11-10-2021 2:00 PM

Assessment of Executive Function Using a Series of Operant Conditioning Based Tasks in T1DM Rodents

Kevin T. Murphy, *The University of Western Ontario*

Supervisor: Melling, Jamie CW, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Kinesiology

Abstract

This study examined the impact of Type 1 Diabetes Mellitus (T1DM) on executive function using a series of operant conditioning based tasks in rats. Sprague Dawley rats were randomized to either non-diabetic (n = 12; 6 male) or diabetic (n = 14; 6 male) groups. Diabetes was induced using multiple low-dose streptozotocin injections. All diabetic rodents were insulin-treated using subcutaneous insulin pellet implants. At week 14 of the study, rats were placed on a food restricted diet to induce 5 - 10% weight loss. Rodents were familiarized and tested on a series of tasks that required continuous adjustments to novel stimulus-reward paradigms in order to receive food rewards. No differences were observed in the number of trials, nor number / type of errors made to successfully complete each task between groups. Therefore, we report no differences in executive function, or more specifically set-shifting abilities between non-diabetic and diabetic rodents.

Keywords

Type 1 diabetes mellitus, cognition, executive function, set-shifting, reversal learning, brain, rat, insulin

Summary for Lay Audience

Type 1 Diabetes Mellitus (T1DM) is a disease in which the body loses the ability to produce insulin, a hormone that helps regulate blood sugar and provides energy to bodily tissues. Although there is no cure, patients with T1DM can lead a relatively normal life thanks to the invention of pharmaceutical insulin. However, T1DM increases risk of both short- (seizure, diabetic coma) and long-term (heart disease, vision and nerve problems) health complications. There have also been several studies that have demonstrated that patients with T1DM have minor brain abnormalities that may impair cognition. Research in both humans and animals has shown that T1DM is associated with decreased performance in tests of intelligence, information processing, and cognitive flexibility. Cognitive flexibility (also referred to as set-shifting) measures one's ability to adapt to changing circumstances or "outside of the box thinking". Many prior studies in animals have examined cognition, however very few have used tests that specifically assessed cognitive flexibility. Furthermore, very few animal studies have used rodents that were insulin-treated, the standard treatment for patients with T1DM. Therefore, the purpose of this study was to test the set-shifting abilities of insulin-treated T1DM rats compared to non-diabetic rats. Rats were randomly divided into two groups: twelve non-diabetic (six male, six female) and fourteen diabetic (six male, eight female). At week 14 of the study, rats were put on a food restricted diet to help motivate them to complete the set-shifting tasks. Once they lost 5% of their body weight, rats were familiarized with the testing apparatus and then progressed through three unique tasks. Each task corresponded to a specific rule that the rat must learn in order to receive a food reward. The number of trials it took for the rat to fully learn the rule, as well as the number of errors they accrued in learning the rule were recorded. There were no differences in either measure across the three tasks that were completed. Therefore, we conclude that insulin-treated T1DM rats do not show any decreases in cognitive ability, or more specifically set-shifting, compared to non-diabetic rats.

Co-Authorship Statement

Dr. Jamie Melling of Western University, London, Ontario, Canada was involved in project formulation and implementation, interpretation of results, and revision of thesis.

Dedication

To my entire family: Grandma, Poppa, Grammie, Grampie, Mom, Dad, Heather, Shirley and Madeline. Thank you all for everything you have done for me throughout my entire academic journey.

Acknowledgments

I would like to personally extend my gratitude to everyone who has helped me in completing this dissertation. First and foremost, thank you to my supervisor Dr. Jamie Melling for your continuous support and guidance throughout every stage of this project. These past 24 months have proven extraordinarily unique given the COVID-19 pandemic and I greatly appreciate your unwavering support throughout. I would also like to thank Dr. Raj Rajakumar for his help in formulating and overseeing the project, as well as his tremendous insight assisting in the interpretation of results.

To Sarah Myers, I owe you an immense amount of gratitude for your expertise in facilitating and troubleshooting the operant conditioning chamber based set-shifting aspect of this project. Without your outstanding support, this project would not have been possible. To all of my lab mates, both present and former: David McBey, Justin Camenzuli, Jordan Larocque, Silar Gardy, and Samantha Li: you all were incredibly pleasant to work alongside, and I greatly enjoyed every minute of it whether it be in-person or virtually via video chat. I would like to especially recognize David for his guidance and leadership as a senior Ph.D. student. A special thank you to Justin as well for his assistance in providing animal care when called upon.

Thank you to all of The Animal Care and Veterinary Services (ACVS) and Psychology Animal Facility staff including Asma Al-Sadouni and Francis Boon for their support and care of the animals throughout their lifespan. I greatly appreciate the veterinary staff for the extraordinary care given to the diabetic animals when required.

To all my family: I am greatly appreciative of the continuous support and encouragement you have provided throughout my entire academic career. I would not be here without you. Thank you, Madeline, for your patience and support in completion of my Master's Degree.

Table of Contents

List of Abbreviations

ANOVA – Analysis of Variance

DCCT – Diabetes Control and Complications Trial

EDIC – Epidemiology of Diabetes Interventions and Complications

EF – Executive Function

HbA1c – Glycated Hemoglobin

IR – Insulin Resistance

mmol/L – Millimoles per Litre

mPFC – Medial Prefrontal Cortex

MWM – Morris Water Maze

ORT – Object Recognition Task

PFC – Prefrontal Cortex

RD – Response Discrimination

RL – Reversal Learning

SD – Standard Deviation

SEM – Standard Error of the Mean

STZ – Streptozotocin

T1DM – Type 1 Diabetes Mellitus

T2DM – Type 2 Diabetes Mellitus

VCD – Visual Cue Discrimination

VCR – Visual Cue Retrieval

WCST – Wisconsin Card Sorting Task

List of Figures

List of Appendices

Chapter 1

1 Literature Review

1.1 Overview of Type 1 Diabetes Mellitus

Diabetes mellitus (DM) refers to a category of disorders with the commonality of insulin dysfunction, and resultant chronic hyperglycemia (high blood glucose). Although they may be classified similarly, the two main types of diabetes (type 1 and type 2) are independent diseases with unique etiologies and complications.

Glucose metabolism involves contributions from many different organ systems and pathways including the liver, pancreas, and skeletal muscle [1]. Fundamentally, blood glucose is controlled by two antagonistic hormones. Glucagon acts to release stored glucose into the bloodstream, ultimately raising blood glucose. Conversely, insulin acts to facilitate glucose uptake into target tissues, such as skeletal muscle, lowering blood glucose in the process [2].

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by the destruction of the insulin producing β-cells of the pancreas [3]. This results in a partial, or more often complete inability for the body to produce insulin, leading to unregulated hyperglycemia [4]. As such, patients with T1DM are dependent on exogenous insulin typically administered via injection, or insulin pump. Patients with T1DM must continually monitor and delicately modulate both insulin and blood glucose levels in order to maintain euglycemia (normal blood sugar). If left unregulated, acute hypoglycemia (low blood glucose) can have catastrophic consequences such as seizures, coma, or in rare instances death [5]. T1DM may also result in long-term complications such as cardiovascular disease, neuropathies, and retinopathies, among others [6-8]. The cause(s) of T1DM remain largely unknown, however genetic, immunologic, and environmental contributions are likely factors [9].

In contrast, type 2 diabetes mellitus (T2DM) is a metabolic disease occurring as a result of a combination of genetic and environmental risk factors, some of which are controllable (diet, sedentary behaviour, and/or obesity) [10]. T2DM is characterized by progressive insulin resistance (IR) coupled with chronically elevated insulin levels

(hyperinsulinemia), and hyperglycemia [11]. As target tissues become resistant to the effects of insulin, the pancreas compensates by secreting more insulin, leading to a hyperinsulinemic environment. IR directly affects the body's ability to clear glucose from the bloodstream, and over time will result in a state of chronic hyperglycemia, the hallmark symptom of T2DM. Although several treatment options are available, patients with T2DM can eventually become insulin dependent.

Historically, T1DM was referred to as juvenile diabetes as it was thought to occur only in children. It has since been reclassified as type 1 diabetes mellitus, as onset may occur at any age, although it is most common in children under 14 years old [12]. T1DM onset is usually accompanied by polydipsia, polyphagia, and polyuria (excessive thirst, hunger, and urination, respectively) [13]. Diagnosis is typically confirmed when any of the three following criteria are met: fasting blood glucose > 7 mmol/L, any blood glucose value > 11.1 mmol/L, or an HbA1c value of 6.5% or higher [14, 15]. HbA1c is a form of hemoglobin, an oxygen transporting protein found in red blood cells, that has been bound to glucose in a process called glycation. This is a normally occurring process, however sustained hyperglycemia will lead to a higher concentration of HbA1c. Hemoglobin that has been glycated will remain so for the lifespan of the red blood cell, which is approximately 120 days [16]. As a result, HbA1c concentration serves as an indicator of long-term glycemic control and is a hallmark indicator of both T1DM and T2DM management [17]. In an otherwise healthy individual, HbA1c is below 5.7%. A higher HbA1c value indicates poor glycemic control and even as little as a 1% increase has been shown to correspond to a 30% increase in all-cause mortality [18].

Although tremendous progress has been made in regard to the knowledge and treatment of T1DM, there is presently no cure, nor means of predicting or preventing disease onset. Moreover, the incidence rate of T1DM has increased globally by approximately 3% over the past 30 years and is predicted to continue to trend upwards [19, 20]. It is reported that there are approximately 300,000 Canadians currently living with T1DM, and this number is expected to grow to nearly 400,000 by the year 2030 [21].

In a landmark study that permanently altered the landscape of T1DM patient care, The Diabetes Control and Complications Trial (DCCT) sought to explore the outcome of stricter glycemic control on long-term complications of T1DM [22]. Patients were randomly allocated to receive either conventional insulin therapy consisting of one or two insulin injections per day, or intensive insulin therapy, which included the administration of insulin three or more times per day with the goal of maintaining strict blood glucose values within the range of 4 - 7 mmol/L. At the end of the study, it was apparent that intensive insulin treatment significantly reduced several diabetic comorbidities. More specifically, intensive insulin treatment was shown to reduce HbA1c by approximately 2%, as well as reduce the risk of developing retinopathy, nephropathy, and neuropathy, amongst other positive benefits. Intensive insulin therapy has since been adopted into clinical care as a direct result of these findings and has vastly improved the long-term health and wellbeing of those with T1DM.

1.2 Type 1 Diabetes Mellitus and Cognition

Glucose, a simple sugar, is an essential source of energy in humans, playing a critical role in the metabolic processes of nearly every tissue. Perhaps most importantly, the human brain is an obligate glucose user, consuming up to 60% of circulating blood glucose in a fasted, sedentary state [1]. The human brain functions as the core of the central nervous system, controlling nearly every aspect of our body including cognition, sensory processing, and motor control. Due to its critical function, complex control systems regulate blood glucose homeostasis, ensuring it remains within a physiological range of 4 - 6 mmol/L (in a fasted state). Since the consequences of extreme and/or sustained deviations outside of this range can be catastrophic [5], the preservation of a euglycemic state is crucial to survival. Given the sensitivity of the brain and the perturbations in glycemic control caused by metabolic disorders such as T1DM, it is not surprising that there are links between metabolic disorders and brain dysfunction. Indeed, T1DM has been shown to result in varying degrees of impairment across different cognitive domains [23]. Cognitive impairments specifically refer to deficits in neurophysiological processes such as memory, learning, concentration, and/or decision making [24].

As early as 1922, diabetes mellitus has been implicated as a contributor to cognitive impairments. Miles *et al.* [25] demonstrated that patients with diabetes displayed a decrement of approximately 15% in tests of memory and attention compared to healthy individuals. Interestingly, they also noted that with treatment, patients with diabetes rapidly recovered their performance to "near normal" levels. Since this seminal work, many studies have specifically examined the relationship between T1DM and cognition presenting similar findings.

Brands *et al.* [23] conducted a meta-analysis including thirty-three studies comparing the cognitive performance of T1DM adults (minimum 18 years post-diagnosis) to their non-diabetic counterparts. T1DM groups demonstrated significantly lower performance in an array of cognitive domains including: intelligence, information processing, psychomotor efficiency, visual and sustained attention, cognitive flexibility (also referred to as "set-shifting"), and visual perception. However, learning and memory domains were spared. Impaired cognition appeared to be correlated with microvascular complication, but not severe hypoglycemic events, nor poor metabolic control.

Executive function (EF) is an integral component of cognition and is especially pertinent in relation to T1DM management as it requires intricate care [26]. EF encapsulates specific mental processes including inhibition, interference control, working memory, and cognitive flexibility [27] that underlie human behaviour and learning. EF is governed mainly by the frontal lobe of the brain (which includes the prefrontal cortex), with the parietal and cerebellar lobes also serving important roles [28]. Impairments in EF are of particular importance to the patient with T1DM since deficits in these skills are associated with inferior self-regulation, planning, problem-solving and decision making [29]. These behaviours are all important in diabetes management and may be a key factor contributing to clinical outcomes of T1DM. A recent analysis has shown subtle, but significant impairments in groups with T1DM across inhibition, working memory, and set-shifting domains [30]. Several risk factors such as age at onset, prior hypoglycemic episodes, and chronic hyperglycemia were explored in this analysis. Although some significant results were reported, the authors note that there may not be enough data to draw definitive conclusions regarding the effect of specific risk factors on EF. Given this, and the paucity

of data specifically assessing EF / set-shifting in T1DM populations, data pertaining to T1DM and more general measures of cognition will be explored throughout this chapter.

Cognitive dysfunction has also been observed in younger populations. Meta-analysis data in children aged nineteen or younger demonstrated lower performance across multiple cognitive domains amongst those with T1DM compared to non-diabetics [31]. Similar to results from Brands *et al.* [23], learning and memory were unaffected by T1DM. Subgroup analysis of this study determined that children with early-onset diabetes (< 7 years old at diagnosis) performed worse than those with late-onset diabetes. This finding is supported by a subsequent large-scale population-based study in Sweden which found that children with T1DM scored lower grades in school and were more likely to fail their classes [32]. These negative effects appeared to be magnified amongst those with a younger age at diabetes onset, suggesting that the age at onset of diabetes may be a determinant of cognitive function.

Since there is an established consensus across the literature recognizing cognitive impairments as a comorbidity of T1DM, we will next investigate potential risk factors of impaired cognition. Due to the vast amount of literature and the complexity of certain underlying mechanisms, only a handful of landmark studies will be discussed. This is appropriate given the scope of this project and its lack of biochemical and histological analysis. As evidenced above, it appears that age at diabetes onset may be an important risk factor in relation to the development of cognitive dysfunction [31, 32]. This is theorized to be due to chronic hyperglycemia induced structural and functional changes that adversely affect the central nervous system at crucial periods of development in children [33]. More specifically, the medial prefrontal regions, insula, and cerebellum were shown to be negatively altered in children with early onset T1DM [34].

Metadata from Brands *et al.* [23] presented above implicated microvascular complications as a determinant of cognitive dysfunction. This is supported by both cross sectional [35, 36] and longitudinal [37, 38] data which demonstrates that cognitive dysfunction is associated with retinopathies, nephropathies, neuropathies and other microvascular pathologies in adults with T1DM. The reasons for this are complex and not entirely clear,

however in a recent review, impaired vascular tone, capillary plugging, and blood brain barrier disruption were purported as potential mechanisms [39].

