior data from our laboratory (Jolivalt et al. 2008, Jolivalt et al. 2010). Phosphorylation was restored to normal levels by both treatment with AR-A014418 and treatment with TX14(A). Thus both drugs successfully inhibit GSK3 β , confirming what has previously been found about the efficacy of AR-A014418 in inhibiting the enzyme (Bhat et al. 2003) and providing a new potential mechanism of action for TX14(A).

Both tau and A β are downstream targets of GSK3. The level of phosphorylated tau was increased in all of the diabetic mice compared to the control group regardless of

treatment. A similar pattern was seen in the level of A β protein. These data are consistent with prior studies from our laboratory (Jolivald et al. 2010). Other studies have shown the ability of AR-A014418 to significantly reduce phospho-tau levels in 3T3 fibroblast culture and JNPL3 mice (Bhat et al. 2003, Noble et al. 2005). However, in this study we did not see any effect of AR-A014418 on the accumulation of either A β or phospho-tau. This is curious because the AR-A014418 and TX14(A) treated groups displayed definite amelioration of the typical cognitive deficits associated with encephalopathy. Possible interpretations of our data are that GSK3 β is acting on a different downstream target, or on alternative targets that in turn generate the cognitive deficits.

Synaptophysin is a protein found at neuronal synapses and is used as a marker of synaptic integrity (Fletcher et al. 1991). The level of synaptophysin protein was significantly reduced in the non-treated STZ-diabetic group of the prevention study. This level was restored to normal by both treatment with AR-A014418 or TX14(A). Thus it seems that the inhibition of GSK3 β may have neuroprotective effects through the preservation of synaptic integrity. Due to the lack of effect that GSK3 β inhibition had on phospho-tau and A β levels, it seems possible that the prevention of cognitive deficits can be attributed to the preservation of synaptic integrity. Learning and memory are a result of synaptic plasticity, which relies upon the phenomenon of long-term potentiation (Bliss and Collingridge 1993). Thus, the preservation of the integrity of the synapses in the hippocampus likely prevents the loss of synaptic plasticity allowing for continued non-deficient learning and recall.

4.5 Therapeutic implications

The ability to modulate the activity of GSK3 is an exciting development in the field of diabetes. This newfound ability shows great promise and may represent a new therapeutic avenue for the treatment of one of the most devastating complications from diabetes as well as a window to further understand the processes that lead to Alzheimer's disease. Despite this promise, if the GSK3 inhibition is too robust, then numerous harmful side effects can be expected and the therapeutic potential of GSK3 inhibitors may be limited.

Lithium was the first known GSK3 inhibitor (Klein and Melton 1996). It has a long history of use as an effective treatment for bipolar disorder. As it has been proven to be safe for use in humans, its use could be modified for diabetic patients in order to prevent their cognitive decline. Unfortunately, lithium has many biological targets so there is a clinical need for a specific inhibitor of GSK3 (Martinez 2008). This is where drugs like AR-A014418 and TX14(A) could have a role in the clinical setting. A clinical trial examined the use of TX14(A) for the treatment of HIV associated sensory neuropathies and determined short-term administration of TX14(A) in humans to be both safe and well tolerated (Evans et al. 2007). Hopefully more of these drugs can be deemed safe for use in humans. The diabetes epidemic affects so many people that this new research may prove to be a great breakthrough in improving the quality of life for innumerable afflicted persons and their families.

4.6 Future directions

The next experiment to be performed will be western blotting for the tissues from the reversal and DN-GSK3 studies to assess the levels of A β , phospho-tau, and

synaptophysin, as well as the degree of inhibition of GSK3 β . This should provide more insight as to the results seen in these two studies. Further studies with pharmacological modulation of GSK3 activity and endogenous inhibition of GSK3 are warranted to fully analyze the effects of modulation of GSK3. The DN-GSK3 model provides a good way to achieve the aforementioned goal.

There are many other GSK3 β modulating drugs currently available for study (Martinez 2008). Being of different chemical compositions, they likely have differing degrees of modulation of the GSK3 protein. It would be interesting to study the effects of varying degrees of GSK3 modulation on cognition by employing a variety of different drugs in various doses, and various specificities for GSK3 β and/or GSK3 α .

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Figures

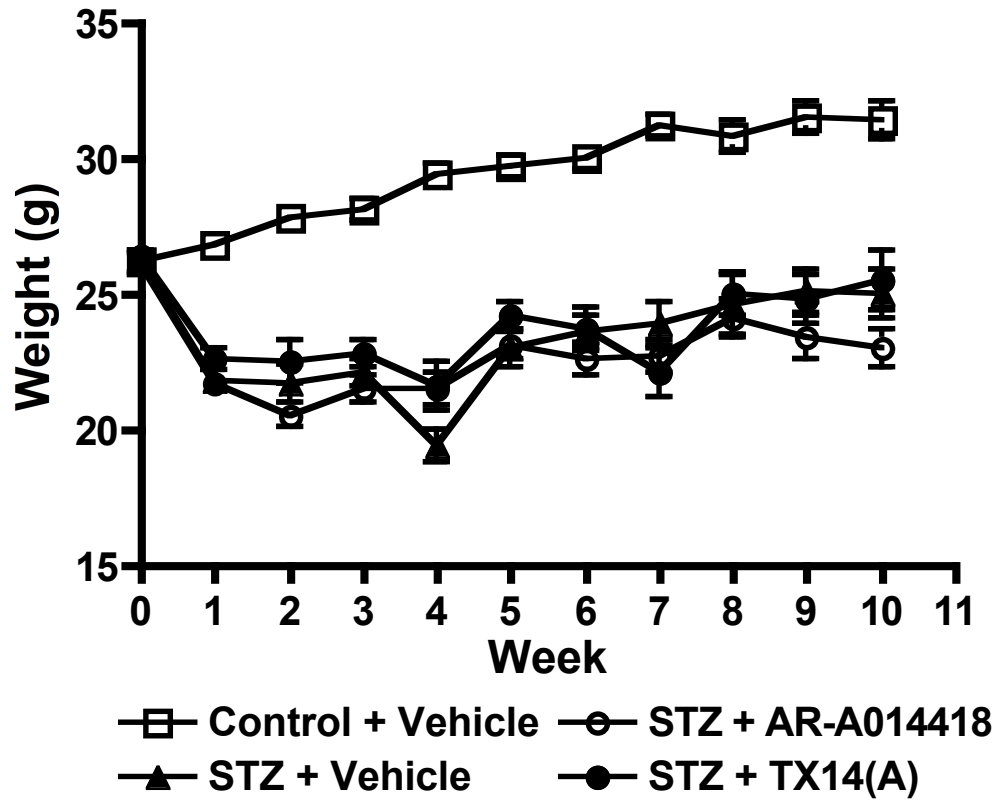


Figure 1: Body weight over the course of the study for control mice receiving vehicle (open squares), STZ-diabetic mice receiving vehicle (closed triangles), STZ-diabetic mice receiving AR-A014418 at 30 μ mol/kg (open circles) and STZ-diabetic mice receiving TX14(A) at 1mg/kg (closed circles). Data are group mean \pm SEM. N= 14 (control), 9-12 (STZ), 8-14 (AR), 5-14 (TX)

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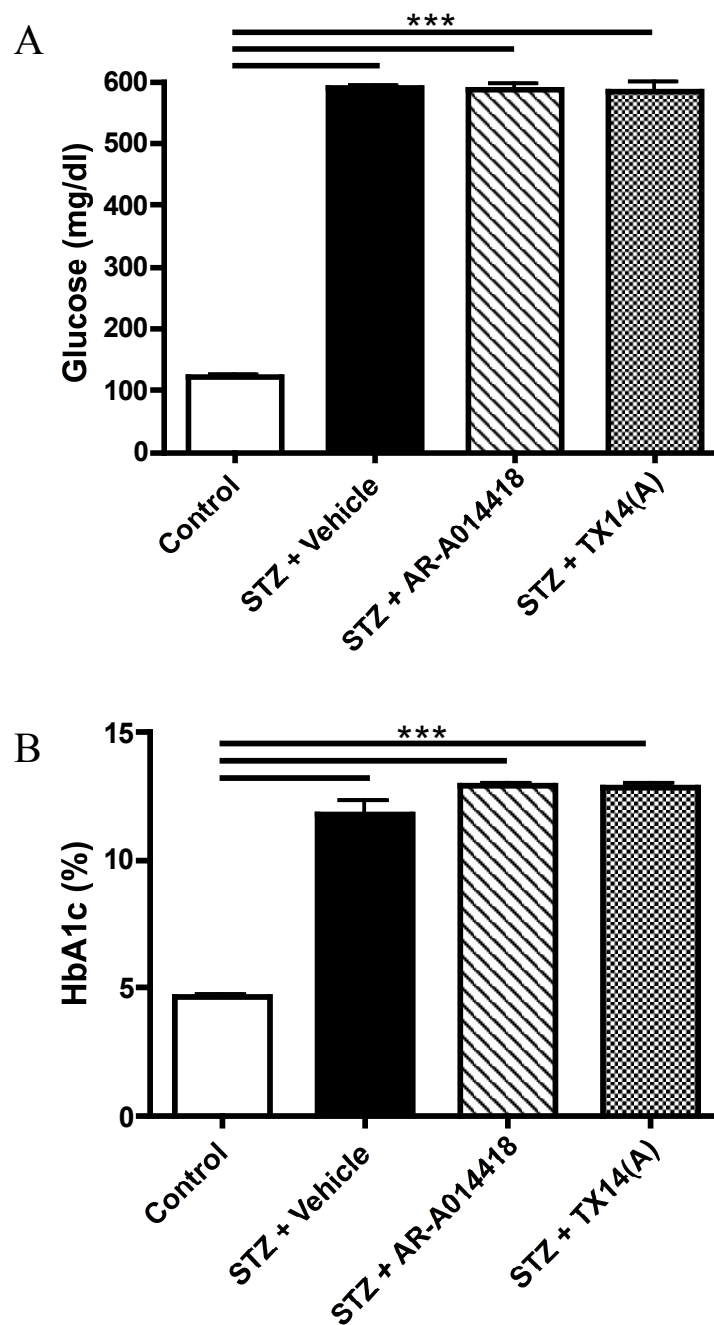


