EFFECTS OF INSULIN ON SYNAPSE FORMATION AND FUNCTION; A POSSIBLE ROLE FOR INSULIN RESISTANCE

by

Manoj Hariharan

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
December 2012

© Copyright by Manoj Hariharan, 2012

DALHOUSIE UNIVERSITY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "EFFECTS OF INSULIN ON SYNAPSE FORMATION AND FUNCTION; A POSSIBLE ROLE FOR INSULIN RESISTANCE" by Manoj Hariharan in partial fulfilment of the requirements for the degree of Master of Science.

Dated: December 5, 2012

Supervisor: _________________________________

Readers: _________________________________

____

____

DALHOUSIE UNIVERSITY

DATE: December 5, 2012

AUTHOR: Manoj Hariharan

TITLE: EFFECTS OF INSULIN ON SYNAPSE FORMATION AND FUNCTION; A POSSIBLE ROLE FOR INSULIN RESISTANCE

DEPARTMENT OR SCHOOL: Department of Biochemistry and Molecular Biology

DEGREE: MSc CONVOCATION: May YEAR: 2013

Permission is herewith granted to Dalhousie University to circulate and to have copied for non-commercial purposes, at its discretion, the above title upon the request of individuals or institutions. I understand that my thesis will be electronically available to the public.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

The author attests that permission has been obtained for the use of any copyrighted material appearing in the thesis (other than the brief excerpts requiring only proper acknowledgement in scholarly writing), and that all such use is clearly acknowledged.

____

Signature of Author

DEDICATION

I would like to dedicate this thesis to my family and my supervisor Dr. Barbara Karten for providing me all the support and valuable advice that helped me so much throughout this research.

TABLE OF CONTENTS

List Of Tables viii

List Of Figures ix

Abstract xi

List Of Abbreviations And Symbols Used xii

Acknoledgements xiv

Chapter 1 :Introduction 1

1.1 Insulin Signaling Pathway 1

1.1.1 Insulin Receptor And Its Substrates 1

1.1.2 Inhibition Of Insulin Receptor Signaling 4

1.1.3 Factors Inducing Insulin Resistance 5

1.2 Insulin Signaling In The Brain 7

1.2.1 Insulin And Insulin Receptor Levels In The Brain 7

1.2.2 Effects Of Insulin In The Brain 8

1.3 Role Of Insulin In Alzheimer's Disease 9

1.4 The Synaptic Vesicle Cycle 10

1.4.1 Exocytosis 11

1.4.2 Endocytosis 12

1.5 Role Of Insulin / Insulin Signaling In Synaptic Function 14

1.6 Insulin And Mitochondrial Function 15

1.6.1 Insulin Resistance And Mitochondrial Dysfunction 15

1.6.2 Mitochondrial Biogenesis In The Brain 15

1.7 Mitochondria In The Synapse ... 16

1.8 Niemann -Pick Type C Disease ... 17

1.8.1 Clinical Symptoms ... 17

1.8.2 Etiology ... 17

1.8.3 Insulin Signaling In Npc Disease ... 18

1.9 Objective Of The Study ... 19

Chapter 2: Materials And Methods ... 20

2.1 Materials ... 20

2.2 Cell Culture And Treatments ... 21

2.3 Synaptic Vesicle Release ... 22

2.4 Image Analysis ... 22

2.5 Cell Collection And Protein Determination ... 24

2.6 SDS-PAGE And Western Blotting ... 24

2.7 Measurement Of Insulin Response ... 25

2.8 Preparation Of Brain Slices ... 25

2.9 Isolation Of mRNA And Quantitative PCR ... 26

2.10 Statistcal Analysis ... 27

Chapter 3: Results ... 29

3.1 Impaired Synaptic Vesicle Release In Rat Hippocampal Neurons Grown In The Absence Of Insulin ... 29

3.2 Decreased Mitochondrial Biogenesis In Neurons Grown In The Absence Of Insulin ... 34

3.3 Impaired Synaptic Vesicle Release In Rat Hippocampal Neurons Grown In The Presence Of Low Insulin Or High Insulin ... 36