An epidemiological follow-up study to the aforementioned DCCT, titled Epidemiology of Diabetes Interventions and Complications (EDIC), followed 1144 patents for 18 years. Neither prior severe hypoglycemic events, nor intensive versus conventional insulin therapy had any effect on the assessed cognitive domains. However, both higher HbA1c levels and the presence of microvascular complications were associated with impaired performance in certain cognitive domains [38]. Participants with poorer metabolic control (HbA1c > 8.8%) performed significantly worse on measures of psychomotor efficiency and speed compared to those with better control (HbA1c < 7.4%). It is thought that poor long-term metabolic control perhaps serves as a proxy to the development of diabetic comorbidities such as microvascular complications, which in turn explains the observed cognitive dysfunction [37]. This may in part explain why metadata from Brands *et al.* [23] implicated microvascular complications but found no independent effect of metabolic control on cognition.

Although the evidence seems to clearly outline a relationship between, long-term glycemic control and cognition, the effect of prior severe hypoglycemic episodes on subsequent cognition remains ambiguous. As outlined above, severe hypoglycemia is a complication of insulin therapy which can have profoundly negative effects on the body [5]. It is well documented that cognition may be impaired during transient states of severe hypoglycemia [40, 41], however it is not clear how these acute states affect future cognitive performance in the long-term. The EDIC [38] showed that the cumulative number of severe hypoglycemic events did not affect performance in any cognitive domain. This finding is replicated by high quality data from several other works [23, 36 , 42 ,43]. However, some cross-sectional studies [44-46], as well as a recent, large scale perspective cohort study [47] demonstrated evidence that prior severe hypoglycemic episodes do not affect cognition.

Given the constraints and potential ramifications of manipulating long-term glycemic control in humans, the majority of research presented thus far is observational in nature. Experimental work in pre-clinical animal models can provide important insight into

certain factors that are not feasible to manipulate in humans. Several studies in rats and mice have also shown that cognitive decrements exist in a T1DM population relative to respective non-T1DM counterparts [48-51]. An important consideration is the effect of insulin, since it is not always utilized as a treatment strategy in diabetic animal models. In a landmark paper, Biessels *et al.* [52] demonstrated that insulin treatment was effective at preventing cognitive decrements in streptozotocin (STZ)-induced T1DM rats. Interestingly, insulin was effective at preserving cognitive performance if immediately administered following diabetes induction, however if insulin treatment was deferred to later in the course of the disease (administered ten weeks following diabetes induction), cognitive deficits were observed. A more recent study found similar results [53], showing that insulin treatment administered immediately following diabetes induction preserved the performance of rats in cognitive assessments when compared to non-insulin treated diabetic rats. However, insulin treatment only partially prevented adverse structural and hormonal changes.

Although there is mixed evidence over which specific domains are affected and the impact of specific risk factors, it is clear that the literature provides strong evidence for a relationship between T1DM and cognitive impairment. Age at diabetes onset, long-term glycemic control, microvascular complications, as well as the administration of insulin all appear to modulate the presence and severity of both cognitive dysfunction and structural alterations.

1.3 Methods of Assessing Cognitive Function

There are many valid methods of assessing cognitive function in a wide variety of animal models and in humans, each requiring the use of various cognitive domains and corresponding brain areas. Given the abundance of assessment methods available throughout the literature, only a few relevant cognitive assessment tools will be discussed. The majority of research carried out in T1DM rodent models to date has utilized tests that assess predominantly hippocampal function such as the Morris Water Maze (MWM), or the Object Recognition Task (ORT). The ORT is sometimes referred to as “Novel ORT”

or “Object-placement Recognition Task”, but will be addressed simply as ORT in this review. The hippocampus is comma-shaped component of the brain found deep within the medial temporal lobe [54]. It serves important roles in learning and memory consolidation, especially in spatial tasks [55].

The MWM, pioneered by Dr. Richard Morris in 1981 [56, 57], is a hippocampal-dependent assessment of spatial learning and memory. This is evidenced by data showing attenuated MWM performance among rats with induced hippocampal lesions [58]. In the MWM, rodents are placed in a large circular pool of opaque water and must search for a hidden platform that allows them to escape and be rescued by research personnel. Escape latency and total distance travelled are typically measured, with lower values indicating better performance [57]. Several experimental studies in a variety of T1DM rodent models have assessed cognition using this task, showing decrements in performance, and when measured, hippocampal abnormalities in comparison to non-diabetic groups [48-50, 59-63]. Notably however, none of these studies attempted to regulate glycemia with the use of insulin or other compounds. As a result, many studies were conducted in rodent populations with extreme levels of hyperglycemia (> 20 mmol/L). Work from Biessels *et al.* [52] outlined above demonstrated that insulin-treated rodents did not show any differences in MWM performance compared to non-diabetic controls. Subsequent work from Biessels *et al.* [64] followed a similar theme, demonstrating that only severely hyperglycemic rats (25.6 ± 1.0 mmol/L) showed decrements in MWM performance, but not moderately hyperglycemic rats (18.9 ± 1.8 mmol/L). The MWM is a valid, well-established cognitive assessment that can be applied to a variety of rodent populations. However, performance is heavily dictated by hippocampal function, and spatial learning and memory are among the only cognitive domains assessed [65].

Another common cognitive assessment used in rodent models is the ORT, first developed by Ennaceur & Delacour [66] in 1988. The ORT involves visual exploration of two or more objects, relying on rodents’ unconditioned preference for exploring novel objects [67]. The greater amount of time that a rodent spends exploring the novel object signifies that they recognize it as novel, serving as a positive measure of learning and memory. Although there are subtle differences compared to the MWM, the ORT primarily

assesses learning and memory, which is mainly governed by the hippocampus [68, 69]. More specifically, the ORT does not rely as heavily on spatial learning, is modifiable to evaluate different types of memory (spatial, working, short-, or long-term), and is less stressful than other cognitive tests [70]. The ORT has been used in several induced T1DM-rodent model studies, consistently demonstrating that non-T1DM animals outperform T1DM animals [49, 51, 53, 71]. In accordance with earlier aforementioned studies [52, 53], Kassab *et al.* [71] demonstrated that insulin treatment ameliorated deficits amongst T1DM rodents in the ORT. Overall, the ORT is a flexible and simple assessment of cognitive function in animal models. However, it is limited in the amount of information it can yield and may not serve as a complete measure of overall cognitive function [70].

As outlined above, EF refers to higher-level cognitive skills that enable us to regulate behaviour within the context of goal setting or rules, and includes specific set-shifting skills [72]. Developed by Grant *et al.* [73] in 1948, the Wisconsin Card Sorting Task (WCST) is perhaps the most classic assessment of EF / set-shifting ability in humans. In this task, unacquainted subjects must learn to sort cards based on one of three distinct dimensions (number, colour, or shape). The paradigm will eventually shift unannounced, and subjects must adapt their sorting to align with one of the other two previously irrelevant dimensions. This test is mainly governed by frontal lobe function [74], although other, more specific areas have been implicated more recently [75, 76]. The WCST generates a rich data set that includes insight into the number and type of errors committed.

Most of the literature in rodent models outlined thus far has utilized cognitive assessments that rely primarily on spatial learning and memory-based tasks (MWM, ORT). Although less commonly used, there are a handful of tasks that assess EF / set-shifting developed for use in rodents [77]. Set-shifting ability can be measured using tasks conducted in radial-arm or cross-mazes that assess rodents' ability to transition from one stimulus-response strategy to another, inhibiting the former strategy in the process [78-81]. Birrell & Brown [82] developed a more intricate procedure resembling the WCST where rodents are trained to dig for a food reward based on three separate dimensions: odour, digging medium, or texture. Rodents are assessed on their ability to complete both intradimensional and extradimensional shifts, as well as reversal learning (RL) tasks. Disruptions to the

medial prefrontal cortex (mPFC) have been shown to impair set-shifting performance, but not reversal learning, indicating that the mPFC may specifically underlie set-shifting ability [83]. In the same study, inhibitions of the orbitofrontal region of the PFC resulted in decrements in reversal learning, but not set-shifting ability, providing evidence that distinct regions of the PFC regulate different domains of EF.

The aforementioned tests of set-shifting provide a highly valid assessment of EF and mPFC function in rodents. However, they can require complicated set-up and are limited by the overall throughput, as each animal must typically be individually monitored. In 2008, Floresco *et al.* [84] established an automated set-shifting procedure utilizing similar paradigms to the maze- and digging-based tests described above. In this procedure, rodents are placed in an operant conditioning chamber fitted with two retractable levers and progress through a series of learned contingencies, intradimensional and extradimensional shifts. Taken as a whole, this assessment produces many meaningful metrics that serve as detailed indicators of the different components of EF. Similar to other tests of behavioural flexibility [83], Floresco and colleagues [84] demonstrated using this task that inactivation of the mPFC impaired set-shifting, but not reversal learning performance.

To our knowledge, no prior work has examined the effects of T1DM on executive function / set-shifting abilities using rodent models. In what is perhaps the only other body of work to examine this area of research, the set-shifting abilities of rats subjected to recurrent hypoglycemia were assessed using a maze-based test [85]. They found that multiple episodes of insulin-induced antecedent hypoglycemia led to impaired set-shifting performance, accompanied by reduced PFC function. Although recurrent hypoglycemia is a symptom of T1DM that may have potentially deleterious consequences, the rodents in this experiment were never subjected to sustained hyperglycemia, a cornerstone symptom of T1DM that appears to be implicated more often in cognitive dysfunction [38]. Work from Kaleeswari *el al.* [86] explored the impact of early stage T1DM on bar pressing in an operant chamber task and found no differences in performance between control and T1DM rats. Although this closely resembles the testing procedures used in the present study, there was no visual cueing nor shifting of stimuli, making this task far less intricate. Also of relevance, recent work demonstrated that the offspring of pregnant rats induced with

gestational diabetes show attenuated set-shifting performance using the operant chamber based procedure pioneered by Floresco *et al.* [84]. Multiple works from Sharma *et al.* [87-89] have examined the effects of vascular dementia induced by experimental diabetes on behavioural flexibility using an attentional set-shifting digging based task, consistently showing deficits amongst the experimental groups compared to the controls.

There are several highly valid methods of assessing cognition in both humans and animal models. The majority of research conducted on T1DM rodent models has utilized hippocampal-dependent assessments such as the MWM or iterations of the ORT. It would appear there is a consensus across the literature in that T1DM rodent models consistently demonstrate inferior performance on these tasks when compared to non-T1DM counterparts. Although tests of EF have existed for many years, very few animal studies have specifically examined the impact of T1DM on EF or more specifically set-shifting.

1.4 Rationale

Over 300,000 Canadians and millions more globally are currently living with T1DM [21]. Although great strides have been made in improving disease related outcomes, persons with diabetes remain at elevated risk for several comorbidities [18] including cognitive dysfunction [23, 90]. EF is a vital component of cognition, as it includes mental processes that allow for complex problem solving, self-regulation, and higher level thinking [27]. EF is of particular importance amongst T1DM populations, as impairments in this area have been linked to poorer glycemic control [90]. In turn, poor glycemic control (indicated by higher HbA1c) is well established as a causative factor leading to increased risk of comorbidity development and all-cause mortality [18].

Several studies have examined the relationship between T1DM and cognitive function in both humans [23] and animal models [91]. More specifically, studies in humans have examined EF in patients with T1DM [30], however to our knowledge, no studies have examined this relationship using pre-clinical animal models. Assessing EF using a rodent model of T1DM may allow us to validate an experimental model to measure EF and

ascertain any potential underlying mechanisms of impaired EF. Although valid assessments of EF have been adapted for use in rodents [66, 82, 84], they have not been utilized in T1DM rodents. Furthermore, many of the studies that have examined cognition in rodent models have not featured an insulin-treated group [48, 49, 51, 59, 60, 92, 93]. In many of these studies, rodents are maintained at levels of extreme hyperglycemia (> 20 mmol/L) that may potentially drive or further exacerbate cognitive dysfunction. Hence, this study serves to address two main gaps in the literature: a) the lack of assessment of EF conducted in T1DM rodent models, and b) the lack of general cognitive assessment in insulin-treated rodent models.

1.5 Purpose and Hypothesis

Given the lack of prior work examining the executive function of T1DM rodents, the purpose of the study was to evaluate the effect of T1DM on EF through the use of a series of automated operant conditioning based tasks developed by Floresco *et al.* [84]. Specifically, we examined the ability of rodents in three tasks: visual cue discrimination, response discrimination (set-shift), and reversal learning. Based on the literature demonstrating reductions in measures of EF in humans [23, 30], and studies in rodents showing attenuated MWM and/or ORT performance [48-53] we hypothesized that T1DM rodents would show impairments in measures of EF. Given that the response discrimination task best assesses set-shifting, a core pillar of EF, we specifically hypothesized that T1DM rodents would show increases in both the number of trials, and the number of errors to criterion (i.e. inferior performance).

1.6 Bibliography

1. Wasserman, D. H. Four grams of glucose. *Am. J. Physiol. - Endocrinol. Metab.* **296**, 11–21 (2009).

2. Cryer, P. E. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes* **54**, 3592–3601 (2005).

3. Daneman, D. Type 1 diabetes. *Lancet* **367**, 847–858 (2006).

4. DiMeglio, L. A., Evans-Molina, C. & Oram, R. A. Type 1 diabetes. *Lancet* **391**, 2449–2462 (2018).

5. Wood J, P. A. *The Type 1 Diabetes Self-Care Manual*. *The Autoimmune Diseases* (2006). doi:10.2337/9781580406208

6. Sarwar, N. *et al.* Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: A collaborative meta-analysis of 102 prospective studies. *Lancet* **375**, 2215–2222 (2010).

7. Hicks, C. W. & Selvin, E. Epidemiology of Peripheral Neuropathy and Lower Extremity Disease in Diabetes. *Curr. Diab. Rep.* **19**, 86 (2019).

8. Bourne, R. R. A. *et al.* Causes of vision loss worldwide, 1990-2010: A systematic analysis. *Lancet Glob. Heal.* **1**, 339–349 (2013).

9. Acharjee, S., Ghosh, B., Al-Dhubiab, B. E. & Nair, A. B. Understanding type 1 diabetes: Etiology and models. *Can. J. Diabetes* **37**, 269–276 (2013).

10. Wu, Y., Ding, Y., Tanaka, Y. & Zhang, W. Risk factors contributing to type 2 diabetes and recent advances in the treatment and prevention. *Int. J. Med. Sci.* **11**, 1185–1200 (2014).

11. Cefalu, W. T. Insulin resistance: Cellular and clinical concepts. *Proc. Soc. Exp. Biol. Med.* **226**, 13–26 (2001).

12. Haller, M. J., Atkinson, M. A. & Schatz, D. Type 1 diabetes mellitus: Etiology, presentation, and management. *Pediatr. Clin. North Am.* **52**, 1553–1578 (2005).

13. Atkinson, M. A., Eisenbarth, G. S. & Michels, A. W. Type 1 diabetes. *Lancet* **383**, 69–82 (2014).

14. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **33 Suppl 1**, S62-9 (2010).

15. Nathan, D. M. *et al.* International expert committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes Care* **32**, 1327–1334 (2009).