Figure 2: A: Blood glucose levels at the conclusion of the study (10 weeks). B: HbA1c levels at the conclusion of the study (10 weeks). Data are group mean + SEM. *** $p < 0.001$ by one-way ANOVA versus control, followed by Tukey's *post-hoc* test. N= 14 (control), 9 (STZ), 8 (AR), 8 (TX)

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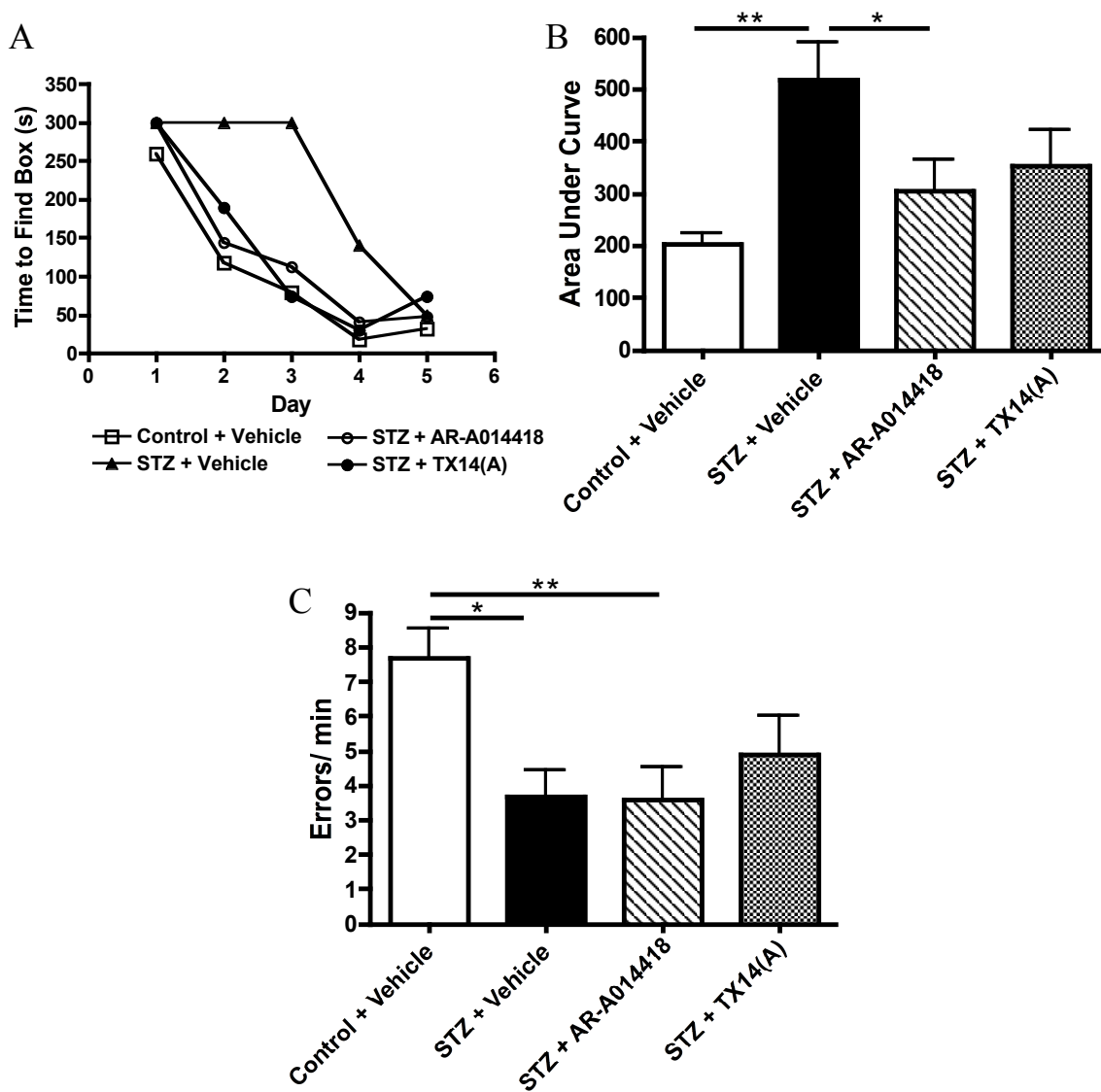


Figure 3: Effect of AR-A014418 or TX14(A) treatment on the Barnes maze test. A: Time to find the escape box. B: Area under the curve for A. C: Rate of errors made during the test. Data are group mean + SEM. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA versus control, followed by Tukey's *post-hoc* test. N= 14 (control), 9 (STZ), 11 (AR), 7 (TX)

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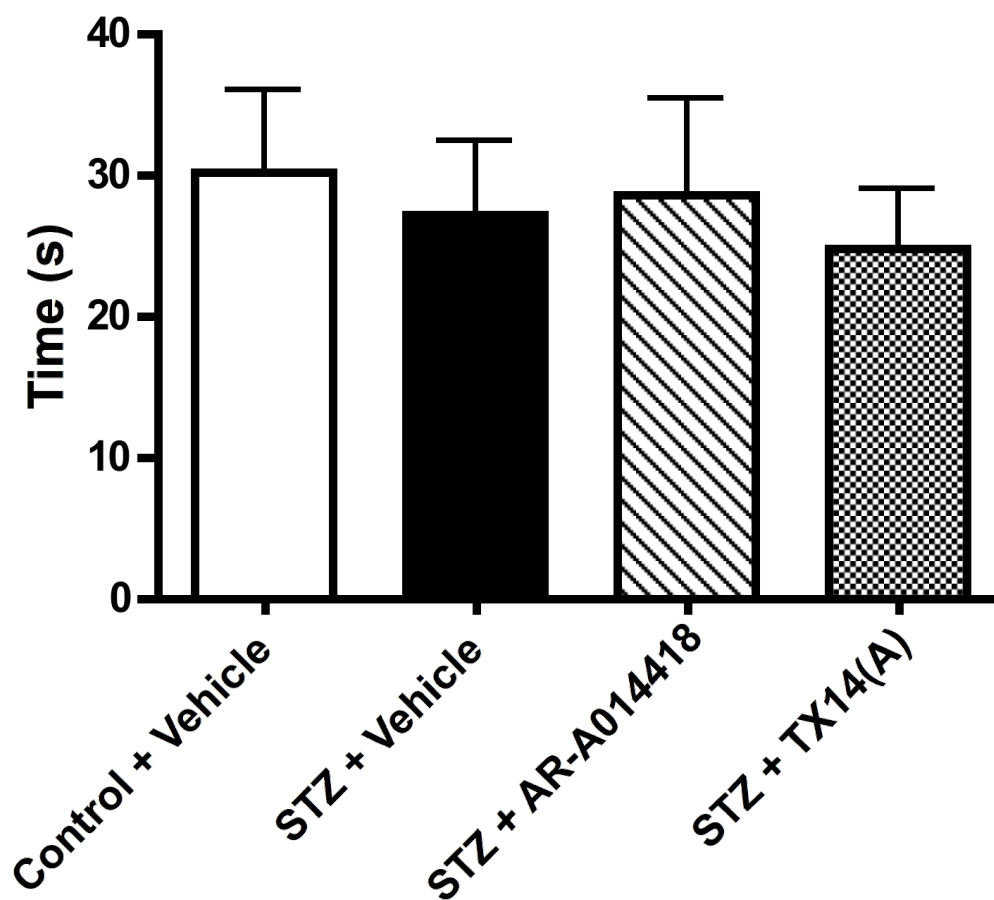


Figure 4: Effect of AR-A014418 or TX14(A) treatment on rotarod performance. Data are group mean + SEM. N= 8 (control), 9 (STZ), 7 (AR), 10 (TX)

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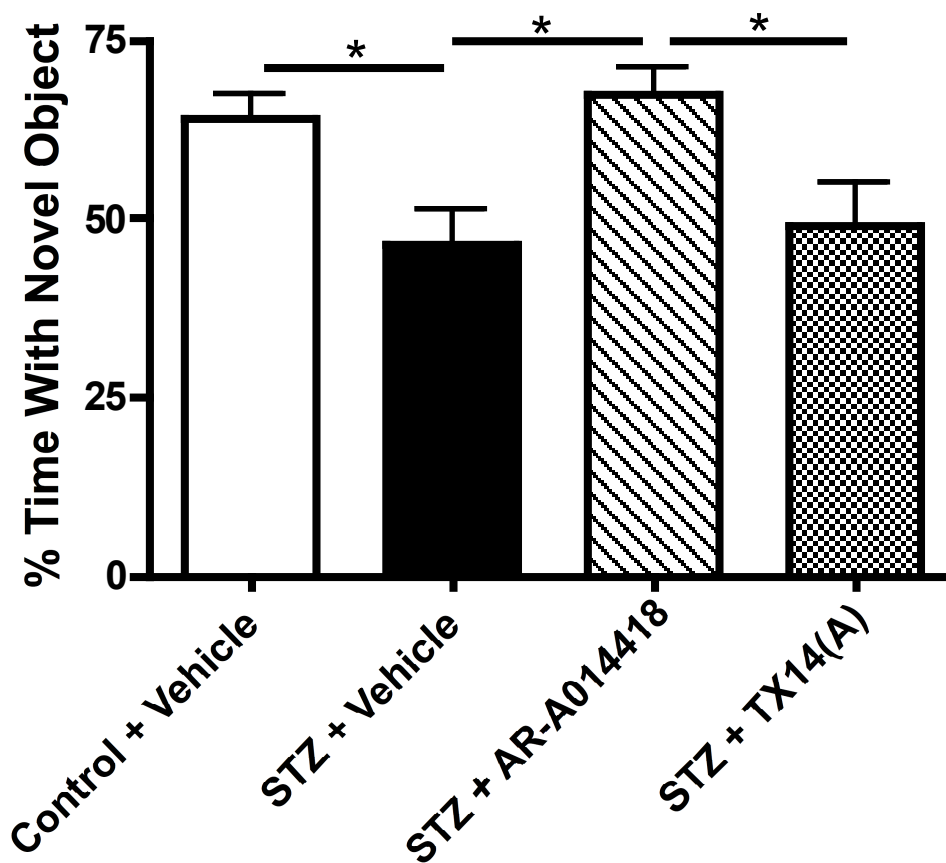