3.4 PPAR Gamma Agonist Increases Synaptic Vesicle Release In Rat Hippocampal Neurons ... 41

3.5 Chronic PPAR Gamma Agonist Treatment Has No Effect On PGC1 Alpha mRNA And Protein Levels In Neurons ... 43

3.6 Acute Insulin Exposure Leads To Activation Of Akt In A Dose Responsive Manner In Rat Hippocampal Neurons ... 46

3.7 Treatment With Leucine Or TNFα Downregulates The Ability Of Rat Hippocampal Neurons To Respond To Acute Insulin Stimulation And Leads To A High Basal Akt Phosphorylation ... 49

3.8 Synaptic Vesicle Release Is Impaired In Neurons Treated With Leucine, TNFα Or Low Antioxidants ... 51

3.9 Decreased Synaptotagmin 1 In Rat Hippocampal Neurons Treated With High Insulin And Leucine ... 56

3.10 AICAR Rescues Synaptic Vesicle Release And Prevented The Defect Caused By Leucine ... 59

3.11 NPC1 Deficient Neurons Show A Trend Towards Decreased Insulin Sensitivity In The Hippocampus And Cerebral Cortex ... 61

Chapter 4: Discussion ... 63

4.1 Neurons Require An Optimum Amount Of Insulin For Normal Synaptic Function ... 63

4.2 Decreased Mitochondrial Biogenesis In Neurons ... 64

4.3 Insulin Resistance May Contribute To Impaired Synaptic Vesicle Release In Neurons ... 67

4.4 Role Of Mitochondria In Synaptic Transmission ... 71

4.5 Role Of AICAR In Modulating The Defects In Synaptic Vesicle Release Caused By Leucine ... 72

4.6 Conclusion ... 74

References ... 75

LIST OF TABLES

Table 2.1 List Of Primers Used In Thesis ……………………………………….. 28

LIST OF FIGURES

Figure 1.1 Insulin Signaling Pathway 3

Figure 1.2 Synaptic Vesicle Cycle 13

Figure 3.1 Poor Growth Of Neurons In The Absence Of Insulin 32

Figure 3.2 Synaptic Vesicle Release Is Impaired In Neurons Grown In The Absence Of Insulin 33

Figure 3.3 Mitochondrial Biogenesis Is Decreased In Neurons Grown In The Absence Of Insulin 35

Figure 3.4 Morphology Of Neurons Grown Under Different Concentrations Of Insulin 38

Figure 3.5 Synaptic Vesicle Release Is Impaired In Neurons Grown In The Presence Of Low Insulin Or High Insulin Representing All Synapses 39

Figure 3.6 Synaptic Vesicle Release Is Impaired In Neurons Grown In The Presence Of Low Insulin Or High Insulin Representing Responsive Synapses 40

Figure 3.7 Synaptic Vesicle Release Is Improved In Neurons With The Addition Of A PPAR Gamma Agonist Grown In The Presence Of Low Insulin 42

Figure 3.8 PGC1α mRNA Levels Were Unaltered Even After The Addition Of PPAR Gamma Agonist In Neurons 44

Figure 3.9 Decreased Mitochondrial Biogenesis In Neurons Grown In The Presence Of Low Insulin 45

Figure 3.10 Acute Insulin Stimulation Leads To The Activation Of Akt In A Dose Responsive Manner In Rat Hippocampal Neurons 48

Figure 3.11 Neurons Are Less Insulin Sensitive Upon Treatment With Leucine, High Insulin Or TNFα 50

Figure 3.12 Morphology Of Neurons Grown In The Presence Of Leucine, Leucine +High Insulin, Low Antioxidants, Low Antioxidants + High Insulin Or TNFα 53

Figure 3.13 Impaired Synaptic Vesicle Release In Neurons Treated With Leucine, TNFα Or Low Antioxidants Representing All Synapses 54

Figure 3.14 Impaired Synaptic Vesicle Release In Neurons Treated With Leucine, TNFα Or Low Antioxidants Representing Responsive Synapses 55

Figure 3.15 Decreased Synaptotagmin 1 Levels In Neurons Treated With High Insulin And Leucine But No Changes In The Levels Of VDAC1 In Neurons Treated With Leucine, TNFα Or Low Antioxidants........ 58

Figure 3.16 AICAR Improves Synaptic Vesicle Release In Neurons Treated With Leucine 60