16. Wintrobe, M. M. Clinical Hematology. *Lea Febiger, Philadelphia* (1965).

17. Weykamp, C. HbA1c: A review of analytical and clinical aspects. *Ann. Lab. Med.* **33**, 393–400 (2013).

18. Khaw, K. T. *et al.* Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk). *BMJ* **322**, 15–8 (2001).

19. Patterson, C. C. *et al.* Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989–2013: a multicentre prospective registration study. *Diabetologia* **62**, 408–417 (2019).

20. Norris, J. M., Johnson, R. K. & Stene, L. C. Type 1 diabetes—early life origins and changing epidemiology. *Lancet Diabetes Endocrinol.* **8**, 226–238 (2020).

21. Diabetes Canada. Diabetes in Canada: Backgrounder. *Diabetes.Ca* 1–6 (2020).

22. Diabetes Control and Complications Trial Research Group *et al.* The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **329**, 977–86 (1993).

23. Brands, A. M. A., Biessels, G. J., de Haan, E. H. F., Kappelle, L. J. & Kessels, R. P. C. The effects of type 1 diabetes on cognitive performance: a meta-analysis. *Diabetes Care* **28**, 726–35 (2005).

24. CDC. Cognitive Impairment: A Call for Action, Now! (2011).

25. Miles, W. R. & Root, H. F. Psychologic Tests Applied to Diabetic Patients. *Arch. Intern. Med.* **30**, 767 (1922).

26. Duke, D. C., Raymond, J. K. & Harris, M. A. The Diabetes Related Executive Functioning Scale (DREFS): Pilot results. *Child. Heal. Care* **43**, 327–344 (2014).

27. Diamond, A. Executive functions. *Annu. Rev. Psychol.* **64**, 135–68 (2013).

28. Nowrangi, M. A., Lyketsos, C., Rao, V. & Munro, C. A. Systematic review of neuroimaging correlates of executive functioning: converging evidence from different clinical populations. *J. Neuropsychiatry Clin. Neurosci.* **26**, 114–25 (2014).

29. Goschke, T. Dysfunctions of decision-making and cognitive control as transdiagnostic mechanisms of mental disorders: advances, gaps, and needs in current research. *Int. J. Methods Psychiatr. Res.* **23 Suppl 1**, 41–57 (2014).

30. Broadley, M. M., White, M. J. & Andrew, B. A Systematic Review and Meta-analysis of Executive Function Performance in Type 1 Diabetes Mellitus. *Psychosom. Med.* **79**, 684–696 (2017).

31. Gaudieri, P. A., Chen, R., Greer, T. F. & Holmes, C. S. Cognitive function in children with type 1 diabetes. *Diabetes Care* **31**, 1892–1897 (2008).

32. Dahlquist, G. & Källén, B. School performance in children with type 1 diabetes-a population-based register study. *Diabetologia* **50**, 957–964 (2007).

33. Ryan, C. M. Why is cognitive dysfunction associated with the development of diabetes early in life? The diathesis hypothesis. *Pediatr. Diabetes* **7**, 289–297 (2006).

34. Marzelli, M. J. *et al.* Neuroanatomical correlates of dysglycemia in young children with type 1 diabetes. *Diabetes* **63**, 343–353 (2014).

35. Chaytor, N. S. *et al.* Clinically significant cognitive impairment in older adults with type 1 diabetes. *J. Diabetes Complications* **33**, 91–97 (2019).

36. Ferguson, S. C. *et al.* Cognitive ability and brain structure in type 1 diabetes: Relation to microangiopathy and preceding severe hypoglycemia. *Diabetes* **52**, 149–156 (2003).

37. Ryan, C. M., Geckle, M. O. & Orchard, T. J. Cognitive efficiency declines over time in adults with Type 1 diabetes: Effects of micro- and macrovascular complications. *Diabetologia* **46**, 940–948 (2003).

38. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research Group *et al.* Long-term effect of diabetes and its treatment on cognitive function. *N. Engl. J. Med.* **356**, 1842–52 (2007).

39. Sharma, S. & Brown, C. E. Microvascular basis of cognitive impairment in type 1 diabetes. *Pharmacol. Ther.* (2021). doi:10.1016/j.pharmthera.2021.107929

40. Wright, R. J., Frier, B. M. & Deary, I. J. Effects of acute insulin-induced hypoglycemia on spatial abilities in adults with type 1 diabetes. *Diabetes Care* **32**, 1503–1506 (2009).

41. McAulay, V., Deary, I. J., Sommerfield, A. J. & Frier, B. M. Attentional functioning is impaired during acute hypoglycaemia in people with Type 1 diabetes. *Diabet. Med.* **23**, 26–31 (2006).

42. Reichard, P. & Pihl, M. Mortality and treatment side-effects during long-term intensified conventional insulin treatment in the Stockholm Diabetes Intervention Study. *Diabetes* **43**, 313–317 (1994).

43. Kramer, L. *et al.* Previous episodes of hypoglycemic coma are not associated with permanent cognitive brain dysfunction in IDDM patients on intensive insulin treatment. *Diabetes* **47**, 1909–1914 (1998).

44. Deary, I. J. *et al.* Severe hypoglycemia and intelligence in adult patients with insulin-treated diabetes. *Diabetes* **42**, 341–344 (1993).

45. Gold, A. E. *et al.* Severe Deterioration in Cognitive Function and Personality in Five Patients with Long-standing Diabetes: A Complication of Diabetes or a Consequence of Treatment? *Diabet. Med.* **11**, 499–505 (1994).

46. Perros, P., Deary, I. J., Sellar, R. J., Best, J. J. K. & Frier, B. M. Brain abnormalities demonstrated by magnetic resonance imaging in adult IDDM patients with and without a history of recurrent severe hypoglycemia. *Diabetes Care* **20**, 1013–1018 (1997).

47. Lacy, M. E. *et al.* Severe hypoglycemia and cognitive function in older adults with type 1 diabetes: The Study of Longevity in Diabetes (SOLID). *Diabetes Care* **43**, 541–548 (2020).

48. Chen, G., Wang, Y., Li, Y., Zhang, L. & Dong, M. A novel hippocampus metabolite signature in diabetes mellitus rat model of diabetic encephalopathy. *Metab. Brain Dis.* (2020). doi:10.1007/s11011-020-00541-2

49. Ahmed, A. *et al.* Time-dependent impairments in learning and memory in Streptozotocin-induced hyperglycemic rats. *Metab. Brain Dis.* **34**, 1431–1446 (2019).

50. Zhu, B., Jiang, R. Y., Yang, C. & Liu, N. Adenosine monophosphate-activated protein kinase activation mediates the leptin-induced attenuation of cognitive impairment in a streptozotocin-induced rat model. *Exp. Ther. Med.* **9**, 1998–2002 (2015).

51. Revsin, Y. *et al.* Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice. *Neuropsychopharmacology* **34**, 747–758 (2009).

52. Biessels, G. J. *et al.* Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: Effects of insulin treatment. *Brain Res.* **800**, 125–135 (1998).

53. Marissal-Arvy, N. *et al.* Insulin treatment partially prevents cognitive and hippocampal alterations as well as glucocorticoid dysregulation in early-onset insulin-deficient diabetic rats. *Psychoneuroendocrinology* **93**, 72–81 (2018).

54. Green, J. D. The Hippocampus. *Physiol. Rev.* **44**, 561–608 (1964).

55. Squire, L. R. Memory and the Hippocampuss: A Synthesis From Findings With Rats, Monkeys, and Humans. *Psychol. Rev.* **99**, 195–231 (1992).

56. Morris, R. G. M. Spatial localization does not require the presence of local cues. *Learn. Motiv.* **12**, 239–260 (1981).

57. Morris, R. Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods* **11**, 47–60 (1984).

58. R. G. M. Morris, P. Garrud, J. N. P. Rawlins & J. O'Keefe. Place navigation impaired in rats with hippocampal lesions. *Nature* **297**, 681–683 (1982).

59. Zhang, S., Yuan, L., Zhang, L., Li, C. & Li, J. Prophylactic use of troxerutin can delay the development of diabetic cognitive dysfunction and improve the expression of Nrf2 in the hippocampus on STZ diabetic rats. *Behav. Neurol.* **2018**, (2021).

60. Manschot, S. M. *et al.* Angiotensin converting enzyme inhibition partially prevents deficits in water maze performance, hippocampal synaptic plasticity and cerebral blood flow in streptozotocin-diabetic rats. *Brain Res.* **966**, 274–282 (2003).

61. Ahmadi, M., Rajaei, Z., Hadjzadeh, M. A., Nemati, H. & Hosseini, M. Crocin improves spatial learning and memory deficits in the Morris water maze via attenuating cortical oxidative damage in diabetic rats. *Neurosci. Lett.* **642**, 1–6 (2017).

62. Kamal, A., Biessels, G. J., Duis, S. E. J. & Gispen, W. H. Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: Interaction of diabetes and ageing. *Diabetologia* **43**, 500–506 (2000).

63. Li, Z. G., Zhang, W., Grunberger, G. & Sima, A. A. F. Hippocampal neuronal apoptosis in type 1 diabetes. *Brain Res.* **946**, 221–231 (2002).

64. Biessels, G. J. *et al.* Place learning and hippocampal synaptic plasticity in streptozotocin-induced diabetic rats. *Diabetes* **45**, 1259–66 (1996).

65. Gehring, T. V., Luksys, G., Sandi, C. & Vasilaki, E. Detailed classification of swimming paths in the Morris Water Maze: Multiple strategies within one trial. *Sci. Rep.* **5**, 1–15 (2015).

66. Ennaceur, A. & Delacour, J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav. Brain Res.* **31**, 47–59 (1988).

67. Ennaceur, A. One-trial object recognition in rats and mice: Methodological and theoretical issues. *Behav. Brain Res.* **215**, 244–254 (2010).

68. Dere, E., Huston, J. P. & De Souza Silva, M. A. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci. Biobehav. Rev.* **31**, 673–704 (2007).

69. Winters, B. D., Saksida, L. M. & Bussey, T. J. Object recognition memory: Neurobiological mechanisms of encoding, consolidation and retrieval. *Neurosci. Biobehav. Rev.* **32**, 1055–1070 (2008).

70. Lueptow, L. M. Novel object recognition test for the investigation of learning and memory in mice. *J. Vis. Exp.* **2017**, 1–9 (2017).

71. Kassab, S. *et al.* Cognitive dysfunction in diabetic rats is prevented by pyridoxamine treatment. A multidisciplinary investigation. *Mol. Metab.* **28**, 107–119 (2019).

72. Mahone, E. M. *et al.* Validity of the behavior rating inventory of executive function in children with ADHD and/or Tourette syndrome. *Arch. Clin. Neuropsychol.* **17**, 643–662 (2002).

73. Grant, D. A. & Berg, E. A behavioral analysis of degree of reinforcement and ease of shifting to new responses in a Weigl-type card-sorting problem. *J. Exp. Psychol.* **38**, 404–411 (1948).

74. Milner, B. Effects of Different Brain Lesions on Card Sorting. *Arch. Neurol.* **9**, 90 (1963).

75. Gläscher, J., Adolphs, R. & Tranel, D. Model-based lesion mapping of cognitive control using the Wisconsin Card Sorting Test. *Nat. Commun.* **10**, (2019).

76. Buchsbaum, B. R., Greer, S., Chang, W. L. & Berman, K. F. Meta-analysis of neuroimaging studies of the Wisconsin Card-Sorting Task and component processes. *Hum. Brain Mapp.* **25**, 35–45 (2005).

77. Bizon, J. L., Foster, T. C., Alexander, G. E. & Glisky, E. L. Characterizing cognitive aging of working memory and executive function in animal models. *Front. Aging Neurosci.* **4**, 1–14 (2012).

78. Ragozzino, M. E., Detrick, S. & Kesner, R. P. Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. *J. Neurosci.* **19**, 4585–4594 (1999).

79. Seamans, J. K., Floresco, S. B. & Phillips, A. G. Functional Differences Between the Prelimbic and Anterior Cingulate Regions of the Rat Prefrontal Cortex. *Behav. Neurosci.* **109**, 1063–1073 (1995).

80. Stefani, M. R., Groth, K. & Moghaddam, B. Glutamate receptors in the rat medial prefrontal cortex regulate set-shifting ability. *Behav. Neurosci.* **117**, 728–737 (2003).

81. Ragozzino, M. E. The effects of dopamine D1 receptor blockade in the prelimbic-infralimbic areas on behavioral flexibility. *Learn. Mem.* **9**, 18–28 (2002).

82. Birrell, J. M. & Brown, V. J. Medial frontal cortex mediates perceptual attentional set shifting in the rat. *J. Neurosci.* **20**, 4320–4324 (2000).

83. Dias, R., Robbins, T. W. & Roberts, A. C. Dissociation in prefrontal cortex of affective and attentional shifts. *Nature* **380**, 69–72 (1996).

84. Floresco, S. B., Block, A. E. & Tse, M. T. L. Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. *Behav. Brain Res.* **190**, 85–96 (2008).

85. Jahagirdar, V., Ramcharitar, J., Cotero, V. E. & McNay, E. C. Moderate recurrent hypoglycemia markedly impairs set-shifting ability in a rodent model: Cognitive and neurochemical effects. *Open Diabetes J.* **5**, 1–7 (2012).

86. Kaleeswari, R., Thombre, D. P. & Chakrabarty, A. S. Acute effect of streptozotocin induced diabetes on bar pressing for food reward in albino rats. *Indian J. Physiol. Pharmacol.* **30**, 319–21 (1986).

87. Jain, S., M Sharma, B. & Sharma, B. Calcium Channel Blockade and Peroxisome Proliferator Activated Receptor γ Agonism Diminish Cognitive Loss and Preserve Endothelial Function During Diabetes Mellitus. *Curr. Neurovasc. Res.* **13**, 33–44 (2016).

88. Jain, S. & Sharma, B. Effect of ruthenium red, a ryanodine receptor antagonist in experimental diabetes induced vascular endothelial dysfunction and associated dementia in rats. *Physiol. Behav.* **164**, 140–150 (2016).

89. Sharma, B. & Singh, N. Pitavastatin and 4-Hydroxy-3-Methoxyacetophenone (HMAP) Reduce Cognitive Dysfunction in Vascular Dementia During Experimental Diabetes. *Curr. Neurovasc. Res.* **7**, 180–191 (2010).

90. Hill-Briggs, F. & Gemmell, L. Problem solving in diabetes self-management and control: A systematic review of the literature. *Diabetes Educ.* **33**, 1032–1050 (2007).

91. Biessels, G. J. & Gispen, W. H. The impact of diabetes on cognition: What can be learned from rodent models? *Neurobiol. Aging* **26**, 36–41 (2005).

92. Zarrinkalam, E., Ranjbar, K., Salehi, I., Kheiripour, N. & Komaki, A. Resistance training and hawthorn extract ameliorate cognitive deficits in streptozotocin-induced diabetic rats. *Biomed. Pharmacother.* **97**, 503–510 (2018).

93. De Senna, P. N. *et al.* Effects of physical exercise on spatial memory and astroglial alterations in the hippocampus of diabetic rats. *Metab. Brain Dis.* **26**, 269–279 (2011).