Figure 5: Effect of AR-A014418 or TX14(A) treatment on the object recognition test, expressed as percent of the total time spent with the novel object. Data are group mean + SEM. * $p < 0.05$ by one-way ANOVA, followed by Tukey's *post-hoc* test. N= 19 (control), 14 (STZ), 13 (AR), 13 (TX)

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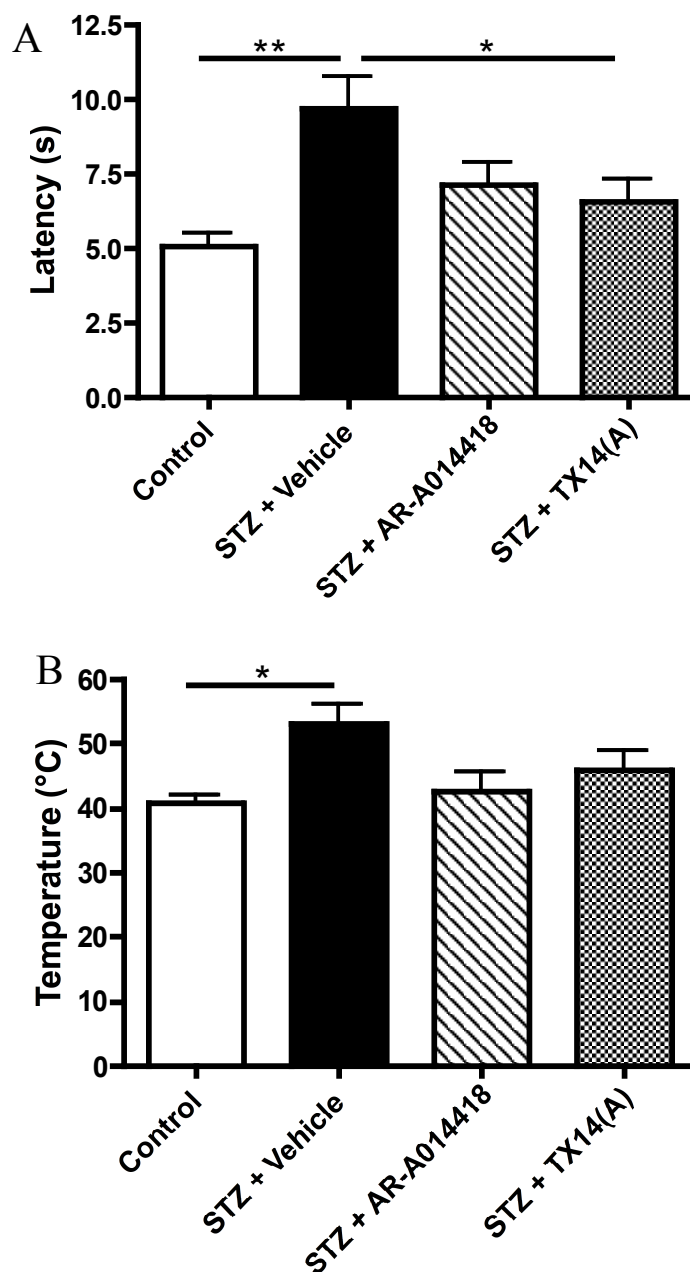


Figure 6: Effect of AR-A014418 or TX14(A) treatment on thermal withdrawal. A: Thermal withdrawal latency. B: Temperature at time of withdrawal. Data are group mean + SEM. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA, followed by Tukey's *post-hoc* test. N= 8 (control), 9 (STZ), 10 (AR), 11 (TX)

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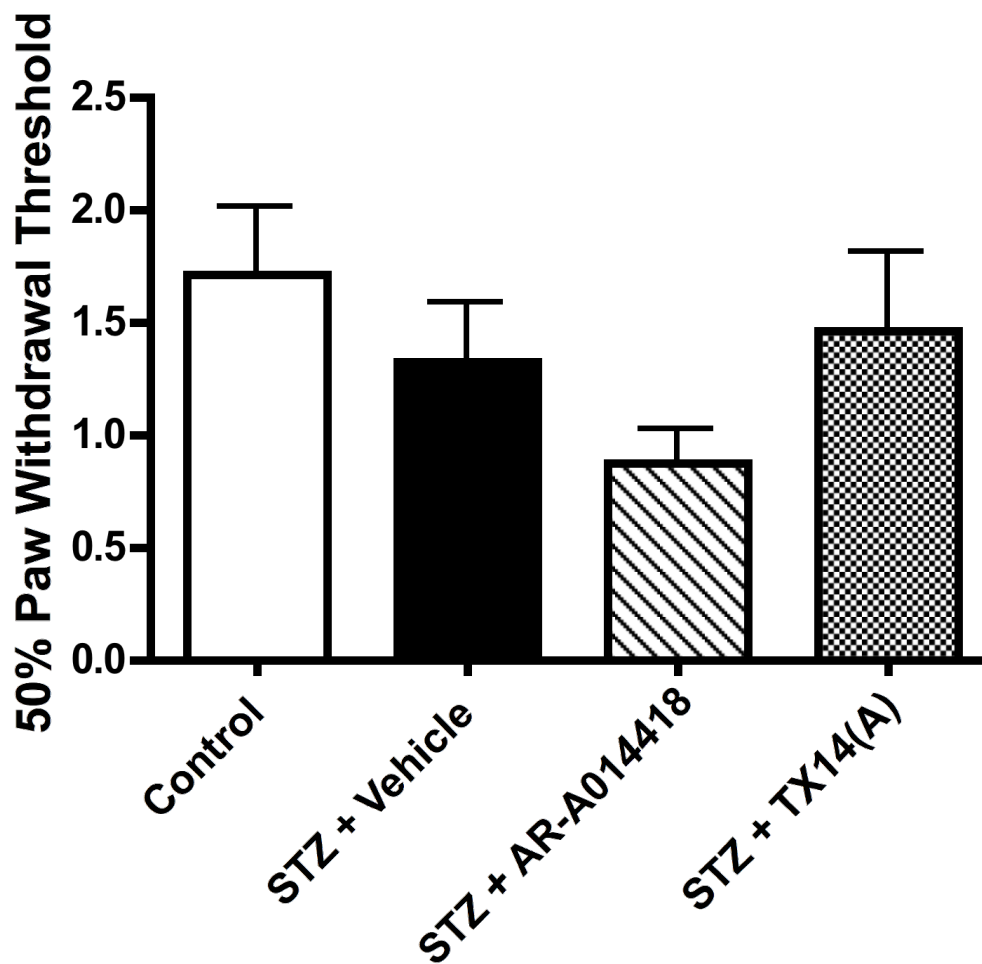


Figure 7: Effect of AR-A014418 or TX14(A) treatment on 50% paw withdrawal threshold. Data are group mean + SEM. N= 8 (control), 9 (STZ), 11 (AR), 11 (TX)

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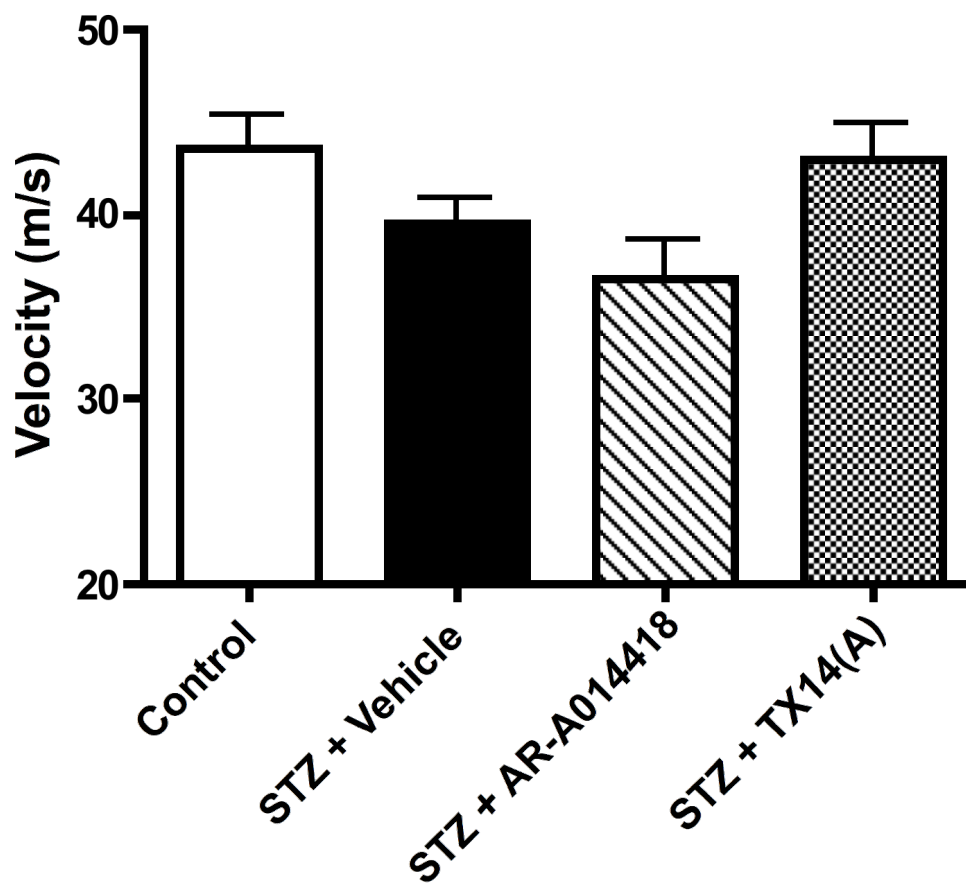


Figure 8: Effect of AR-A014418 or TX14(A) treatment on nerve conduction velocity. Data are group mean + SEM. N= 6 (control), 7 (STZ), 5 (AR), 9 (TX)