Figure 3.17 Insulin Response In Wild Type NPC1 And NPC1- Deficient Neurons 62

Figure 4.1 Leucine-Induced Insulin Resistance 69

Figure 4.2 Hypothesis 73

ABSTRACT

Insulin resistance is a major risk factor for metabolic syndrome, including Type II diabetes (T2D) and obesity. Most studies on insulin resistance focus on peripheral tissues, but little is known about insulin resistance in the brain. T2D is associated with a higher risk for cognitive decline. Here, we examined the effects of insulin on synapse formation and function using primary rat hippocampal neurons as a model system. Our aim was to characterize the effects of low or high levels of insulin on synapse maturation and synaptic vesicle release using live cell imaging. We also wanted to examine the factors responsible for causing insulin resistance in neurons and their role in synapse formation and function. We found impaired synaptic vesicle exocytosis in neurons grown under either low or high levels of insulin compared to control. A significant reduction in the levels of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) were observed by Western blotting in neurons grown under conditions of insufficient insulin indicating decreased mitochondrial biogenesis. To investigate factors that may cause neuronal insulin resistance, we measured insulin responsiveness in neurons treated with leucine, high insulin or TNFα, all of which are known to contribute to insulin resistance in the periphery. Chronic treatment with leucine, high insulin or TNFα decreased insulin sensitivity and increased basal Akt phosphorylation in neurons deprived of insulin for 30 min. In addition, impaired vesicle exocytosis was observed in neurons treated with leucine but this defect was improved by 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), an AMP-dependent protein kinase (AMPK) activator. Overall, our results suggest that neurons require an optimum amount of insulin to undergo normal synaptic vesicle release. In addition, high levels of insulin can cause insulin resistance in neurons accompanied with impaired synaptic vesicle exocytosis. We also demonstrate that supplementation with leucine causes the neurons to become less insulin sensitive with defects in synaptic vesicle release. This provides an insight into the link between T2D and cognitive decline.

LIST OF ABBREVIATIONS AND SYMBOLS USED

FBS - fetal bovine serum
FM1-43 - N- (3-triethylammoniumpropyl)- 4-(4-(dibutylaminostyryl) -pyridinium dibromide
MEM – minimum essential media
DIV - days *in vitro*
qPCR – Quantitative polymerase chain reaction
HBS2/1 - Hepes buffered saline perfusion buffer
PBS - Phosphate buffered saline
^{0}C - degree Celsius
E17 - 17 days of gestation
Ins- Insulin
AO- Antioxidants
Low insulin- 0.05 μg/ml insulin
High insulin- 4 μg/ml insulin
h - hours
min - minutes
mOsm – milliosmole
NPC – Niemann –Pick type C
NPC1- Niemann –Pick type C 1
T2D- type 2 diabetes
TNFα- Tumor necrosis factor alpha
AMPK- 5' adenosine monophosphate-activated protein kinase
MAPK- Mitogen-activated protein (MAP) kinase
GLUT 4- glucose transporter protein
IRS- insulin receptor substrate
s – seconds
GSK3- glycogen synthase kinase 3
BBB- blood brain barrier
CSF- Cerebrospinal fluid
CNS- Central nervous system
AD- Alzheimer's disease
PM- plasma membrane
CME- clathrin mediated endocytosis
GTPase - guanoside triphosphate hydrolase
IGF1 - Insulin-like growth factor 1
AP- adapter proteins
GABA- gamma-aminobutyric acid
NMDA- N-Methyl-D-aspartic acid
TZD- Thiazolidinediones
DRM- detergent resistant membrane
SIRT1- Silent mating type information regulation 2 homolog
BDNF- brain-derived neurotropic factor
PPARγ- Peroxisome proliferator-activated receptor gamma

SEM – standard error of the mean
PMSF – phenyl methane sulfonyl fluoride
v/v – volume/volume
SDS–PAGE – sodium do dedecyl sulfate-polyacrylamide gel electrophoresis
VDAC – voltage dependent anion channel
PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
w/v – weight / volume
DRG- dorsal root ganglion
TFAM- mitochondrial transcription factor A
RXR- retinoid X receptors

ACKNOWLEDGEMENTS

This degree is indeed special for me as it's the first one out of my home country. I have really enjoyed each and every part of it for the last two years. It gave me lot of self-confidence and immense knowledge in the field of Biochemistry & Molecular biology. I am really thankful to many people who have played a major role in the completion of this degree.