Chapter 2

2 Assessment of Executive Function in T1DM Rodents

2.1 Introduction

Type 1 Diabetes Mellitus is a chronic condition which occurs as a result of an autoimmune mediated destruction of the insulin producing β-cells of the pancreas [1]. As a result, patients with T1DM are unable to produce insulin and are typically reliant on exogenous pharmaceutical insulin for survival. Insulin is a peptide hormone that is responsible for facilitating the uptake of glucose from the bloodstream into target tissues such as skeletal muscle. Without insulin, blood glucose levels remain chronically elevated, and tissues are unable to receive glucose that they require for metabolism. Conversely, patients with T1DM are also at risk of hypoglycemia that may be caused by overcorrections in insulin treatment, sustained periods of fasting, or prolonged exercise. If untreated, extreme hypoglycemia can cause severe symptoms such as seizures, coma, or in rare instances death [2]. Prior to the invention of insulin in 1921 by Frederick Banting and Charles Best, it was uncommon for those diagnosed with diabetes to live for more than a few years. Despite the great advances that have been made in regard to the understanding and treatment of T1DM, there is still no cure presently available. Although patients with T1DM can effectively live a normal life, they face an elevated risk of comorbidities such as cardiovascular disease, neuropathies, and retinopathies [3-5].

The human brain is an obligate glucose consumer and functions as the core of the central nervous system, governing bodily processes including cognition, sensory processing, and motor control. Despite comprising only ~ 2% of body weight, the brain consumes up to 60% of circulating blood glucose in a fasted, sedentary state [6]. Due to the critical function of the brain and its reliance on glucose, intricate mechanisms tightly regulate blood glucose. Given the perturbations in glycemic control caused metabolic diseases such as diabetes, it is not surprising that brain dysfunction may arise. A link between cognitive dysfunction and patients with T1DM was first identified as early as 1922 when Miles *et al.* [7] found that patients with diabetes scored approximately 15% lower on tests of memory and attention compared to non-diabetic counterparts. Many subsequent works [8-11] have reported similar findings and provide much greater insight into the risk factors and mechanisms that may lead to cognitive dysfunction in patients with T1DM.

A 2005 meta-analysis of thirty-three studies comparing cognitive performance of T1DM to non-T1DM adults reported that patients with T1DM demonstrated minor, but significant decrements in specific cognitive domains including: intelligence, information processing, psychomotor efficiency, visual and sustained attention, cognitive flexibility, and visual perception [8]. Similar findings have also been reported in younger populations [12, 13]. There are several risk factors and underlying mechanisms that have been identified that may contribute to cognitive dysfunction in T1DM populations. Several high-quality works have implicated microvascular complications as a contributing factor in cognitive dysfunction [9, 14-16]. Although a causal relationship was not definitively established in these works, a recent review has proposed several mechanisms that may be responsible [17]. Earlier age at diabetes onset, as well as poor long-term metabolic control have also been correlated with greater decrements in cognition in patients with T1DM [9, 12, 13, 16].

Several experimental works in non-human subjects have also examined the relationship between cognitive dysfunction and T1DM. In a seminal study, Biessels *et al.* [18] found that insulin therapy was able to preserve cognitive function in T1DM rats, but only if it was initiated immediately following diabetes onset. A more recent study reached a similar conclusion, demonstrating that insulin treatment was able to rescue cognitive impairments, however it was not able to fully prevent structural nor hormonal changes [19].

Executive function is a domain of cognition that encapsulates specific mental processes such as inhibition, working memory, and cognitive flexibility [20]. EF is controlled primarily by the frontal, parietal, and cerebellar areas of the brain [21]. Similar to results on general cognition presented above, patients with T1DM score lower on specific assessments of EF [22]. Several experimental works in rodents have demonstrated that cognition is generally attenuated amongst T1DM groups using tests such as the Morris Water Maze [23-30] or the Object Recognition Task [19, 24, 31, 32]. However, both of these tests are measures of spatial learning and memory that are primarily hippocampal-dependent [33, 34]. Although less commonly implemented, specific tests exist that assess EF in rodents [35]. EF, or more specifically behavioural flexibility can be assessed in rodents using maze-based tasks [36, 37] or a digging based task [38] using similar concepts to the Wisconsin Card Sorting Task [39]. In 2008, Floresco *et al.* [40] developed an automated set-shifting task that

solves some of the shortcomings of the aforementioned tests. This task utilizes an operant conditioning chamber fitted with two levers where rodents must continually adjust to shifting paradigms in order to receive food rewards.

Although a handful of studies in humans have specifically examined executive function and set-shifting abilities of patients with T1DM [22], to our knowledge, no studies have specifically examined this relationship in animal models. By assessing EF in a rodent model of T1DM, we aim to validate an experimental model to measure EF, and ascertain any potential underlying mechanisms of impaired EF. Hence, the purpose of this study was to compare the EF of non-diabetic rats to insulin-treated diabetic rats using a series of operant conditioning based tasks outlined above [40-42]. Both male and female rats were randomly assigned to either a non-diabetic group (n = 12), or an insulin-treated diabetic group (n = 14). Rats underwent a series of tasks that assessed learning, inhibitory control, behavioural flexibility, and reversal learning. We hypothesized that diabetic rats would show performance decrements on these assessments of executive function. Specifically, diabetic rats would require more trials to criterion, and incur more errors in reaching criterion on the response discrimination (set-shifting) task.

2.2 Materials and Methods

2.2.1 Ethics Approval

All protocols utilized in this study were approved without stipulation by The University Council of Animal Care of Western University (London, Ontario, Canada) and conducted in accordance with the standards outlined by The Canadian Council on Animal Care (CCAC).

2.2.2 Animals

Twenty-eight Sprague Dawley rats (fourteen male, fourteen female) were procured from Charles River Laboratories (St. Constant, Quebec, Canada) at eight weeks of age. One male rodent died from complications relating to diabetes induction (week 8), and a second male died from complications relating to food restriction (week 14), leaving a final

cohort of twelve males and fourteen females. Upon arrival at the animal holding facility, all rats were acclimatized for a minimum of 72 hours before handling. Rats were housed together in same sex pairs until food restriction, at which point all animals were housed individually for the duration of the study. All rats were maintained on a 12:12 hr alternating light/dark cycle (lights on at 07:00 AM). Room temperature was held at a constant 21 ± 2°C and relative humidity was maintained at 40 - 50% throughout the duration of rodents' lifespan. All rats were provided *ad libitum* access to standard rat chow (until food restriction occurred) and tap water.

2.2.3 Experimental Groups

Rodents were randomly assigned to non-diabetic (*n* = 12; 6 male, 6 female) or diabetic (*n* = 14; 6 male, 8 female) groups. Diabetes induction occurred in the diabetic group at week 8 of the study. Food restriction commenced at week 14 of the study and continued until each animal completed all operant conditioning based protocols. Following the completion of reversal learning (week 16), all animals were sacrificed via anaesthetization with isoflurane, followed by cardiac exsanguination in accordance with our animal use protocol.

2.2.4 Experimental Procedures

2.2.4.1 Diabetes Induction

At week 8 of the study, diabetes was induced using a standardized protocol (Appendix A) via multiple low-dose injections of streptozotocin (STZ; Sigma-Aldrich). All animals were injected intraperitoneally with 20 mg/kg/day of STZ (dissolved in citrate buffer; 0.1M, pH = 4.5) for 7 consecutive days. All injections were administered within 10 minutes of final preparation of STZ. T1DM was confirmed with a blood glucose measurement of ≥ 15 mmol/L taken at approximately 48 hours following the final injection.

2.2.4.2 Insulin Pellet Modulation

Approximately 72 hours following the final injection of STZ and confirmation of T1DM, all animals in the diabetic group were given a single insulin pellet (1 pellet = 2 IU insulin/per day; Linshin). Insulin pellets were surgically implanted (Appendix B) by

trained personnel into the abdomen through a small subcutaneous incision (~ 0.75 cm). Insulin dosing was modulated in response to blood glucose levels via removal or insertion of additional pellets. Blood glucose was intended to be maintained within a range of 9 - 15 mmol/L.

2.2.4.3 Food Restriction

In week 14 of the study, all animals were separated into individual cages and placed on a food restricted diet according to a fixed schedule dictating minimum feeding values (Appendix C). Animals were closely monitored for abnormalities and weighed daily to calculate the appropriate amount of food to induce weight loss. All animals were fed at either ~ 08:00 AM daily, or upon completion of their set-shifting protocol for the day (if applicable). Diabetic animals had their daily allotment of food split into two boluses given approximately 12 hours apart in order to limit hypoglycemic episodes. Once each animal had reached ~ 95% of free-feeding body weight (5% weight loss), operant conditioning protocols commenced. Animals were maintained at 90 - 95% free-feeding weight for the duration of the study. Water was provided *ad libitum* throughout the food restriction period.

2.2.4.4 Operant Conditioning Protocols

2.2.4.4.1 Apparatus

All procedures were conducted in an operant conditioning chamber (Med Associates) housed within a sound and light attenuating box. The chamber was fitted with two retractable response levers (on the same wall) with a stimulus light above each. The levers were separated by a food pellet receptacle in the middle. A house light was located on the top portion of the opposite wall, serving to illuminate the entire chamber. All aspects of testing were controlled via MED-PC software (Med Associates). In response to a correct lever press, a 45 mg sucrose pellet (BioServ) was released into the receptacle from a dispenser housed outside the chamber.

2.2.4.4.2 Acclimatization and Pre-Training

On each of the 3 days preceding pre-training, each animal was given ten sucrose pellets in their home cage (in addition to their daily allotment of rat chow) in order to

familiarize them with the taste and texture. On the day that 95% free-feeding weight was achieved, rats were placed in the operant chamber for 20 minutes each in order to acclimatize them. Randomly throughout their time in the chamber, five pellets were released in order to establish a contingency between the receptacle and a food reward (sucrose pellet). On the day following acclimatization, each rat commenced a manual training protocol (pre-training) in order to establish a contingency between the pressing of the levers and a food reward. To start, the left lever was extended, and when the rat came into close proximity or interacted with it, the lever was manually retracted and a pellet was dropped into the receptacle. This process was repeated until the rat learned to press the lever, at which point it would automatically retract, and a sucrose pellet would be released. This continued until the rat achieved fifteen presses, at which time the left lever was retracted and the right lever was deployed. Pre-training was satisfied when fifteen consecutive left lever presses, fifteen consecutive right lever presses, and thirty alternating lever presses were achieved within the same session. If unsuccessful after 90 minutes, the animal was returned to their home cage, and the entire process was repeated the following day until all sixty presses were achieved within the same session. The house light remained switched on throughout the entire duration of pre-training.

2.2.4.4.3 Training

On the day following successful completion of pre-training, animals began an automated training protocol to establish a temporal contingency whereby they learned to press the lever within a timely (10 s) manner to receive a food reward. Animals were placed in the operant chamber, this time with the house light switched off. Upon commencement of the protocol, the house light would illuminate and simultaneously, either the left or right lever would be randomly extended. The lever would remain extended for 10 s or until the rat pressed it. If the rat did not press the lever, it was retracted and the house light automatically switched off. No food reward was given in this instance and it was recorded as an omission. If the rat did press the lever, it was automatically retracted immediately, and at the same time, a sucrose pellet was dropped into the receptacle. The house light remained on for an additional 4 s whilst the rat consumed the pellet. This was counted as a successful press. Regardless of the outcome, a 10 s inter-trial period with the house light

remaining off would ensue, after which the entire process would be repeated. This period of lever deployment and the inter-trial period immediately following constitutes one trial. Training consisted of ninety successive trials and took approximately 30 minutes to complete. In order to progress to the next task, rats must have achieved eighty-five or more successful presses (i.e. five or fewer omissions). If unsuccessful, rats were returned to their home cage and the entire process was repeated the following day until the criterion was satisfied.

2.2.4.4.4 Side Bias Determination

Immediately following successful completion of training, a protocol was initiated to determine the rat's side bias. The first trial began with the illumination of the house light and the presentation of both levers. If either of the levers were pressed, they would both be immediately retracted and a pellet was awarded. The house light would remain on for an additional 4 s, after which the 10 s inter-trial period would occur (house light off). If neither of the levers were pressed after 10 s, they would both be immediately retracted with no pellet awarded and the house light would immediately extinguish. The same 10 s inter-trial period would occur, after which both levers would present again and the same process would occur. In the trial directly following a successful press (either lever yielded a reward in the initial trial), both levers would be presented, however this time, only the lever opposite the one pressed in the trial prior would trigger a reward. If the rat pressed the same lever as the one in their initial trial, no pellet was deployed, the house light was turned off, and the inter-trial period occurred. Trials would continue until the rat successfully pressed the opposite lever; at which time they would be awarded a pellet. Once two correct presses had been achieved (i.e. either lever initially, then the opposite lever), the entire process would reset, and now either lever would again yield a pellet. This protocol ended when the rat had completed seven correct pairs (i.e. fourteen total correct presses). The side bias was determined to be the side that initiated the majority of the presses (i.e. responses to the trials where either lever yielded a reward), or if one lever was pressed twice as many times (or more) than the other lever, this side would automatically be determined as the rat's side bias.

2.2.4.4.5 Visual Cue Discrimination (VCD)

Once training had successfully been completed and a side bias was ascertained, a VCD task was initiated on the following day. This task was the first to utilize the cue lights located above each lever. In this paradigm, rats would only receive a food reward if they pressed the lever that corresponded to the illuminated stimulus (cue) light. A trial began with the random illumination of either the left or right cue light. After 3 s of the cue light being on, the house light was automatically switched on and both levers were presented. Only the lever positioned directly under the illuminated cue light would produce a sucrose pellet. If the incorrect lever was pressed (i.e. the lever opposite the illuminated cue light), both levers were retracted, no pellet was awarded, and the inter-trial period began. If the correct lever was pressed, both levers were retracted, a pellet would be released, and the house light would remain on for an additional 4 s, after which the inter-trial period occurred. If neither lever was pressed after 10 s, no pellet was awarded, both levers were retracted, and the inter-trial period began. This was scored as an omission. All rats underwent 100 consecutive trials in one session. The performance criterion was set as ten consecutive correct trials (omissions were not counted against this). If rats did not achieve criterion on the first session, they were returned to their home cage and retested the following day until criterion was reached. Both the total number of trials to criterion, and the total number of errors to criterion were measured. Errors to criterion were the total number of errors that were incurred until criterion was achieved. Errors that occurred after criterion had been achieved were not scored against this.

2.2.4.4.6 Set-Shifting: Response Discrimination (RD)

On the day following successful completion of VCD, rats were provided a brief visual cue retrieval (VCR) test consisting of twenty VCD trials (identical to the previous day) to ensure that they retained the visual cue paradigm. In order to proceed to the RD task, rats must have successfully completed at least sixteen of the twenty VCR trials (≥ 80%; Appendix D). If rats did not achieve this, they were then given 100 more VCD trials and returned to their home cage to attempt VCR the following day. Immediately following successful completion of the recall test (VCR), the paradigm shifted to a RD task in which the visual cue became irrelevant. In the RD trials, a random cue light preceded the

presentation of both levers and the illumination of the house light by 3 s (same as VCD). However, now only the lever opposite the rat's pre-determined side bias produced a reward, regardless of the cue light. Rats had to un-learn the old VCD contingency (i.e. follow the light) and adopt the new RD one (i.e. only press lever opposite side bias). Correct trials, incorrect trials, and omissions all invoked the same respective responses outlined above in prior tasks. Performance criterion was again set as ten consecutive trials with omissions having no impact. All rats underwent 120 trials per session. If rats did not achieve criterion on the first session, they were returned to their home cage and retested the following day until criterion was reached. Trials and errors to criterion, as well as total number and type of errors were measured.