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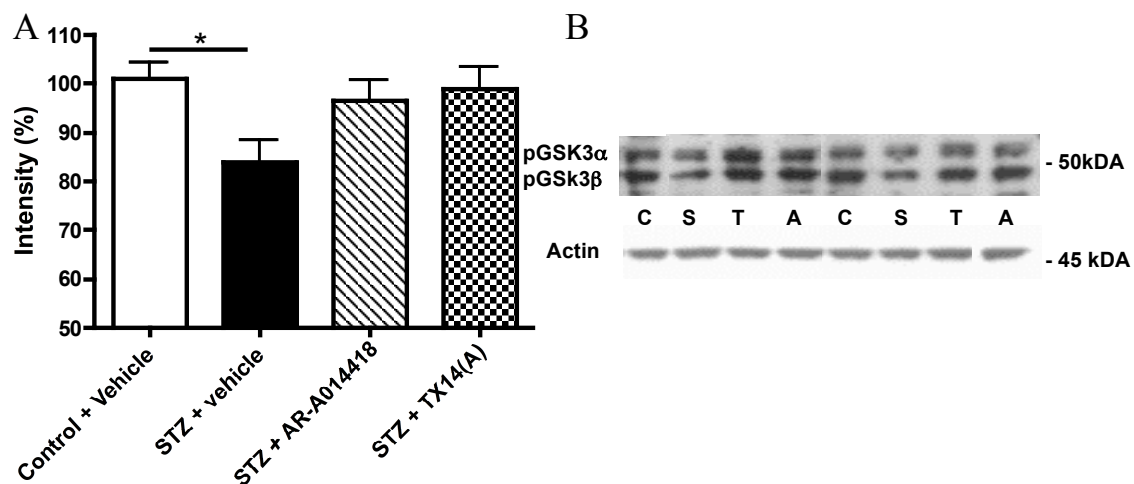


Figure 9: Effect of AR-A014418 or TX14(A) treatment on GSK3 β phosphorylation. A: Intensity of bands from mouse brain homogenates for phosphorylated GSK3 β at serine 9 (p-GSK3 β) over actin. Phosphorylation of serine 9 of GSK3 β isoforms results in inactivation of the enzyme. B: Western blots were performed with brain homogenates obtained from control (C) and STZ-diabetic (S) mice receiving vehicle and STZ-diabetic mice receiving AR-A014418 (A) or TX14(A) (T). Data are group mean + SEM. * $p < 0.05$ by one-way ANOVA versus control, followed by Dunnett's Multiple Comparison *post-hoc* test. N= 10 (control), 10 (STZ), 10 (AR), 7 (TX)

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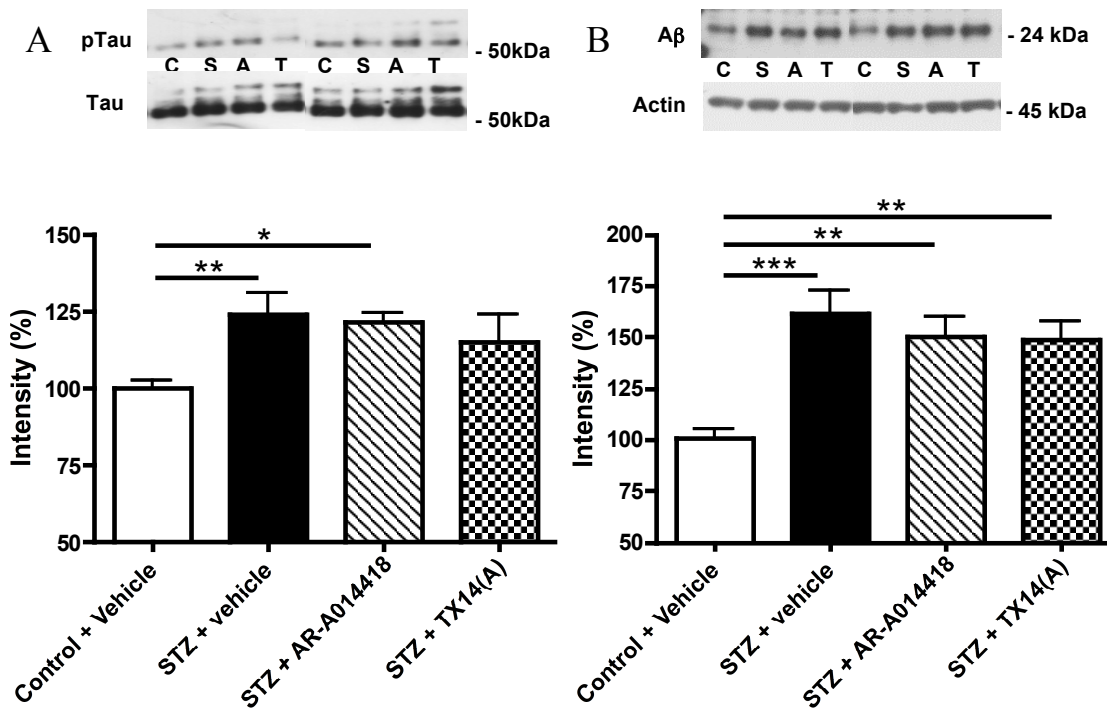


Figure 10: Effect of AR-A014418 or TX14(A) treatment on tau phosphorylation and amyloid β levels. A: Representative Western blot image and intensity of bands corresponding to phosphorylated tau and total tau. The data represent the ratio ptau/tau of the intensity of the bands corresponding to phosphorylated tau at threonine 231 over the intensity of the bands corresponding to total tau. Data are group mean + SEM. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA versus control, followed by Dunnett's Multiple Comparison *post-hoc* test. N= 8 (control), 8 (STZ), 8 (AR), 3 (TX). B: Representative Western blot image and intensity of bands corresponding to amyloid β over actin. Data are group mean + SEM. ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA versus control, followed by Dunnett's Multiple Comparison *post-hoc* test. N= 7 (control), 8 (STZ), 6 (AR), 6 (TX).

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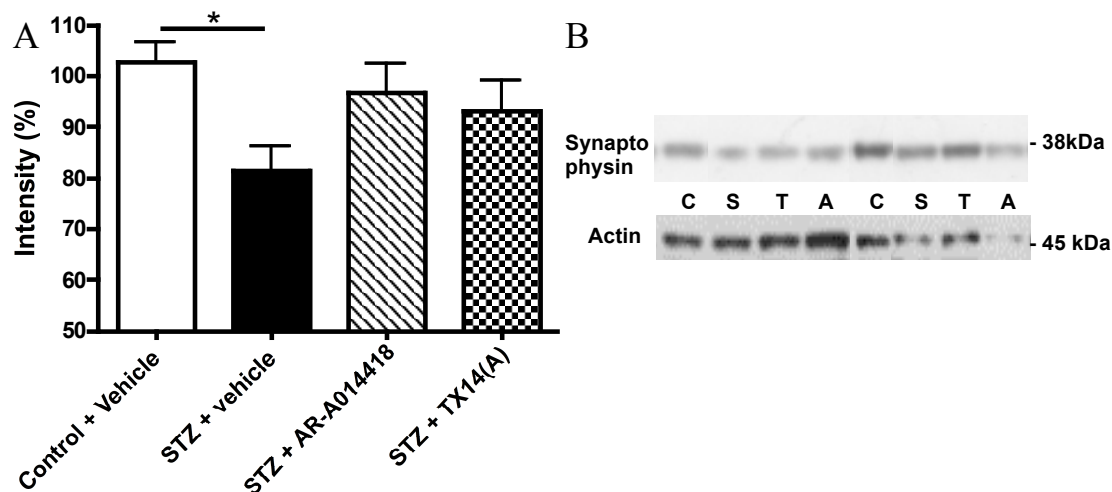


Figure 11: Effect of AR-A014418 or TX14(A) treatment on synaptophysin protein levels in mouse hippocampus. A: Intensity of bands corresponding to synaptophysin normalized to the intensity of bands corresponding to actin. Data are represented as group mean + SEM. * $p < 0.05$ versus control + vehicle group by one-way ANOVA followed by Dunnett's Multiple Comparison *post-hoc* test. N= 8 (control), 8 (STZ), 8 (AR), 3 (TX). B: Representative image of Western blots of brain homogenates from control (C) and STZ-diabetic (S) mice receiving vehicle and STZ-diabetic mice receiving AR-A014418 (A) or TX14(A).

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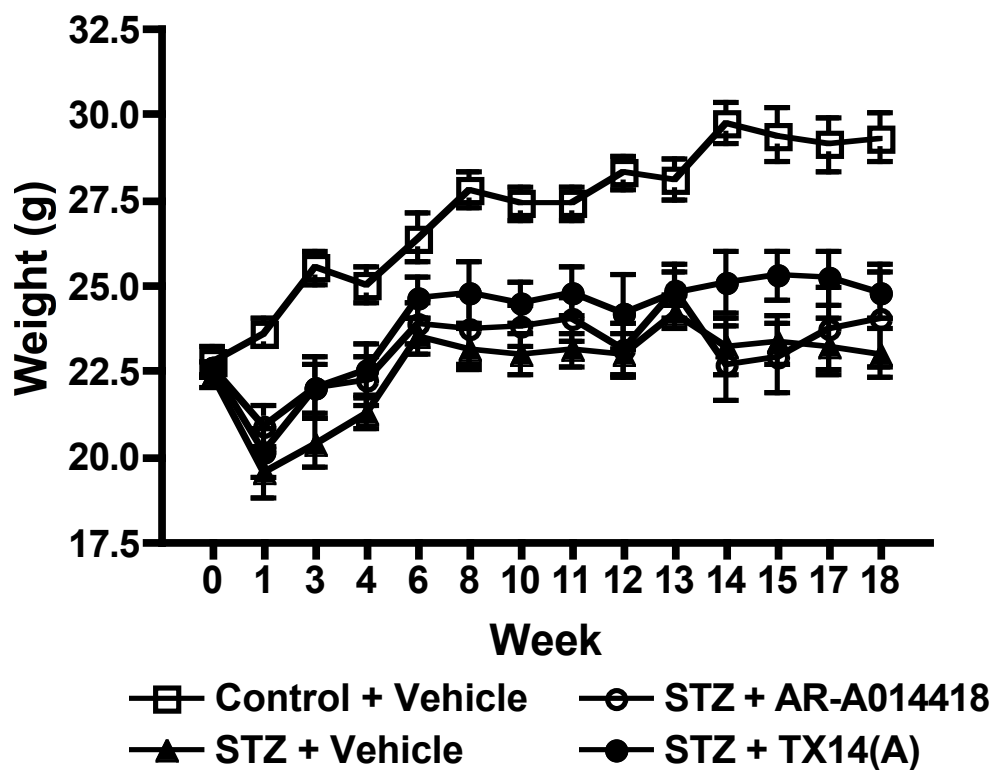