I would first like to thank my supervisor, Dr. Barbara Karten for giving me this opportunity to undertake a Master's program in her lab and for all the advice and immense support throughout my research. She has been a steady influence throughout my Masters and has been a moral support in each and every aspect of my professional career over the last two years. I cannot express how much I appreciate all of the confidence and guidance she has given me during this degree. She is really a great supervisor and would never forget her for all the efforts she has taken in making me a potential researcher.

I would also like to thank my committee members Dr. Stefan Krueger and Dr. Neale Ridgway for their useful advice and feedback during the committee meetings. My special thanks goes to all the Karten lab members especially to Barry and Debby who have been with me throughout the Masters program. I have had a great time with them in the lab, with Barry being the constant source of conversation in the lab. He had always been patient to listen all of my stories from India! In fact, I finally convinced him to make a trip to India after the completion of his PhD program. Thanks very much Barry, you have

been a really good buddy of mine and without you it would have been very tough to complete this degree.

Thanks to Dr. Eileen M Denovan-Wright for her useful suggestions and feedback in the combined lab meetings. I would also like to thank her a lot for providing me with reference letters for the next big step in my career. My special thanks to Annette Kolar from the Krueger lab for providing me with the neurons every monday. Without you this project wouldn't have been possible. Thanks so much! Also, thanks to all of my friends in the department (Samir, Mandar, Aditya, Mitesh, Eric) for being so nice to me throughout the program.

Finally, thank you to my family who has been a great support through this wonderful journey. It was really nice of my parents to come over to Canada to spend some time with me and share all their love and affection. That really helped me not getting homesick. Thank you Mom and Dad! Thanks to my brother and sister-in-law who are the one and only family I have in North America and you both have really been a great source of inspiration for me. How can I forget my fiancé who has been an incredible form of encouragement and support through this expedition, you are a very important person in my life. Thanks for all your love and support.

CHAPTER 1

INTRODUCTION

1.1 Insulin signaling pathway

1.1.1 *Insulin receptor and its substrates*

Insulin, a peptide hormone is produced by the pancreatic beta cells and regulates carbohydrate and fat metabolism in the body. Glucose uptake and storage is mainly controlled through the initiation of a signal transduction cascade by insulin. Insulin is secreted from the pancreas following the rise of glucose levels in the blood and in turn is responsible for removing excess glucose from the bloodstream. Insulin increases energy storage and glucose uptake by stimulating the translocation of glucose transporter protein (GLUT4) to the plasma membrane (1). The insulin signaling cascade is triggered by the binding of insulin to the insulin receptor, a receptor tyrosine kinase which consists of two α subunits and two β subunits that are disulfide linked into an $\alpha_2\beta_2$ heterotetrameric complex (2,3). The receptor then undergoes a series of transphosphorylation reactions during which it phosphorylates specific tyrosine residues. Activation of the insulin receptor leads to tyrosine phosphorylation of different isoforms of insulin receptor substrates (IRS) and binding of other signaling molecules like Shc adapter protein isoforms and Grb2. This in turn triggers two separate branches of signaling, one involving the Shc, Grb2 and the mitogen-activated protein (MAP) kinase (MAPK) cascade and the other involving activation of phosphatidylinositol 3-kinases (PI3K) by

the IRS (2,4). Activation of PI3K is followed by the phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2) leading to the formation of phosphatidylinositol-3,4,5-trisphosphate (Ptd(3,4,5)P_3) by the catalytic subunit of PI 3-kinase, p110 as shown in **Figure 1.1**. AKT or protein kinase B (PKB), an important downstream effector of Ptd(3,4,5)P3, is recruited to the plasma membrane. Akt is activated by 3-phosphoinositide dependent protein kinase 1 (PDK1), which is indirectly regulated by PI3K by phosphorylating phosphatidylinositols. The phosphorylation of Akt leads to the phosphorylation of the Rab GTPase activating protein AS160 that facilitates the translocation of GLUT4 transporters to the plasma membrane. Activation of AKT leads to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3) (5). Glycogen synthase is a substrate of GSK3 and catalyses the final step in glycogen synthesis. Glycogen synthesis is inhibited by GSK3 via phosphorylation of glycogen synthase (2).