One key difference in the RD task was that trials were delivered in blocks of sixteen featuring eight congruent and eight non-congruent trials randomly dispersed throughout. A congruent trial was one in which the cue light corresponded to the correct lever. In these trials, the rat would be rewarded regardless of which paradigm they were adhering to, since the old VCD strategy was still correct in these trials. An incongruent trial was one in which the cue light did not correspond to the correct lever, meaning that the rat must follow the new RD strategy in order to receive a sucrose pellet (i.e. only the lever opposite the cue light yielded a reward). As such, incongruent trails indicated which strategy rats were adhering to (VCD or RD).

2.2.4.4.7 Reversal Learning (RL)

Once criterion had been reached on the RD task, rats were next assessed on their ability to adopt the opposite RD strategy the following day. The protocol used was identical to the RD task, except that this time only the lever corresponding to the rat's side bias yielded a reward (i.e. the opposite lever as in the previous day's RD task). All rats received 120 trials and were assessed on trials and errors to criterion, as well as number and type of errors. Note that each animal only underwent a single testing session (120 trials) of RL. This is different than the VCD and RD tasks, in which animals were retested on subsequent days if they did not achieve criterion within the first testing session.

2.2.4.4.8 Error Analysis

In the RD task, errors were categorized as either perseverative, regressive, or never-reinforced. Both perseverative and regressive errors were scored when the incorrect lever was pressed on incongruent trials. Perseverative errors typically occurred earlier in the RD task and were an indication that the rat was adhering to the old VCD strategy. Regressive errors were typically scored later in the task and represented the rat's inability to maintain the new RD strategy. Perseverative and regressive errors were scored in the same way in the RL task. Never-reinforced errors were counted only in the RD task and were scored when the incorrect lever was pressed on congruent trials. These errors indicated that the rat was not adhering to either the VCD nor the RD strategy and was perhaps attempting to learn by filtering out incorrect responses.

2.2.5 Experimental Measures

2.2.5.1 Body Weight and Blood Glucose

Body weight and non-fasted blood glucose were recorded on a weekly basis at a consistent time of day (09:00 AM ± 1 hr) throughout the entire duration of the study. Body weight was measured to the nearest gram using a standard digital scale and weigh basket. In order to analyze blood glucose, a small blood sample (~ 50 µL) was obtained from the saphenous vein of the hind leg via needle prick. Pressure was applied with gauze in order ensure hemostasis was achieved following blood collection. Blood glucose values were obtained using a Freestyle Lite Blood Glucose Monitoring System (Abbott Diabetes Care) and reported in millimoles per litre (mmol/L).

2.2.5.2 Visual Cue Discrimination

All data were captured and analyzed using proprietary MED-PC software (Med Associates) and custom designed protocols see (Appendix E). Total number of trials to criterion, as well as total number of errors to criterion were analyzed. Criterion was set at ten consecutive correct responses for the VCD task. It should be noted that for all tasks, a lower number of trials to criterion is indicative of faster learning (superior performance). Similarly, a lower number of errors to criterion also indicates higher relative performance (fewer errors to learn a new paradigm).

2.2.5.3 Set-Shifting: Response Discrimination

Total number of trials to criterion, total number of errors to criterion, as well as number of perseverative, regressive, and never-reinforced errors were computed. Criterion was set at ten consecutive responses, and any omission that occurred in a string of consecutive trials towards criterion did not reset progress. Errors were analyzed in blocks of sixteen, with each block containing eight congruent and eight non-congruent trials. Perseverative errors were scored as such when rats selected the incorrect lever on six or more of the eight incongruent trials in a block. Conversely, regressive errors were scored as such when rats selected the incorrect lever on five or fewer of the eight incongruent trials in a block. Never-reinforced errors were scored when rats responded incorrectly on any congruent trial, regardless of the block.

2.2.5.4 Reversal Learning

Total number of trials to criterion, total number of errors to criterion, as well as number of perseverative and regressive errors were computed. Similarly to the RD task, criterion was set at ten consecutive responses, and omissions did not affect criterion attainment. Perseverative and regressive errors were analyzed and scored as outlined above (section 2.2.5.3). Since both RD paradigms had now occurred (i.e. both the 'non-biased' lever and the 'biased' lever have yielded a reward) as of RL, never-reinforced errors were no longer applicable, as they were previously reinforced in the RD task. Therefore, they were not analyzed in this task. Animals in the RL task did not undergo more than one testing session (120 trials), and as a result, not all animals achieved criterion.

2.2.6 Data Analysis

Weekly body weight and blood glucose values were analyzed using a two-way repeated measures analysis of variance (ANOVA) with time and diabetic status (non-diabetic vs. diabetic) as factors using GraphPad Prism 8 (GraphPad Software Incorporated). Post-hoc analysis was performed using a Sidak's multiple comparisons test. Trials to criterion and errors to criterion for all three tasks (VCD, RD, and RL), were analyzed using unpaired t-tests. Error types for the RD task (perseverative, regressive, and never-reinforced) were analyzed using a two-way ANOVA with diabetic status and error

type as factors. Post-hoc analysis was performed using Tukey's multiple comparisons test. Error types for the RL task (perseverative and regressive) were analyzed using a two-way ANOVA. Post-hoc analysis was performed using Sidak's multiple comparisons test. Statistical significance was set at a value of $\alpha = 0.05$ for all analyses.

2.3 Results

2.3.1 Animal Characteristics

Body weight and blood glucose were both independently analyzed to determine the impact of diabetic status (non-diabetic vs. diabetic) and time (week of study). Twenty-six rats (non-diabetic = 12, diabetic = 14) were included in final analysis for all measures. The original cohort included sixteen diabetic rats, however two males died throughout the course of the study and data from these animals were removed from all analyses.

For body weight (*Fig. 1a*), there was a significant interaction between diabetic status and time ($P < 0.0001$). The impact of diabetic status on body weight was not statistically significant ($P = 0.1800$). Post-hoc analysis revealed no significant differences between non-diabetic and diabetic groups at any time point. Diabetic animals lost ~ 17% body weight following diabetes induction (week 8 vs. week 9; $P = 0.0003$). This is a typical result of the diabetes induction protocol that has been observed in past works by our laboratory. Diabetic animals recovered their weight by week 13 (week 8 vs. week 13; $P > 0.9999$). A reduction in body weight (non-significant; week 14 vs. week 15 non-diabetic; $P = 0.0796$; week 14 vs. 15 diabetic; $P > 0.9999$) can also be observed at week 15 as a result of the food restriction protocol.

For blood glucose (*Fig. 1b*), there was also a significant interaction between diabetic status and time ($P < 0.0001$). The impact of diabetic status was significant on blood glucose levels ($P < 0.0001$). Post-hoc analysis revealed statistically significant differences in blood glucose levels at weeks 9, and 11 - 15 ($P \leq 0.0003$ for all). There was no blood glucose data obtained during week 8, as diabetic animals were biohazardous for a 10 day period during and following diabetes induction. As a result, biological samples could not

safely be collected. All blood glucose values for week 15 were obtained immediately following each animals operant conditioning task for the day. Values were elevated at this time due to the consumption of up to 140 sucrose pellets (6.3 g total) throughout the duration of testing.

2.3.2 Visual Cue Discrimination

There were no significant differences in the number of trials to criterion (P = 0.1294), nor the number of errors to criterion (P = 0.1244) between the non-diabetic and diabetic groups in the VCD task. Data are visualized in *Fig. 2*.

2.3.3 Set-Shifting: Response Discrimination

No significant differences were observed in the number of trials to criterion (P = 0.2991), nor number of errors to criterion (P = 0.3733). There was no significant interaction observed between diabetic status and error type (P = 0.9350). Post-hoc analysis confirmed that there were no differences in the number of perseverative (P > 0.9999), regressive (P = 0.9852), or never-reinforced (P = 0.9976) errors made between non-diabetic and diabetic groups. There were significantly more perseverative errors made compared to never-reinforced errors (P < 0.0001), and significantly more regressive errors made compared to never-reinforced errors (P < 0.0001). There was no difference between the number of perseverative errors and regressive errors made (P = 0.1541). All RD data are presented in *Fig. 3*.

2.3.4 Reversal Learning

Neither number of trials to criterion (P = 0.0601), nor errors to criterion (P = 0.2004) were different between non-diabatic and diabetic groups. Note that not all animals successfully reached criterion on the RL task, therefore final analysis only included n = 9 non-diabetic and n = 13 diabetic animals for trials to criterion and errors to criterion. Since criterion attainment did not affect either the total number of errors committed, nor the type of errors made, final analysis for RL error type included data from all animals. No significant interaction was found between diabetic status and error type (P = 0.7669). Post-hoc analysis confirmed that there were no differences in the number of perseverative (P = 0.6768), or regressive errors (P = 0.9164) committed between groups. Significantly more

perseverative errors were committed compared to regressive errors within both groups (non-diabetic perseverative versus regressive P = 0.0018; diabetic perseverative versus regressive P = 0.0026). All RL data are visualized in *Fig. 4*.

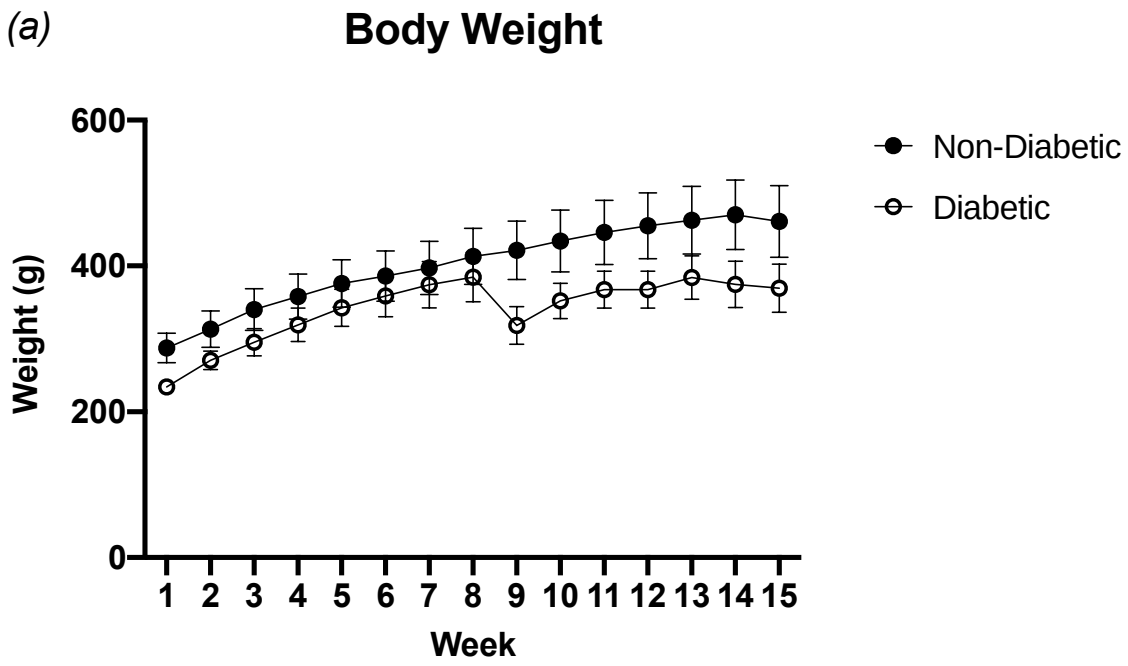
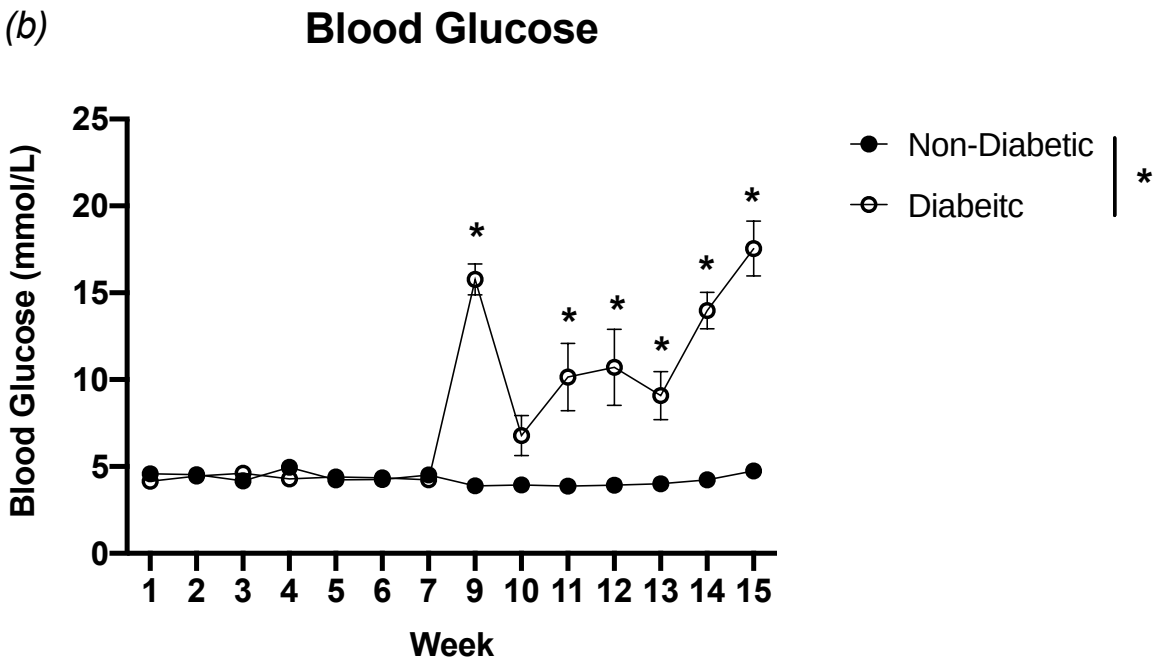


Figure 1: ***(a)*** *Mean body weight (g). Diabetes induction and food restriction occurred at week 8 and 14, respectively. Diabetic animals lost ~ 17% body weight following diabetes induction. This was recovered by week 13 (week 8 vs. week 13; P > 0.9999). Both groups slightly declined in body weight from week 14 to 15 as a result of food restriction. There was no significant impact of diabetic status on overall body weight. Post-hoc analysis revealed no significant differences between groups at any time point. Figure 1 (a) and (b) data are presented as mean ± SEM.*

(b)



(b) *Mean non-fasted blood glucose (mmol/L). Diabetes induction and food restriction occurred at week 8 and 14, respectively. No blood glucose data was obtained for week 8 as animals were biohazardous and biological samples could not safely be collected. Note that there are no error bars displayed for the non-diabetic group as they are too small to be visualized. Analysis revealed a significant impact of diabetic status on blood glucose. Post-hoc analysis revealed significant differences between groups at weeks 9, and 11 - 15. * Denotes P ≤ 0.0003.*