Figure 12: Body weight over the course of the study for control mice receiving vehicle (open squares), STZ-diabetic mice receiving vehicle (closed triangles), STZ-diabetic mice receiving AR-A014418 at 30 $\mu\text{mol/kg}$ (open circles) and STZ-diabetic mice receiving TX14(A) at 1mg/kg (closed circles). Data are group mean \pm SEM. N= 11-12 (control), 11-12 (STZ), 4-11 (AR), 8-13 (TX)

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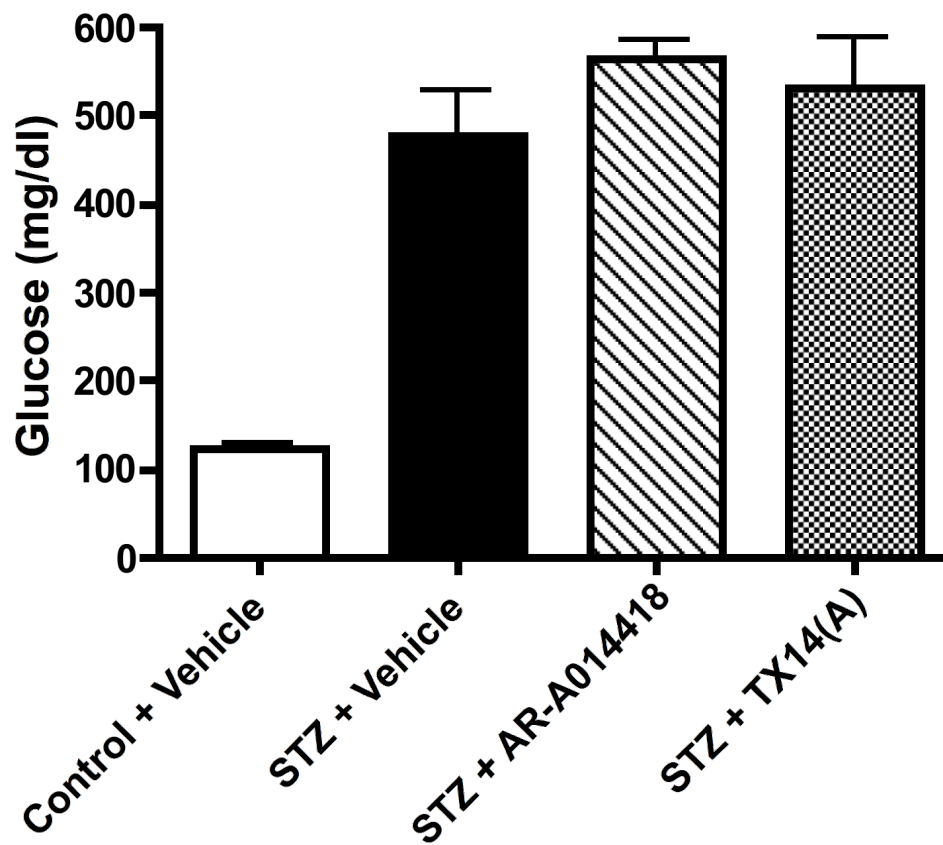


Figure 13: Blood glucose at the conclusion of the study (18 weeks). Data are group mean + SEM. N= 11 (control), 11 (STZ), 4 (AR), 8 (TX)

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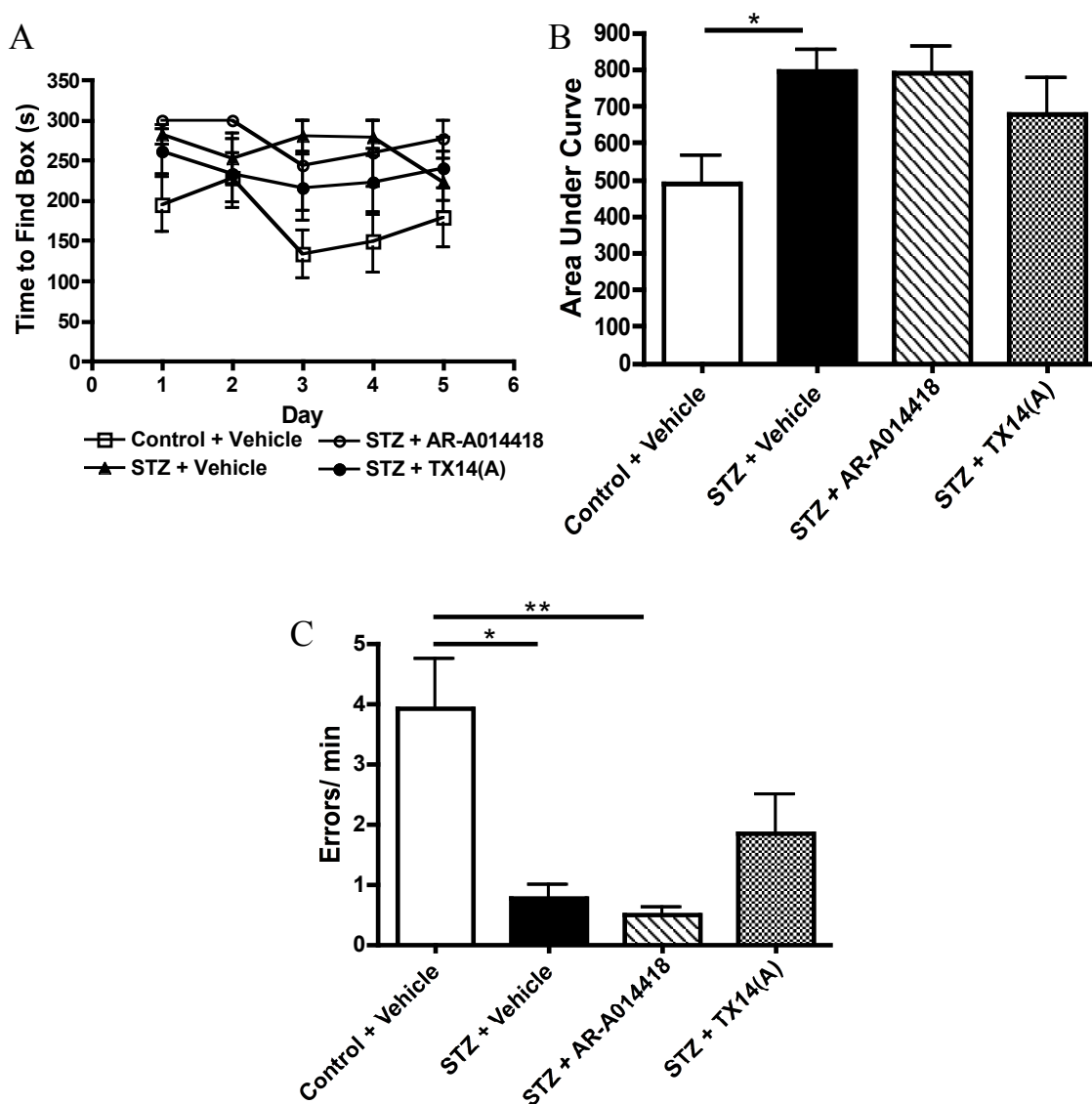


Figure 14: Effect of AR-A014418 or TX14(A) treatment on the Barnes maze test. A: Time to find the escape box for control mice receiving vehicle (open square), STZ-diabetic mice receiving vehicle (closed triangle), STZ-diabetic mice receiving AR-A014418 at 30 μ mol/kg (open circle) and STZ-diabetic mice receiving TX14(A) at 1mg/kg (closed circle). B: Area under the curve for A. C: Rate of errors made during the test. Data are group mean + SEM. * p <0.05 by one-way ANOVA versus control, followed by Tukey's *post-hoc* test. N= 11 (control), 11 (STZ), 4 (AR), 8 (TX)

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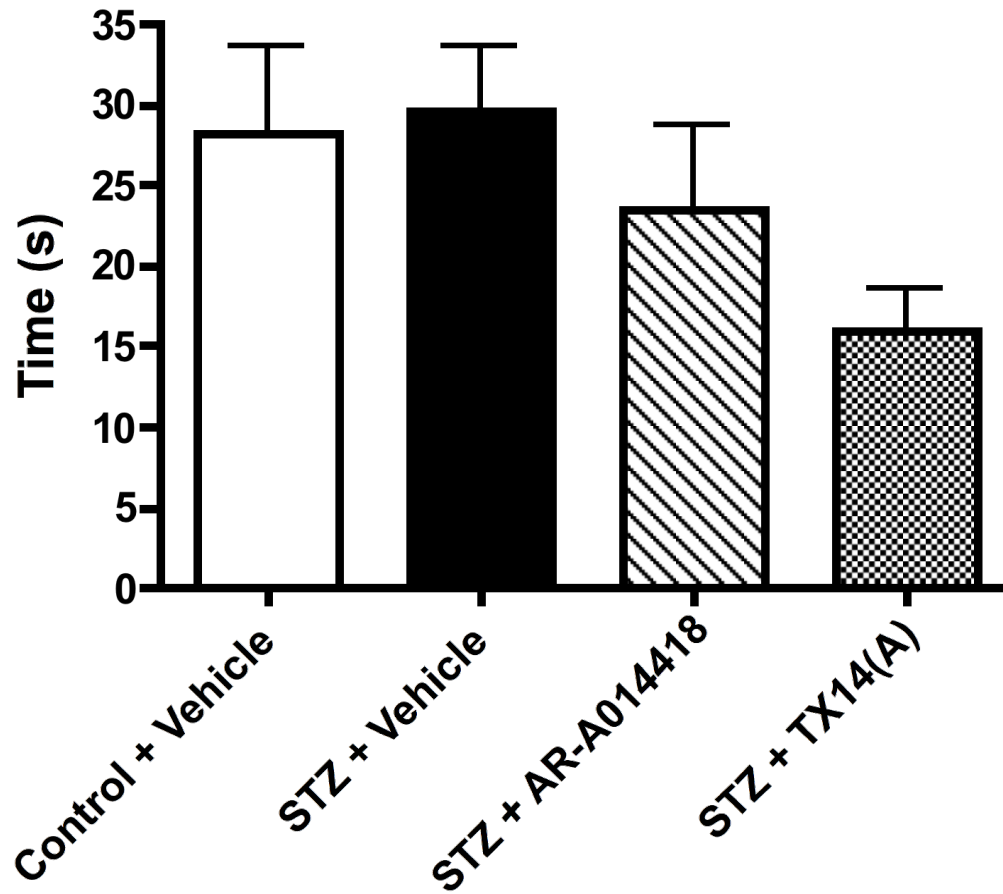