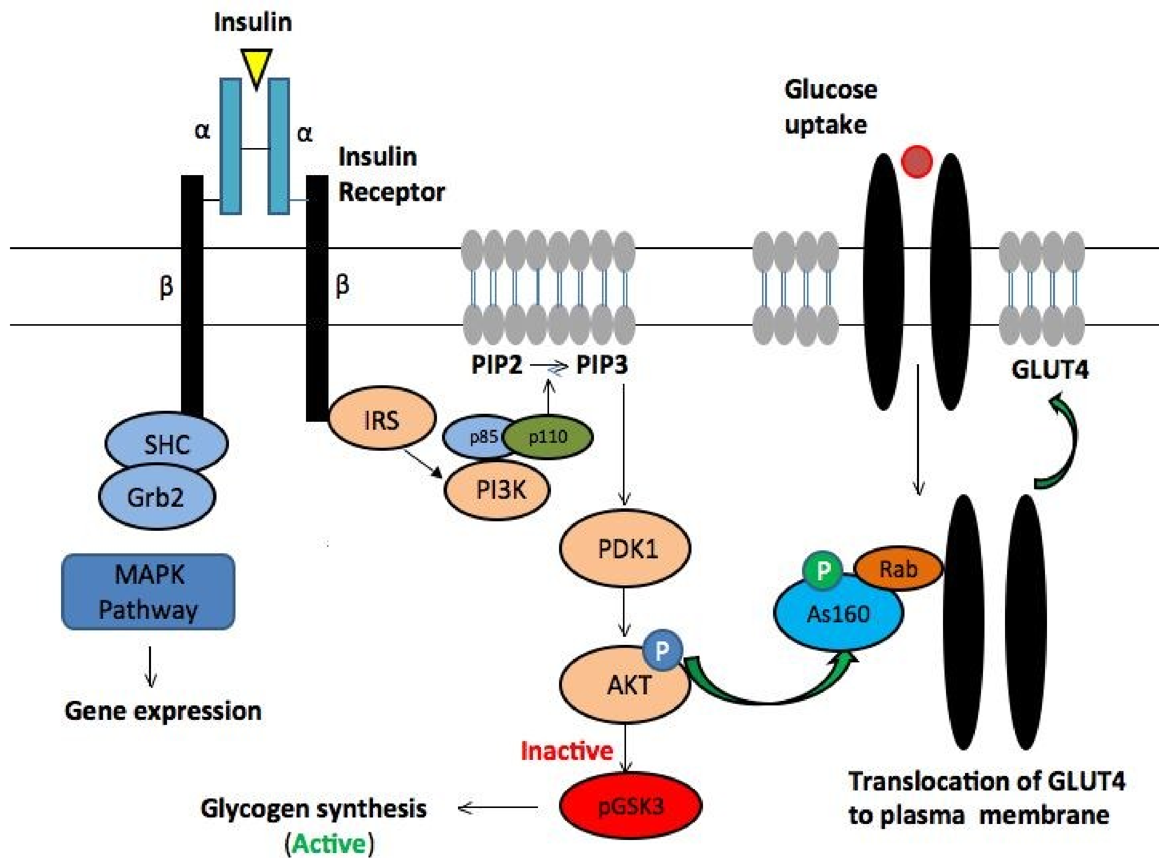

Figure 1.1 Insulin signaling pathway

Insulin binds to its receptor leading to the auto-phosphorylation of the beta subunits and tyrosine phosphorylation of the insulin receptor substrate and binding of other signaling molecules like SHC and Grb2 resulting in a diverse range of signaling pathways including MAPK cascade and PI3K activation. The catalytic subunit of PI 3-kinase, p110, catalyzes the conversion of PtdIns(4,5)P_2 to Ptd(3,4,5)P_3 that in turn activates and phosphorylates Akt through PDK1. Akt phosphorylation of the Rab GTPase activating protein allows the translocation of the GLUT4 transporters to the plasma membrane. Activation of Akt also inactivates and phosphorylates GSK3 that in turn promotes glycogen synthesis.

1.1.2 *Inhibition of insulin receptor signaling*

Impaired insulin signaling contributes to a wide range of metabolic abnormalities including obesity, insulin resistance and diabetes (6). Insulin resistance is central to the pathogenesis of type II diabetes (T2D) and is a major metabolic disorder associated with obesity. Insulin resistance is defined as the inability of cells of the body to efficiently respond to the effects of insulin. Insulin resistance precedes the onset of T2D (7). In the early stage of T2D, β cells located in the islets of Langerhans secrete adequate insulin to compensate for insulin resistance and hence maintain normal glucose levels (8). However at a later stage, insulin deficiency supersedes accelerating diabetes and leads to increased glucose levels in the blood suggesting that destruction of β cells may not be the major defect in this disease (8).