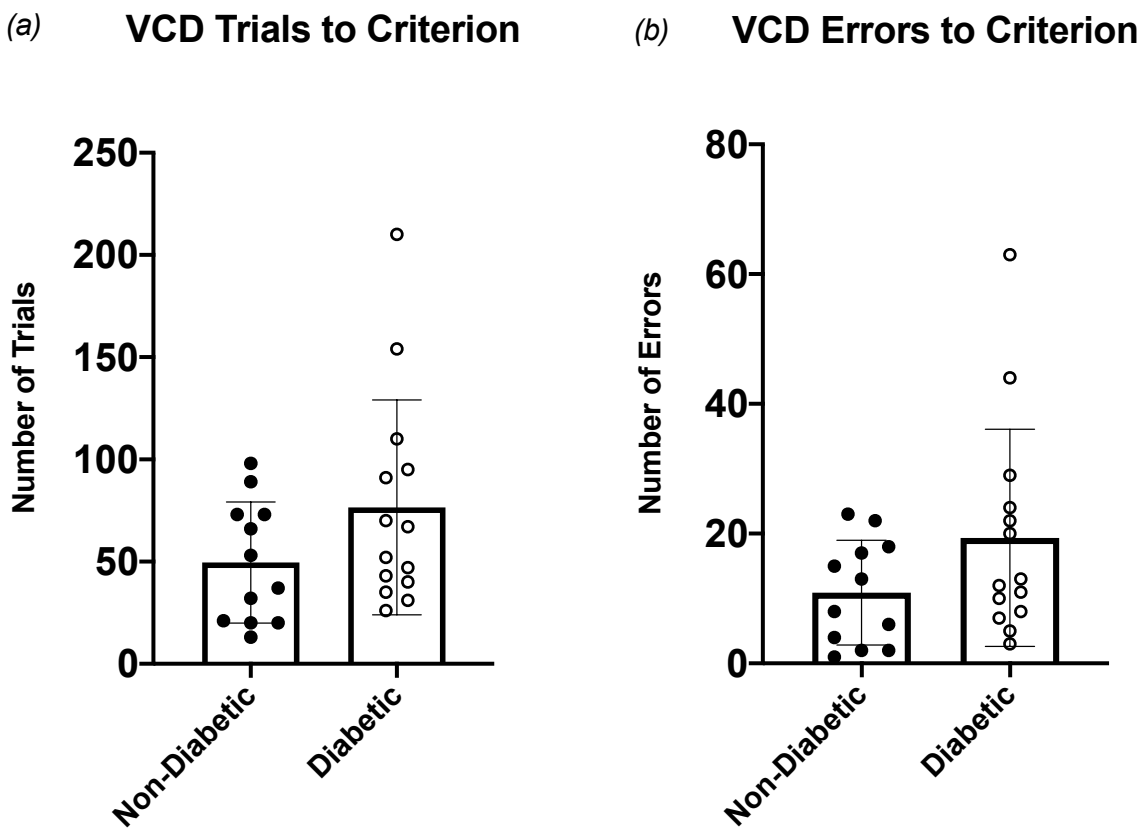


Figure 2: ***(a)*** *Visual cue discrimination (VCD) trials to criterion. No significant differences were observed between groups. Figures 2, 3, and 4 data are presented as mean ± SD with each dot representing an individual data point.*

(b) *VCD errors to criterion. No significant differences were observed between groups.*

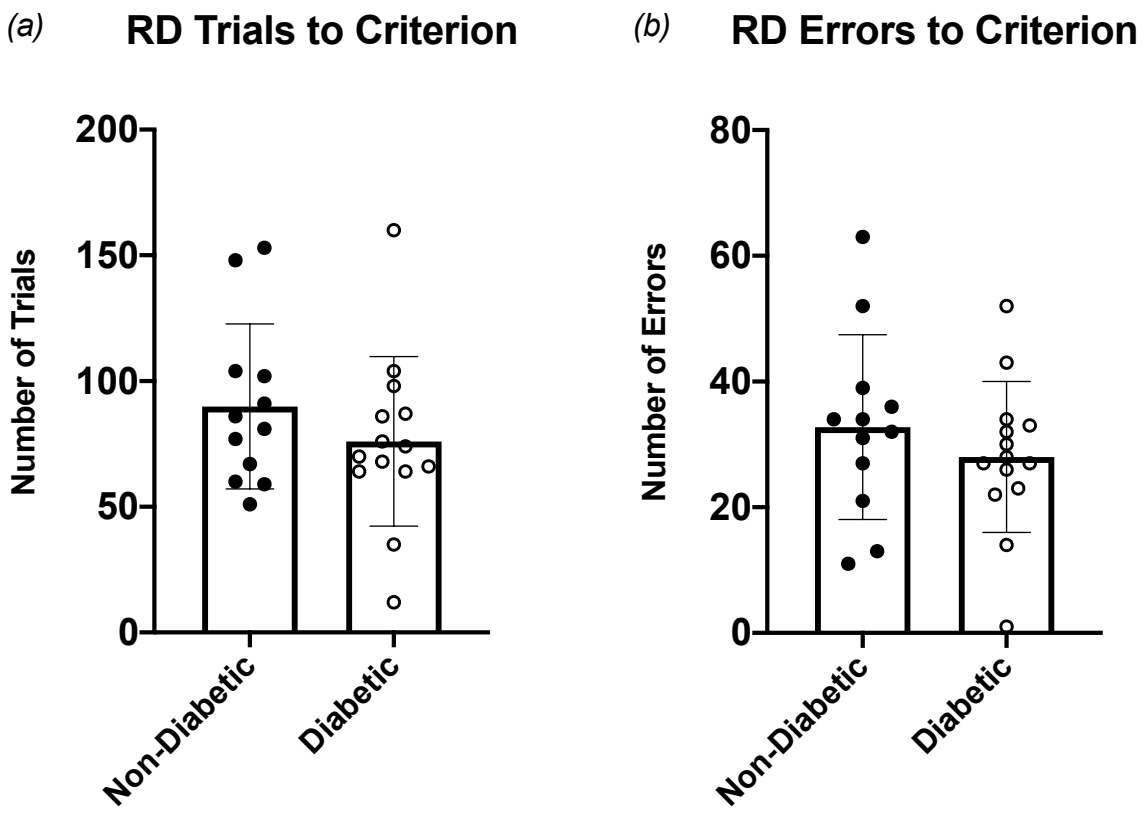
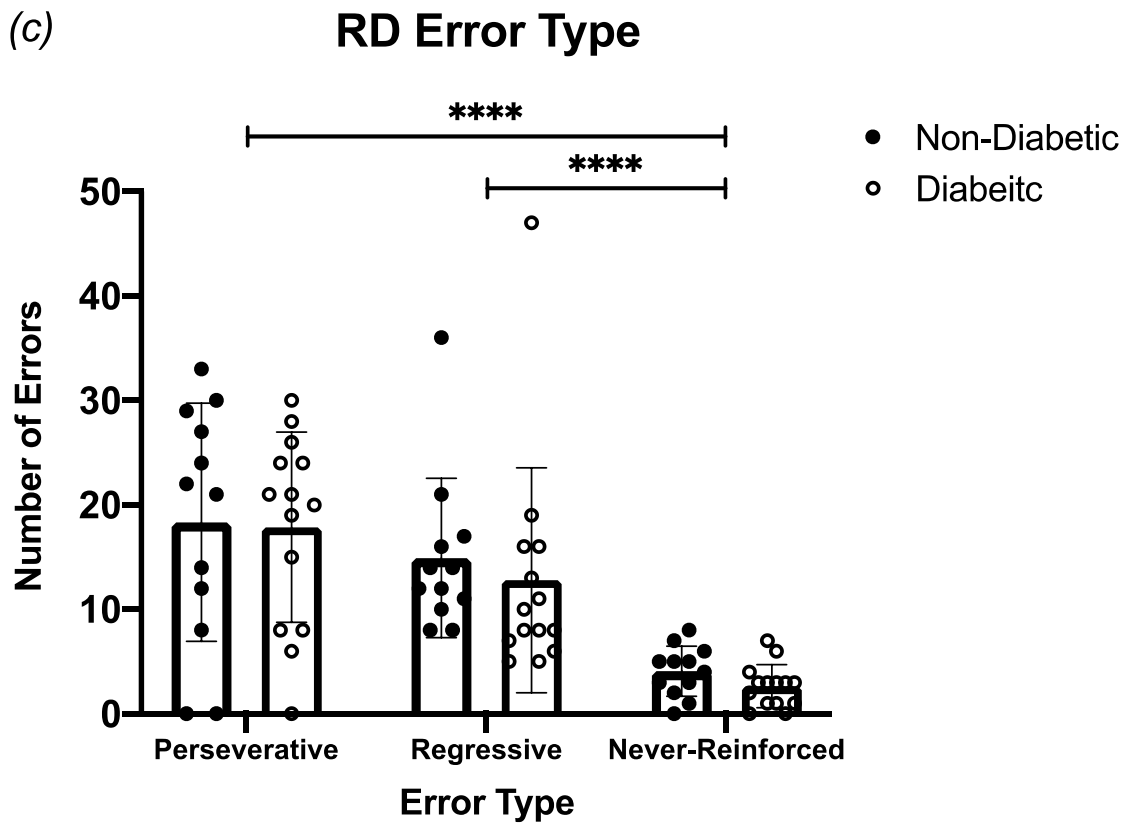


Figure 3: ***(a)*** *Response discrimination (RD) trials to criterion. No significant differences were observed between groups.*

(b) *RD errors to criterion. No significant differences were observed between groups.*



(c) *RD errors sorted by type. No significant differences were found between groups. Significantly more perseverative and regressive errors were committed compared to never-reinforced errors (**** denotes P < 0.0001 for both comparisons).*

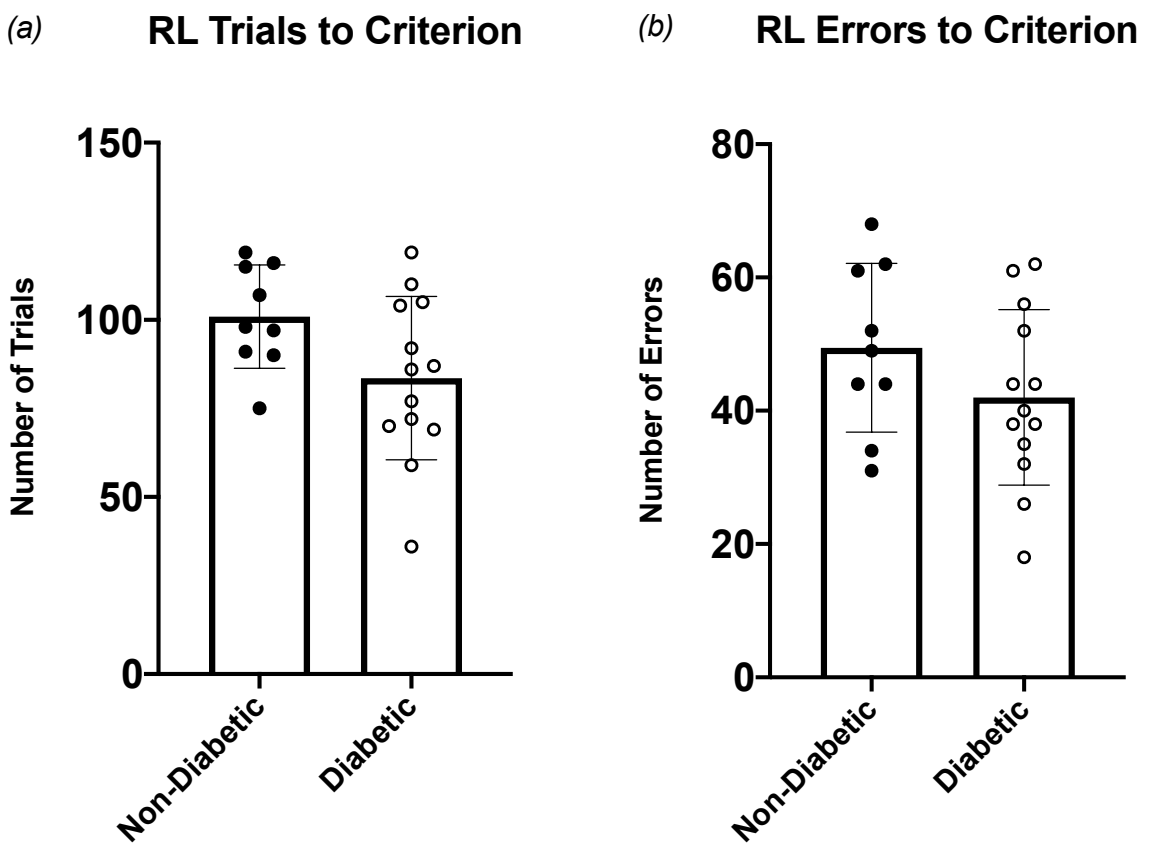
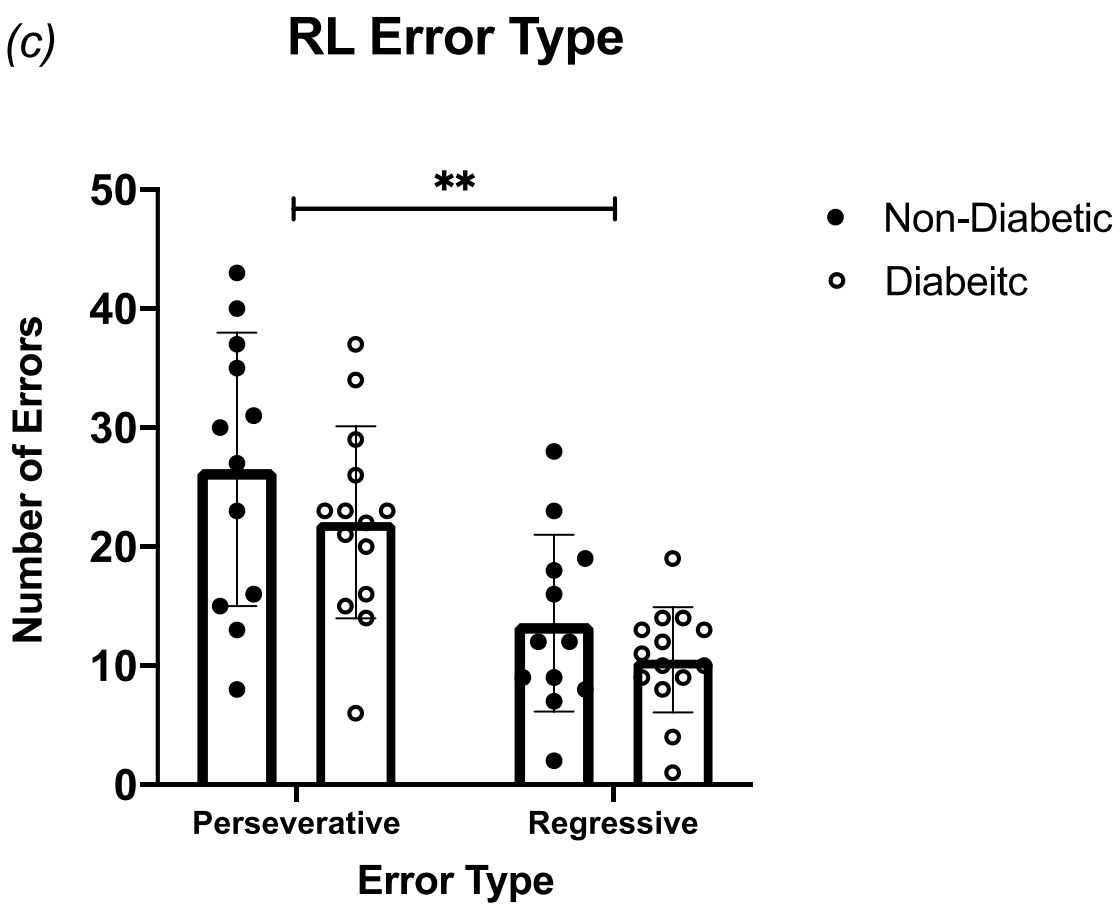


Figure 4: ***(a)*** *Reversal learning (RL) trials to criterion. Note that not all animals reached criterion in this task, therefore final analysis for RL trials to criterion and errors to criterion only included n = 9 non-diabetic and n = 13 diabetic animals. No significant differences were observed between groups.*

(b) *RL errors to criterion. No significant differences were observed between groups.*



(c) *RL errors sorted by type. Note that never-reinforced errors were not applicable in the RL task. No significant differences were observed between groups. Significantly more perseverative errors were committed compared to regressive errors by both groups (** denotes $P < 0.01$).*

2.4 Discussion

In the present study, we examined the impact of T1DM on EF using a series of operant conditioning based tasks. Contrary to our hypothesis, the results presented suggest that T1DM rats do not show decrements on tests of EF nor set-shifting compared to non-diabetic rats. Although this finding may not directly align with similar prior research in humans that demonstrated subtle impairments in EF in patients with T1DM [22], to our knowledge, the present study was the first to specifically analyze the executive function of the T1DM rat.