Figure 15: Effect of AR-A014418 or TX14(A) treatment on rotarod performance. Data are group mean + SEM. N= 11 (control), 11 (STZ), 5 (AR), 7 (TX)

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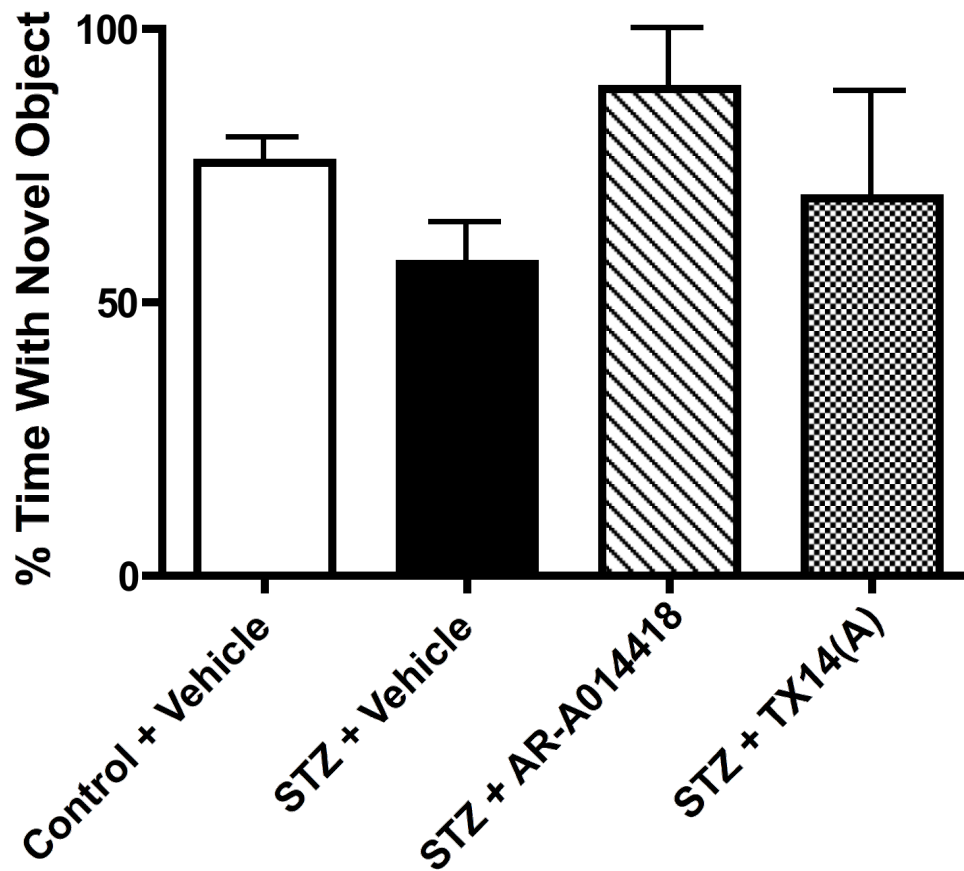


Figure 16: Effect of AR-A014418 or TX14(A) treatment on the object recognition test, percent time spent with the novel object. Data are group mean + SEM. N= 11 (control), 5 (STZ), 3 (AR), 3 (TX)

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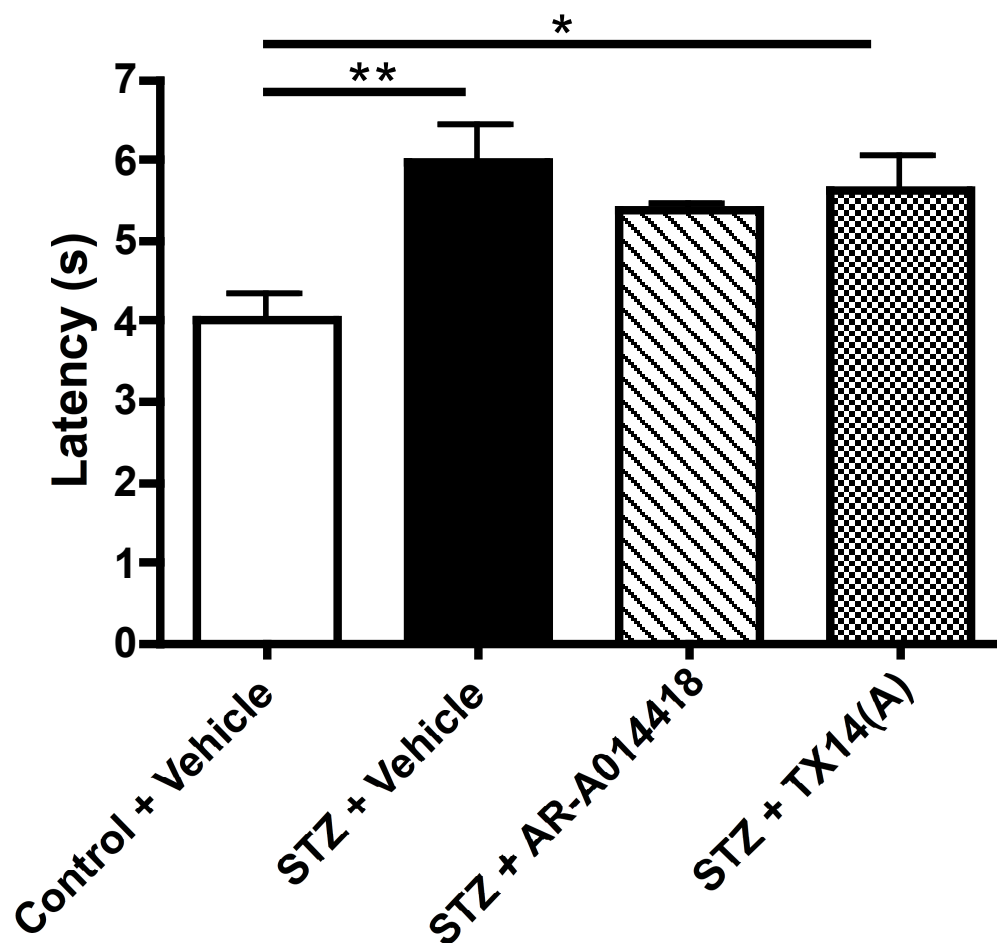


Figure 17: Effect of AR-A014418 or TX14(A) treatment on thermal withdrawal latency. Data are group mean + SEM. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA versus control, followed by Tukey's *post-hoc* test. N= 10 (control), 11 (STZ), 3 (AR), 8 (TX)

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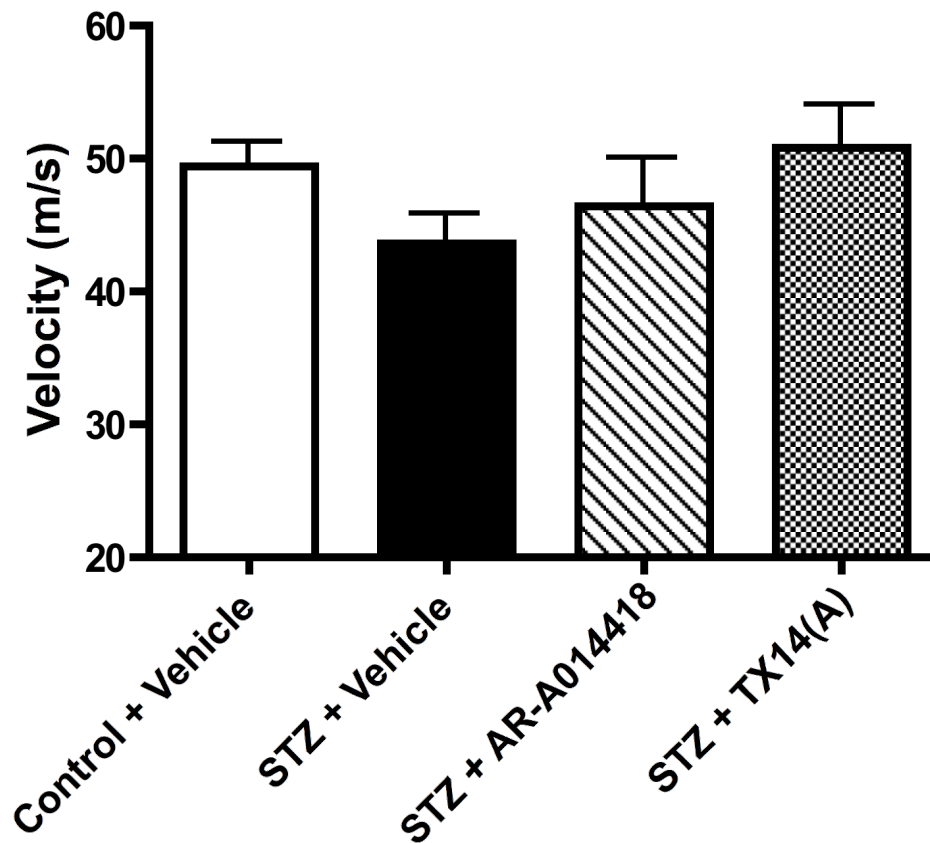