Statistical analysis revealed that diabetic status (i.e. non-diabetic vs. diabetic) had no significant impact on body weight. Although diabetic animals lost on average ~ 17% body weight following diabetes induction (week 8 vs. week 9; $P = 0.0003$), they eventually recovered their body weight to pre-diabetic levels by week 13 (mean week 8 = 384.43 g; mean week 13 = 384.21 g; $P > 0.9999$). As anticipated, both groups declined as a result of food restriction (non-significant; week 14 vs. week 15 non-diabetic; $P = 0.0796$; week 14 vs. 15 diabetic; $P > 0.9999$). There was a significant impact of diabetic status on blood glucose levels ($P < 0.0001$). Post-hoc analysis revealed significant differences between groups at weeks 9, and 11 - 15 ($P \leq 0.0003$ for all). Due to the nature of the insulin therapy used, blood glucose levels are lowest immediately following insulin pellet implantation (week 9), which may explain why no differences were observed between groups at week 10.

In the visual cue discrimination task, no differences were found in the number of trials, nor errors to criterion between non-diabetic and diabetic rats. Statistical analysis of both trials, and errors to criterion, as well as total error number and type in the response discrimination , and reversal learning tasks also revealed no significant differences between the two groups. Taken together, these data suggest that an insulin-treated rodent model of T1DM does not show any decrement in cognitive function, or more specifically executive function.

Several reasons may explain the apparent preservation of EF. Perhaps most significantly, our diabetic group utilized an insulin-treated model of T1DM. Although

rodents in the T1DM group had significantly higher blood glucose than those in the non-diabetic group (*Fig. 1b*), they were maintained within a tighter range compared to other works that did not attempt to regulate glycemia [23-27, 31, 43, 44]. It is plausible that these levels of extreme hyperglycemia were at least partially responsible for the cognitive deficits observed in these works. Further evidence to support this comes from works that have demonstrated the efficacy of insulin treatment at ameliorating cognitive deficits in T1DM rodents when specifically compared to non-insulin treated T1DM groups [18, 19, 32]. Similarly, data from human work has shown that those with higher HbA1c concentrations (indicative of periods of sustained hyperglycemia) showed inferior performance on measures of cognition [9]. It is purported that poor long-term metabolic control (higher HbA1c) increases diabetic comorbidities such as microvascular complications [16] which have been independently correlated to impaired cognition [8].

There is significant research demonstrating the deleterious impact of T1DM on systemic vasculature, much of which has focused on the eyes, kidneys, heart, and extremities [45-48]. Furthermore, previous work from our laboratory has demonstrated reductions in vascular function using the same T1DM model as the present study [49], however cerebral vasculature was not examined. Other studies have specifically explored cerebral vascular function in T1DM, generally reporting attenuated function. More specifically, cerebral blood flow has been shown to be markedly reduced in STZ-induced T1DM rodent models [50, 51]. However, these reductions appear to be region specific and were shown to disproportionately affect the hippocampus, while sparing the 'nontelencephalic' regions (i.e. the cerebrum which includes the mPFC) [50]. This could in part explain why many past works that emphasized mainly tests of hippocampal function reported performance decrements [19, 23-32], while the present work, which mainly assesses mPFC function does not. Future projects that assess executive function should specifically examine cerebral blood flow to different regions the brain, namely the cerebral lobes and prefrontal cortex.

The age at diabetes onset may have been another contributing factor in our results. Diabetes was induced in week 8 of the study, which was approximately week 16 of the rodent's lifespan. At this age, and body weight (*Fig. 1a*) Sprague Dawley rats are sexually

mature and are in adulthood [52]. Research outlined above has shown that those with an earlier age at disease onset, defined as younger than 7 years of age in humans, showed more significant cognitive impairments than those with a later age at onset [12, 13]. Although developmental periods do not align with humans, it is possible that rats were already past the more vulnerable periods of brain development that were theorized to exacerbate cognitive impairments in early onset humans [53]. In accordance with this, several other animal works that reported cognitive dysfunction induced T1DM at a younger age (approximately week 8 of the rodents' lifespan) [23, 26, 54, 55]. However, data from Kamal *et al.* [29] showed that rats induced at both 3 and 22 months of age showed inferior MWM performance compared to age matched controls, indicating that age at diabetes onset may not necessarily play a significant role.

Another important consideration may be the duration of diabetes. In the present study, rats were diabetic for approximately 8 weeks by completion of the final RL task. It is possible that this disease course was not long enough to cause any detectable functional changes. In what is perhaps the most widely cited evidence of cognitive dysfunction in T1DM, Brands *et al.* [8] reported that there was no consistent relationship between disease duration and cognition. However, they only included studies in which T1DM groups had been diagnosed with diabetes for a minimum of 18 years. Perhaps it is possible that had they included studies with shorter disease durations, they may have seen a correlation. However, past animal studies have shown that performance decrements were evident in as few as 3 - 4 weeks of diabetes [19, 55]. Interestingly, Rajashree *et al.* [56] reported that cognitive decrements worsened with disease course, showing that increased cognitive deficits were observed in rats that had been diabetic for 20 days compared to those who were only diabetic for 10 days. Although it is possible that our disease course was not adequate to induce observable cognitive changes, it would appear based on these works that changes are evident even after a few weeks of T1DM. It is perhaps more likely based on the evidence outlined above that other factors such as the role of insulin treatment may explain the lack of EF differences between non-diabetic and diabetic groups observed in the present study.

One significant finding in the present study was that there were significantly more perseverative and regressive errors made compared to never-reinforced errors by both groups in the RD task ($P < 0.0001$ for both comparisons). Similarly, there were more perseverative errors committed than regressive errors in the RL task by both groups (non-diabetic $P = 0.0018$; diabetic $P = 0.0026$). This aligns with the work of others using the same operant conditioning based tasks [40-42]. Floresco *et al.* [40] hypothesized that due to the fixed spatial orientation of the chamber, rats may have fewer strategies at their disposal compared to other assessments of behavioral flexibility such as the radial arm maze [57]. Given that perseverative errors are indicative of a failure to adopt a new strategy, it is plausible that rats have a greater propensity to adhere to the former cue rather than seeking out the novel, correct cue [40].

2.5 Limitations and Future Directions

To our knowledge, this study is the first to assess the EF abilities of an insulin-treated T1DM rodent model. Furthermore, we included both male and female rodents to incorporate a representative sample of the T1DM population. In summary, we have demonstrated that non-diabetic and insulin-treated T1DM rodents show no differences in their executive function, nor set-shifting abilities. Given the exploratory nature and novelty of this project, it is important to highlight certain limitations. Firstly, we were limited by our sample size due to constraints in the number of rodents that could be procured and properly managed within the project timeframe. Furthermore, we recognize that the rodents in the non-diabetic group were not subjected to the same experimental stressors as those in the diabetic group (i.e. injections, anesthetization, and surgery in non-diabetic animals may have impacted our results). Another limitation may have stemmed from potential visual impairments amongst the diabetic rodents. As outlined in the literature review, retinopathies are a common comorbidity of T1DM and may adversely affect vision. Although we did not specifically test for visual deficits, all rodents were carefully monitored by both study investigators and veterinary staff for any abnormalities, especially throughout the testing period. Given that neither party noted any visual abnormalities in

the diabetic rodents in the present study, we feel confident that visual disturbances did not impact our results.

Future work should aim to employ a larger sample size, ensuring each group receives identical treatment by subjecting non-diabetic rodents to saline injections and sham surgeries. Future studies that adjust variables such as the age at diabetes induction, disease duration, and treatment interventions (e.g. insulin) in order to gauge their impact on executive function are also warranted. Post-mortem analysis of the brain, cerebral blood flow, and other measures of vascular health may provide valuable insight into the mechanisms of EF in T1DM rats. More specifically, structural and biochemical analysis of the underlying brain regions that have been shown to govern EF such as the mPFC may be warranted.

2.6 Conclusion

In conclusion, this study has demonstrated that there are no significant differences in the executive function nor set-shifting ability of non-diabetic and T1DM rats. We hypothesized that insulin-treated T1DM rats would show impairments in EF, specifically manifested as an increased number of trials and errors to criterion in the response discrimination task. However, data from the present study demonstrates that there were no significant differences between groups in any of the operant conditioning measures obtained. It is possible that the age at diabetes induction, diabetes duration, and treatment interventions (insulin) prevented us from observing any cognitive decrements in the T1DM group. Future work may look to alter these variables, while taking into consideration some of the limitations of the present study. The adoption of additional experimental measures such as biochemical and/or histological analysis of the mPFC may provide valuable insight into the physiology of the T1DM rodent brain.

2.7 Bibliography

1. Daneman, D. Type 1 diabetes. *Lancet* **367**, 847–858 (2006).

2. Wood J, P. A. *The Type 1 Diabetes Self-Care Manual*. *The Autoimmune Diseases* (2006). doi:10.2337/9781580406208

3. Sarwar, N. *et al.* Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: A collaborative meta-analysis of 102 prospective studies. *Lancet* **375**, 2215–2222 (2010).

4. Hicks, C. W. & Selvin, E. Epidemiology of Peripheral Neuropathy and Lower Extremity Disease in Diabetes. *Curr. Diab. Rep.* **19**, 86 (2019).

5. Bourne, R. R. A. *et al.* Causes of vision loss worldwide, 1990-2010: A systematic analysis. *Lancet Glob. Heal.* **1**, 339–349 (2013).

6. Wasserman, D. H. Four grams of glucose. *Am. J. Physiol. - Endocrinol. Metab.* **296**, 11–21 (2009).

7. Miles, W. R. & Root, H. F. Psychologic Tests Applied to Diabetic Patients. *Arch. Intern. Med.* **30**, 767 (1922).

8. Brands, A. M. A., Biessels, G. J., de Haan, E. H. F., Kappelle, L. J. & Kessels, R. P. C. The effects of type 1 diabetes on cognitive performance: a meta-analysis. *Diabetes Care* **28**, 726–35 (2005).

9. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research Group *et al.* Long-term effect of diabetes and its treatment on cognitive function. *N. Engl. J. Med.* **356**, 1842–52 (2007).

10. Tonoli, C. *et al.* Type 1 diabetes-associated cognitive decline: A meta-analysis and update of the current literature. *J. Diabetes* **6**, 499–513 (2014).

11. Nunley, K. A. *et al.* Clinically relevant cognitive impairment in Middle-Aged adults with childhood-onset type 1 diabetes. *Diabetes Care* **38**, 1768–1776 (2015).

12. Gaudieri, P. A., Chen, R., Greer, T. F. & Holmes, C. S. Cognitive function in children with type 1 diabetes. *Diabetes Care* **31**, 1892–1897 (2008).

13. Dahlquist, G. & Källén, B. School performance in children with type 1 diabetes-a population-based register study. *Diabetologia* **50**, 957–964 (2007).

14. Chaytor, N. S. *et al.* Clinically significant cognitive impairment in older adults with type 1 diabetes. *J. Diabetes Complications* **33**, 91–97 (2019).

15. Ferguson, S. C. *et al.* Cognitive ability and brain structure in type 1 diabetes: Relation to microangiopathy and preceding severe hypoglycemia. *Diabetes* **52**, 149–156 (2003).

16. Ryan, C. M., Geckle, M. O. & Orchard, T. J. Cognitive efficiency declines over time in adults with Type 1 diabetes: Effects of micro- and macrovascular complications. *Diabetologia* **46**, 940–948 (2003).

17. Sharma, S. & Brown, C. E. Microvascular basis of cognitive impairment in type 1 diabetes. *Pharmacol. Ther.* (2021). doi:10.1016/j.pharmthera.2021.107929

18. Biessels, G. J. *et al.* Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: Effects of insulin treatment. *Brain Res.* **800**, 125–135 (1998).

19. Marissal-Arvy, N. *et al.* Insulin treatment partially prevents cognitive and hippocampal alterations as well as glucocorticoid dysregulation in early-onset insulin-deficient diabetic rats. *Psychoneuroendocrinology* **93**, 72–81 (2018).

20. Diamond, A. Executive functions. *Annu. Rev. Psychol.* **64**, 135–68 (2013).

21. Nowrangi, M. A., Lyketsos, C., Rao, V. & Munro, C. A. Systematic review of neuroimaging correlates of executive functioning: converging evidence from different clinical populations. *J. Neuropsychiatry Clin. Neurosci.* **26**, 114–25 (2014).

22. Broadley, M. M., White, M. J. & Andrew, B. A Systematic Review and Meta-analysis of Executive Function Performance in Type 1 Diabetes Mellitus. *Psychosom. Med.* **79**, 684–696 (2017).

23. Chen, G., Wang, Y., Li, Y., Zhang, L. & Dong, M. A novel hippocampus metabolite signature in diabetes mellitus rat model of diabetic encephalopathy. *Metab. Brain Dis.* (2020). doi:10.1007/s11011-020-00541-2

24. Ahmed, A. *et al.* Time-dependent impairments in learning and memory in Streptozotocin-induced hyperglycemic rats. *Metab. Brain Dis.* **34**, 1431–1446 (2019).

25. Zhu, B., Jiang, R. Y., Yang, C. & Liu, N. Adenosine monophosphate-activated protein kinase activation mediates the leptin-induced attenuation of cognitive impairment in a streptozotocin-induced rat model. *Exp. Ther. Med.* **9**, 1998–2002 (2015).

26. Zhang, S., Yuan, L., Zhang, L., Li, C. & Li, J. Prophylactic use of troxerutin can delay the development of diabetic cognitive dysfunction and improve the expression of Nrf2 in the hippocampus on STZ diabetic rats. *Behav. Neurol.* **2018**, (2021).

27. Manschot, S. M. *et al.* Angiotensin converting enzyme inhibition partially prevents deficits in water maze performance, hippocampal synaptic plasticity and cerebral blood flow in streptozotocin-diabetic rats. *Brain Res.* **966**, 274–282 (2003).

28. Ahmadi, M., Rajaei, Z., Hadjzadeh, M. A., Nemati, H. & Hosseini, M. Crocin improves spatial learning and memory deficits in the Morris water maze via attenuating cortical oxidative damage in diabetic rats. *Neurosci. Lett.* **642**, 1–6 (2017).

29. Kamal, A., Biessels, G. J., Duis, S. E. J. & Gispen, W. H. Learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: Interaction of diabetes and ageing. *Diabetologia* **43**, 500–506 (2000).

30. Li, Z. G., Zhang, W., Grunberger, G. & Sima, A. A. F. Hippocampal neuronal apoptosis in type 1 diabetes. *Brain Res.* **946**, 221–231 (2002).

31. Revsin, Y. *et al.* Glucocorticoid receptor blockade normalizes hippocampal alterations and cognitive impairment in streptozotocin-induced type 1 diabetes mice. *Neuropsychopharmacology* **34**, 747–758 (2009).

32. Kassab, S. *et al.* Cognitive dysfunction in diabetic rats is prevented by pyridoxamine treatment. A multidisciplinary investigation. *Mol. Metab.* **28**, 107–119 (2019).

33. R. G. M. Morris, P. Garrud, J. N. P. Rawlins & J. O'Keefe. Place navigation impaired in rats with hippocampal lesions. *Nature* **297**, 681–683 (1982).

34. Dere, E., Huston, J. P. & De Souza Silva, M. A. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci. Biobehav. Rev.* **31**, 673–704 (2007).

35. Bizon, J. L., Foster, T. C., Alexander, G. E. & Glisky, E. L. Characterizing cognitive aging of working memory and executive function in animal models. *Front. Aging Neurosci.* **4**, 1–14 (2012).

36. Ragozzino, M. E. The effects of dopamine D1 receptor blockade in the prelimbic-infralimbic areas on behavioral flexibility. *Learn. Mem.* **9**, 18–28 (2002).

37. Stefani, M. R., Groth, K. & Moghaddam, B. Glutamate receptors in the rat medial prefrontal cortex regulate set-shifting ability. *Behav. Neurosci.* **117**, 728–737 (2003).

38. Birrell, J. M. & Brown, V. J. Medial frontal cortex mediates perceptual attentional set shifting in the rat. *J. Neurosci.* **20**, 4320–4324 (2000).

39. Grant, D. A. & Berg, E. A behavioral analysis of degree of reinforcement and ease of shifting to new responses in a Weigl-type card-sorting problem. *J. Exp. Psychol.* **38**, 404–411 (1948).

40. Floresco, S. B., Block, A. E. & Tse, M. T. L. Inactivation of the medial prefrontal cortex of the rat impairs strategy set-shifting, but not reversal learning, using a novel, automated procedure. *Behav. Brain Res.* **190**, 85–96 (2008).

41. Levit, A. *et al.* Behavioural inflexibility in a comorbid rat model of striatal ischemic injury and mutant hAPP overexpression. *Behav. Brain Res.* **333**, 267–275 (2017).

42. Desai, S. J., Allman, B. L. & Rajakumar, N. Combination of behaviorally sub-effective doses of glutamate NMDA and dopamine D1 receptor antagonists impairs executive function. *Behav. Brain Res.* **323**, 24–31 (2017).

43. Zarrinkalam, E., Ranjbar, K., Salehi, I., Kheiripour, N. & Komaki, A. Resistance training and hawthorn extract ameliorate cognitive deficits in streptozotocin-induced diabetic rats. *Biomed. Pharmacother.* **97**, 503–510 (2018).

44. De Senna, P. N. *et al.* Effects of physical exercise on spatial memory and astroglial alterations in the hippocampus of diabetic rats. *Metab. Brain Dis.* **26**, 269–279 (2011).

45. Donnelly, R., Emslie-Smith, A. M., Gardner, I. D. & Morris, A. D. Vascular complications of diabetes. *Bmj* **320**, 1062–1066 (2000).

46. Bursell, S. E. *et al.* Retinal blood flow changes in patients with insulin- dependent diabetes mellitus and no diabetic retinopathy: A video fluorescein angiography study. *Investig. Ophthalmol. Vis. Sci.* **37**, 886–897 (1996).

47. Hink, U., Tsilimingas, N., Wendt, M. & Münzel, T. Mechanisms underlying endothelial dysfunction in diabetes mellitus: Therapeutic implications. *Treat. Endocrinol.* **2**, 293–304 (2003).

48. Fowler, M. J. Microvascular and macrovascular complications of diabetes. *Clin. Diabetes* **29**, 116–122 (2011).

49. Murias, J. M. *et al.* Vessel-specific rate of vasorelaxation is slower in diabetic rats. *Diabetes Vasc. Dis. Res.* **10**, 179–186 (2013).

50. Duckrow, R. B., Beard, D. C. & Brennan, R. W. Regional cerebral blood flow decreases during chronic and acute hyperglycemia. *Stroke* **18**, 52–58 (1987).

51. Knudsen, G. M., Göbel, U., Paulson, O. B. & Kuschinsky, W. Regional density of perfused capillaries and cerebral blood flow in untreated short-term and long-term streptozotocin diabetes. *J. Cereb. Blood Flow Metab.* **11**, 361–365 (1991).

52. Sengupta, P. The laboratory rat: Relating its age with human's. *Int. J. Prev. Med.* **4**, 624–630 (2013).

53. Ryan, C. M. Why is cognitive dysfunction associated with the development of diabetes early in life? The diathesis hypothesis. *Pediatr. Diabetes* **7**, 289–297 (2006).

54. Bhutada, P. *et al.* Ameliorative effect of quercetin on memory dysfunction in streptozotocin-induced diabetic rats. *Neurobiol. Learn. Mem.* **94**, 293–302 (2010).

55. Baydas, G., Nedzvetskii, V. S., Nerush, P. A., Kirichenko, S. V. & Yoldas, T. Altered expression of NCAM in hippocampus and cortex may underlie memory and learning deficits in rats with streptozotocin-induced diabetes mellitus. *Life Sci.* **73**, 1907–1916 (2003).

56. Rajashree, R., Kholkute, S. D. & Goudar, S. S. Effects of duration of diabetes on behavioral and cognitive parameters in streptozotocin-induced juvenile diabetic rats. *Malaysian J. Med. Sci.* **18**, 25–30 (2011).

57. Ragozzino, M. E., Detrick, S. & Kesner, R. P. Involvement of the prelimbic-infralimbic areas of the rodent prefrontal cortex in behavioral flexibility for place and response learning. *J. Neurosci.* **19**, 4585–4594 (1999).

Appendices

Appendix A: Multiple Low-Dose Streptozotocin Injections

Purpose: To induce Type 1 Diabetes Mellitus in rats

Materials: Gloves, Lab Coat, Streptozotocin (STZ), 5X Stock Citric Acid/Citrate Buffer, Anhydrous Citric Acid, Sodium Citrate Dihydrate, MilliQ Deionized Water, 13M HCl, Falcon Tubes, Sterile Filter

Equipment: Biological Safety Cabinet Weigh Scale pH Meter

Procedure:

Preparing 5X Citric Acid/Citrate Buffer

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add:
    a. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
    b. 23.8g Sodium Citrate Dihydrate (Sigma), Mix into…
    c. 175mL of MilliQ water the pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)
2. Once the proper pH is attained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

Mixing and dosing STZ for injection (Note: animals should be weighed prior to dosing STZ to ensure accurate amounts)

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM.
2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.
4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15-minute time frame. Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg. 20mg/kg X 0.2kg = 4mg per animal.
5. The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g).
6. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1 min). Sterile filter using a 0.2µm syringe filter. Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution 4mg ÷ 16mg/mL solution = 0.25mL. STZ is time dependent and must be used within 15 minutes.
7. Injecting and Follow-Up of the Animals 1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 15 minutes after it has been

dissolved in the sodium citrate buffer. 2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharp's container. 3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection). 4. Repeat this procedure the following day. 5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of > 15 mmol/L. Diabetes should be achieved after 5-8 injections.

References:

Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols. 2003

O'Brien BA, Harmon B V, Cameron DP, Allan DJ. Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. J Pathol 178: 176–181, 1996.

Melling CWJ, Grisé KN, Hasilo CP, Fier B, Milne KJ, Karmazyn M, Noble EG. A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. Diabetes Metab 39: 226–235, 2013.

Appendix B: Insulin Pellet Implantation

Pellet implantation (for a rat):

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O_2 flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isoflurane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% povidone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.
6. Cleanse a 12g trocar with 10% povidone-iodine solution and insert it through the puncture site at least 2 cm horizontally from the incision site.
7. Using forceps, briefly immerse the pellet in 10% povidone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for up to the first 350g of body weight.
9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % povidone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:

1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% povidone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.

4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

Appendix C: Food Restriction Schedule

Day 1 - 2	Day 3 - 4	Day 5 - 6	Day 7 - 8	Day 9 +
6.0 g / 100 g body weight	5.4 g / 100 g body weight	4.8 g / 100 g body weight	4.5 g / 100 g body weight	4.2 g / 100 g body weight

Note: all values provided are minimums. In certain instances, diabetic animals were allotted slightly more than the minimum allowance in order to stabilize weight and blood glucose levels.

Appendix D: Visual Cue Retrieval Data

	Non-Diabetic	Diabetic
SSF0	85.00%	85.00%
SSF1	80.00%	95.00%
SSF2	80.00%	90.00%
SSF3	95.00%	85.00%
SSF4	95.00%	80.00%
SSF5	80.00%	95.00%
SSF6	N/A	80.00%
SSF7	N/A	85.00%
SSM0	80.00%	N/A
SSM1	85.00%	95.00%
SSM2	100.00%	90.00%
SSM3	75.00%	85.00%
SSM4	90.00%	N/A
SSM5	90.00%	90.00%
SSM6	N/A	90.00%
SSM7	N/A	100.00%
Mean	**86.25%**	**88.93%**
SD	7.72%	5.94%
SEM	2.23%	1.59%

Note: All animals scored ≥ 80% (i.e. at least sixteen correct trials) in order to proceed to the RD task.

Appendix E: MED-PC to Excel Workbook Instructions

MED-PC to Excel Conversion

1. Open MPC2XL.EXE (MED-PC to Excel)
2. Under “Row Transfer” in the “Transfer Data” tab, press “Select”
3. Open “$Visual cue discrimination.MRP” for visual cue discrimination files, open “$Shift Response Discrimination.MRP” for response discrimination files.
4. Make sure both “Column Labels” and “Data” are select under the “Transfer” box
5. Select “Vertical (Column)” under the “Orientation” box
6. Open Excel workbook and ensure that the A1 cell on the first tab (*Input*) is selected; this applies to both workbooks. Transferring data into any other cell can cause irreversible malfunction of the workbooks.
7. Return to MED-PC to Excel, press “Transfer”, select raw data file

Visual Cue Workbook

Once data is transferred into *Visual Cue Workbook.xlsm*, key measures will appear in cells E3-5: Trials to Criterion, Errors to Criterion, Errors after Criterion. Criterion is set to 8 consecutive correct response. Omissions do not reset a series of correct responses. Rows 27-426 show raw data with annotations: Trial number, whether criteria has been met, running total counts of errors, and running counts of consecutive correct responses (performance).

Response Discrimination Workbook

The Excel workbook *Response Discrimination Workbook.xlsm* requires the use of VBA macros. To enable the use of VBA macros in excel, users must first enable the developer tab (see “Show the Developer tab” in Microsoft Office Support documentation) and enable macros (see “Enable or disable macros in Office files” in Microsoft Office Support documentation).

Once Data is transferred into *Response Discrimination Workbook.xlsm*, go to the ‘Developer’ tab and select ‘Macros’. Run *Compiled_Left2* (shortcut key: Ctrl + Shift + E) or *Compiled_Right2* (shortcut key: Ctrl + Shift + R) depending on whether the left or right

lever was selected to be rewarding, respectively. If uncertain which lever was selected to be rewarding in a given experiment, the raw data file can be inspected using a text editor such as *Notepad* or *Word* (Microsoft) and the experiment protocol can be found next to 'MSN:' as either $Shift Response Discrimination LEFT or $Shift Response Discrimination RIGHT corresponding to left or right rewarding levers, respectively. Once the Compiled_Left/Right2 macro is run, Excel should end on the *Summary* tab with error profile data.

To reset the workbook, run the *Clear All* macro (shortcut key: Ctrl + Shift + C).

To produce a list of errors for each incongruent trial, as required for logistic regression modelling, run *Compiled_Left* (shortcut key: Ctrl + E) or *Compiled_Right* (shortcut key: Ctrl + R) on transferred data. Return to the *Input* tab and copy the data under Column D which will indicate '1' for incorrect lever presses or '0' for correct lever presses or omissions for all incongruent trials in order from the top of the column to the bottom.

Appendix F: Animal Use Protocol Approval

PI :	Melling, Jamie
Protocol #	2018-063
Status :	Approved (w/o Stipulation)
Approved :	07/01/2018
Expires :	07/01/2022
Title :	The role of exercise on ameliorating the negative metabolic and vascular effects of Type 1 diabetes.

Table of Contents

Curriculum Vitae

Name: Kevin T. Murphy

Post-secondary Education and Degrees:

Master of Science, Integrative Biosciences
Western University
London, Ontario, Canada
2019 - 2021

Honours Bachelor of Science, Kinesiology
McMaster University
Hamilton, Ontario, Canada
2014 - 2018

Honours and Awards:

Ontario Graduate Scholarship
2020 - 2021

Western Graduate Research Scholarship
2020 - 2021

Natural Sciences and Engineering Research Council of Canada (NSERC) Canada Graduate Scholarship - Master's (CGS-M)
2019 - 2020

Ontario Graduate Scholarship (declined in lieu of CGS-M)
2019 - 2020

Related Work Experience

Teaching Assistant
School of Kinesiology, Faculty of Health Sciences, Western University
2019 - 2021

Research Assistant
Lifestyle Research Team, Department of Family Medicine, Western University
2019 - 2021

Peer-Reviewed Publications:

Stokes T, Tripp TR, **Murphy K**, Morton RW, Oikawa SY, Lam Choi H, McGrath J, McGlory C, MacDonald MJ, Phillips SM. Methodological considerations for and validation of ultrasonographic determination of human skeletal muscle hypertrophy and atrophy. *Physiol Rep*. 9:e14683, 2021.

Stokes T, Timmons JA, Crossland H, Tripp TR, **Murphy K**, McGlory C, Mitchell C, Oikawa SY, Morton R, Phillips BE, Baker SK, Atherton PJ, Wahlestedt C, Phillips SM. Molecular transducers of human skeletal muscle remodeling in different loading states. *Cell Rep*. 32(5): 107980, 2020.

Morton RW, **Murphy KT**, McKellar SR, Schoenfeld BJ, Henselmans M, Helms E, Aragon AA, Devries MC, Banfield L, Krieger JW, Phillips SM. Infographic. The effect of protein supplementation on resistance training-induced gains in muscle mass and strength. Br J Sports Med. 2019.

Jakubowski JS, Wong EPT, Nunes EA, Noguchi KS, Vandeweerd JK, **Murphy KT**, Morton RW, McGlory C, Phillips SM. Equivalent hypertrophy and strength gains with HMB- or leucine-supplemented men. *Med Sci Sports Exerc*. 51(1): 65-74, 2018.

Morton RW, **Murphy KT**, McKellar SR, Schoenfeld BJ, Henselmans M, Helms E, Aragon AA, Devries MC, Banfield L, Krieger JW, Phillips SM. A systematic review, meta-analysis and meta-regression of the effect of protein supplementation on resistance training-induced gains in muscle mass and strength in healthy adults. *Br J Sports Med*. 52: 376-384, 2017.