Figure 18: Effect of AR-A014418 or TX14(A) treatment on motor nerve conduction velocity. Data are group mean + SEM. N= 11 (control), 10 (STZ), 4 (AR), 7 (TX)

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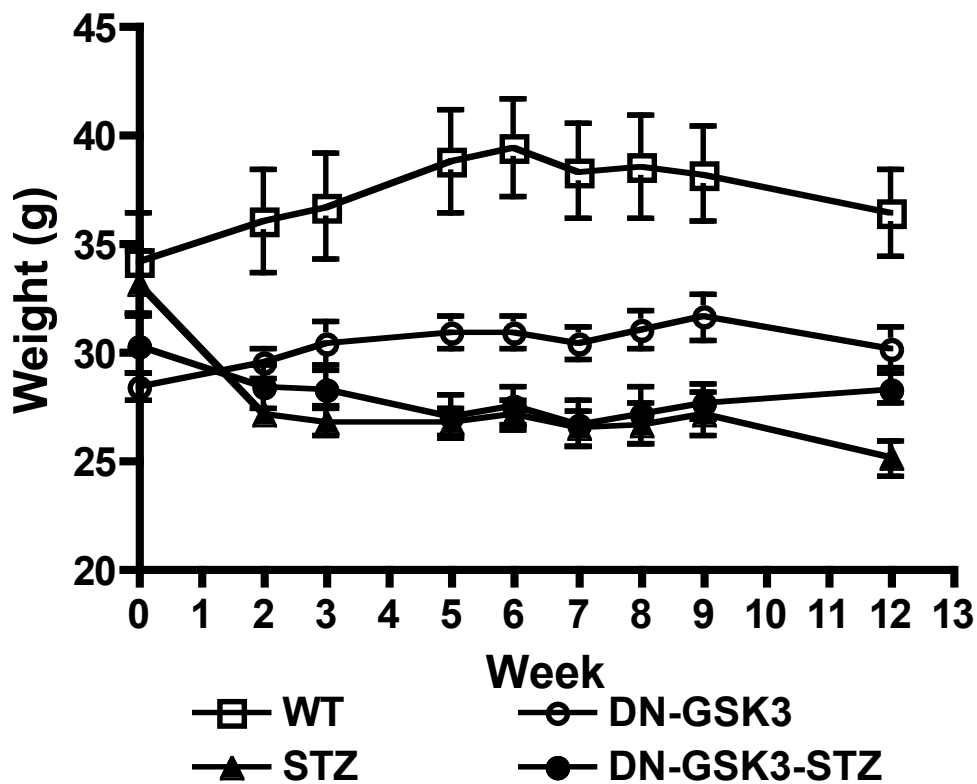


Figure 19: Body weight over the course of the study for wild type (WT) mice (open squares), STZ-diabetic (STZ) mice (closed triangles), DN-GSK3 mice (open circles), and STZ-diabetic DN-GSK3 (DN-GSK3-STZ) mice (closed circles). Data are group mean \pm SEM. N= 13-14 (WT), 15-19 (STZ), 10 (DN-GSK3), 12-15 (DN-GSK3-STZ)

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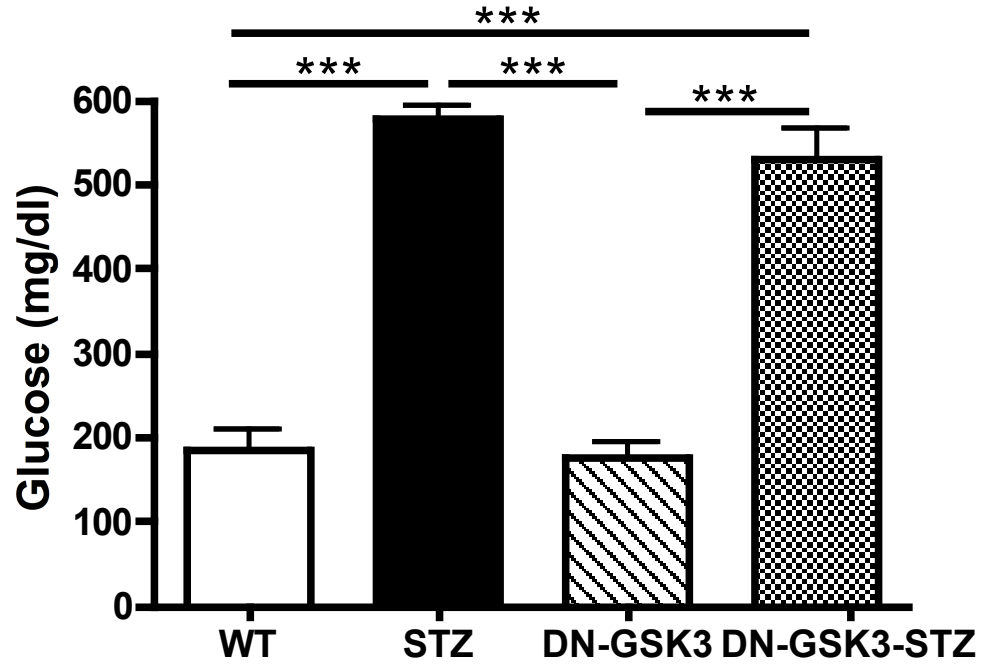


Figure 20: Blood glucose levels at the conclusion of the study. Data are group mean + SEM. N= 7 (WT), 5 (STZ), 5 (DN-GSK3), 4 (DN-GSK3-STZ)

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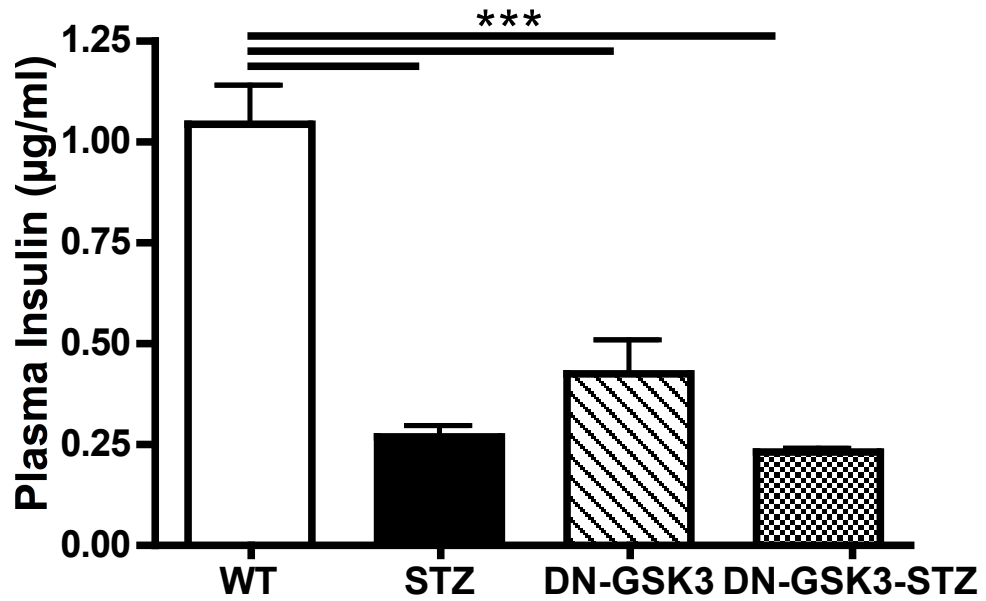


Figure 21: Plasma insulin levels at the conclusion of the study. Data are group mean + SEM. *** $p < 0.001$ by one-way ANOVA versus WT, followed by Tukey's *post-hoc* test. N= 6 (WT), 4 (STZ), 5 (DN-GSK3), 3 (DN-GSK3-STZ)

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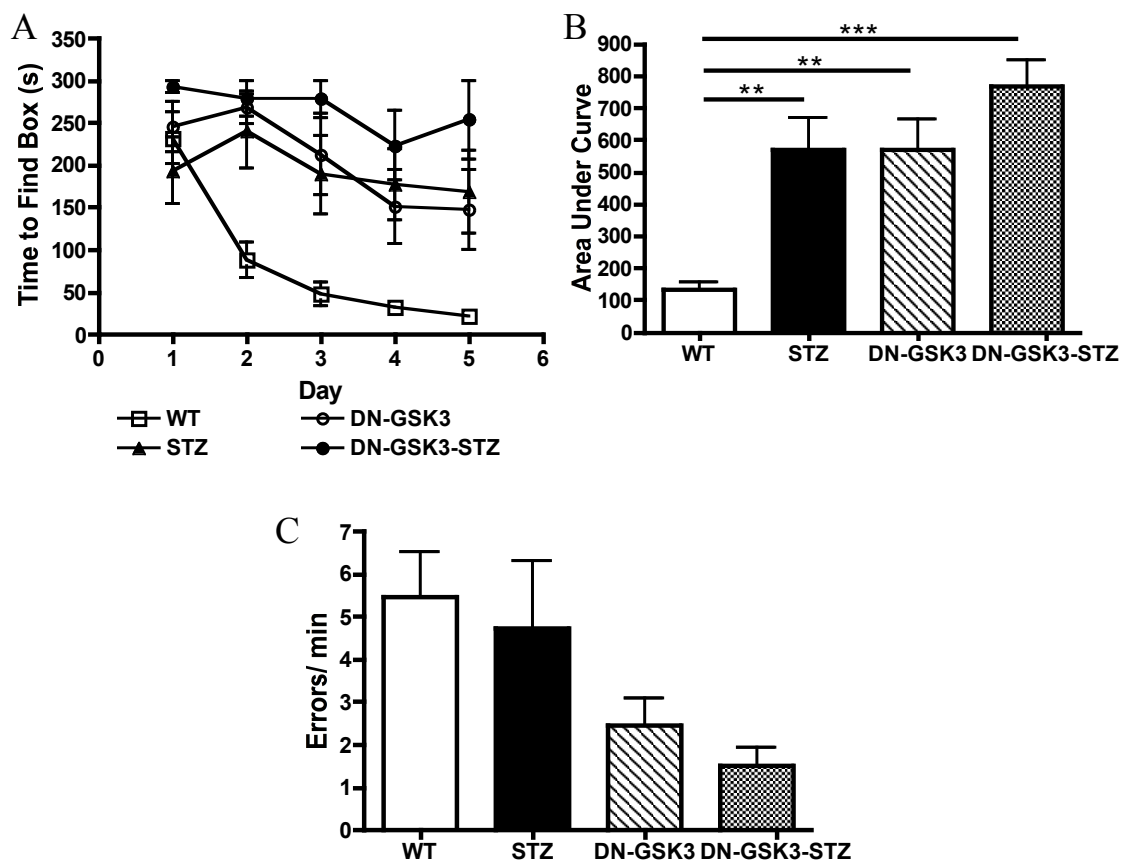


Figure 22: Barnes maze test. A: Time for mice to find the escape box in the Barnes maze test. B: Area under the curve for A. C: Rate of errors made during the test. Data are group mean + SEM. ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA, followed by Tukey's *post-hoc* test. $N = 7$ (WT), 6 (STZ), 5 (DN-GSK3), 5 (DN-GSK3-STZ)

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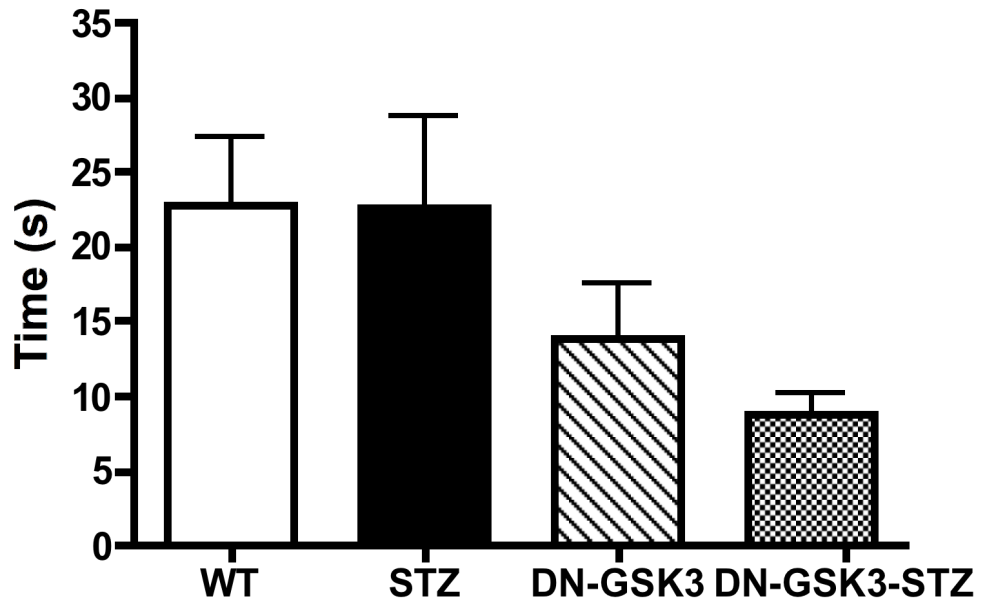


Figure 23: Time spent on Rotarod prior to falling off. Data are group mean + SEM. N= 14 (WT), 17 (STZ), 10 (DN-GSK3), 13 (DN-GSK3-STZ)

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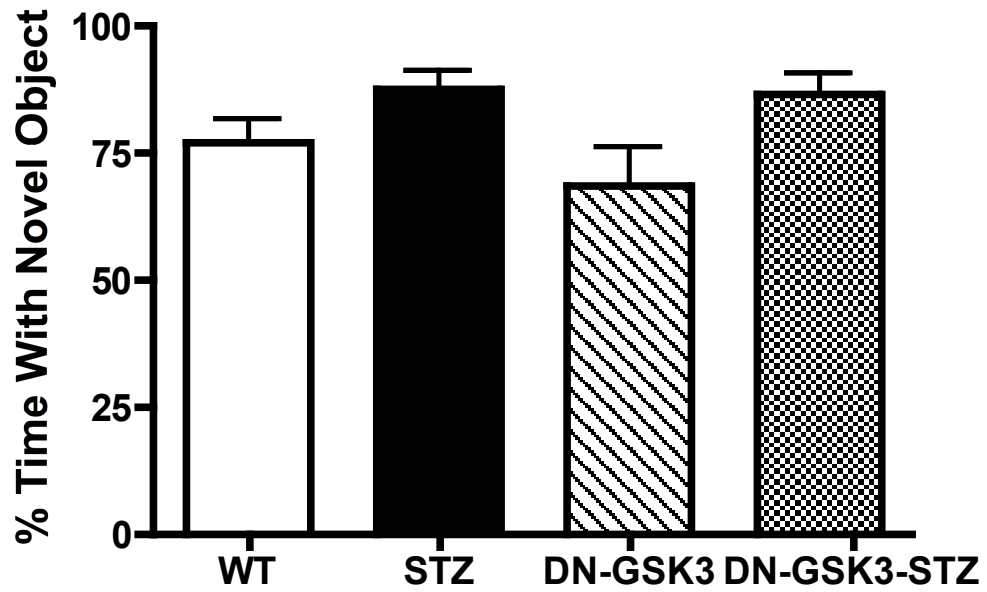


Figure 24: Object recognition test. Percent of the total time mice spent with the novel object. Data are group mean + SEM. N= 7 (WT), 4 (STZ), 4 (DN-GSK3), 5 (DN-GSK3-STZ)

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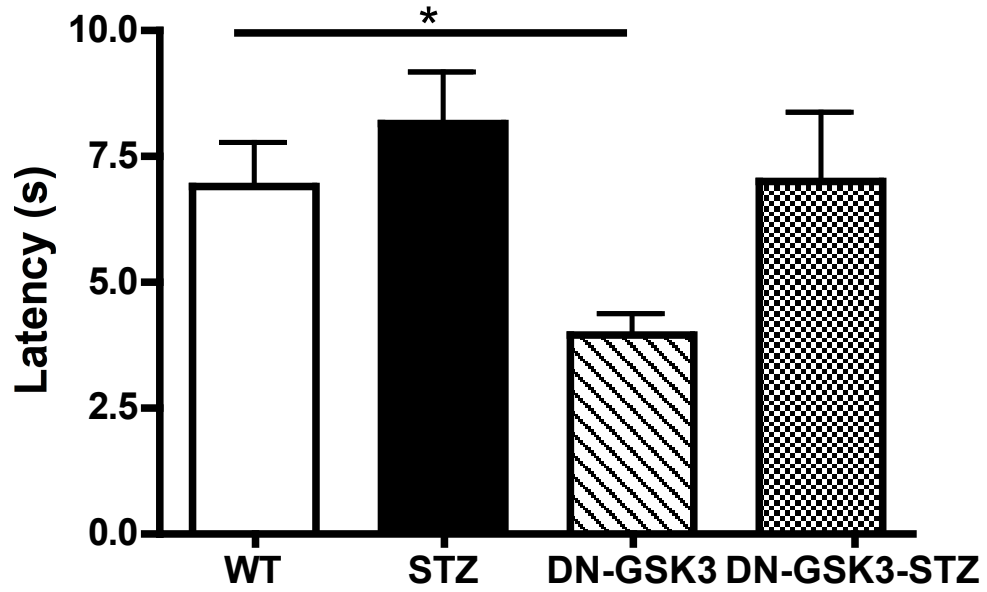


Figure 25: Thermal withdrawal latency. Data are group mean + SEM. * $p < 0.05$ by one-way ANOVA followed by Tukey's *post-hoc* test. N= 7 (WT), 6 (STZ), 5 (DN-GSK3), 5 (DN-GSK3-STZ)

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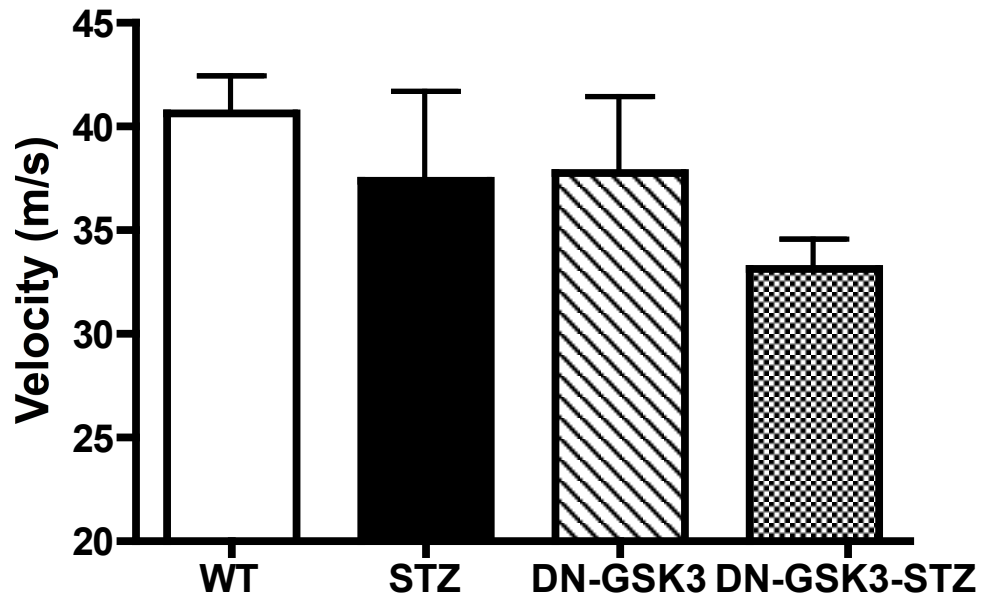


Figure 26: Motor nerve conduction velocity. Data are group mean + SEM. N= 6 (WT), 4 (STZ), 5 (DN-GSK3), 5 (DN-GSK3-STZ)

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