Inflammatory cytokines like TNFα induce serine phosphorylation of IRS-1 and thereby prevent its interaction with PI3K leading to an inhibition of insulin signaling (9-12). Decreased Akt phosphorylation has been reported in skeletal muscle and adipocytes of patients with Type II diabetes (T2D) (13). Defects in insulin signaling are the main cause of insulin resistance and impaired glucose metabolism (14). Although there have been several studies showing defective insulin signaling and insulin resistance in skeletal muscle and liver (13,14), very little is known about insulin resistance in neurons. In 2011, Kim et al reported that hyperinsulinemia caused insulin resistance in cultured dorsal root ganglion neurons by disrupting the Akt pathway (15). The same study showed that chronic insulin treatment lead to a high basal Akt phosphorylation in dorsal root ganglion neurons resulting in a blunted acute phosphorylation of Akt and glycogen synthase

kinase-3β (GSK 3β) (15). Another study reported increased IRS2 expression in dorsal root ganglion neurons of type 2 diabetic mice leading to suppression of insulin signaling (16). Impaired IRS1 signaling was noted in the hippocampi of a transgenic mouse model of Alzheimer's disease (AD), indicating defective insulin signaling in these mouse brains (17). However, the mechanisms behind impaired insulin signaling in neurons are still not completely known and needs further investigation.

1.1.3 *Factors inducing insulin resistance*

A number of factors have been reported to induce insulin resistance in the periphery including high levels of free fatty acids, hyperinsulinemia, increased oxidative stress, inflammatory cytokines and high amino acids (18-20).

The role of free fatty acids (FFA) in causing insulin resistance is widely known in patients with type II diabetes (21). Plasma free fatty acids (FFA) are increased in obese subjects and in turn inhibit insulin's anti-lipolytic action thereby augmenting the FFA release into the circulation even further (22).

Decreased response to insulin accompanied with its increased production leads to hyperinsulinemia. Hyperinsulinemia may be described, as a condition during which there are elevated levels of insulin in the blood circulation. Hyperinsulinemia is common in early stages of T2D and has been considered as a major risk factor for developing insulin resistance (23). Basal plasma insulin levels were increased by two to four fold in mice expressing additional copies of the human insulin gene (23). These mice also exhibited an

augmented postprandial glucose associated with an amplified insulin response to glucose suggesting that hyperinsulinemia in the basal state leads to insulin resistance associated with alterations in glucose metabolism and insulin secretion (23).

Increased oxidative stress has been previously reported to cause defects in insulin signaling and induce insulin resistance in skeletal muscle for example through the Angiotensin II induced reactive oxygen species (ROS) generation by increasing the NADPH oxidase activity (24). Yukihiro Ohta et al (2011) reported that oxidative stress caused insulin resistance due to impaired insulin signaling and increased NADPH oxidase activity in a skeletal muscle of mice with postinfarct heart failure (25). Chronic administration of apocynin (an inhibitor of NAD(P)H oxidase activation) in these mice led to a reduction in the oxidative stress and NADPH activation ultimately causing an improvement in insulin resistance suggesting that oxidative stress was the reason for the development of insulin resistance in these mice (25).

Tumor necrosis factor alpha (TNF α), an important inflammatory cytokine has been associated with insulin resistance through increased free fatty acid secretion and defective insulin signaling at the level of PI3K (26). In cultured human adipocytes, preincubation with TNF-α resulted in a 60-70% reduction of insulin action (26).

Amino acids like leucine have also been implicated in causing insulin resistance in the skeletal muscle by downregulating 5' adenosine monophosphate-activated protein kinase (AMPK) activity and by increasing mTOR/p70S6K signaling (27). Activation of

mTOR/p70S6K signaling causes peripheral insulin resistance through a negative feedback mechanism causing increased serine phosphorylation of the IRS (27-30). Incubation of rat muscles with leucine increased protein synthesis and phosphorylation of mTOR/p70S6K (27). Also high levels of leucine were found in the plasma of 3 weeks old diabetic rat brain indicating its role in diabetes (31). A metabolomic study published by Suhre et al found higher levels of leucine in the plasma of diabetic patients indicating it as biomarker for detecting diabetes-related complications (32). Although there is enough evidence about FFA, hyperinsulinemia, increased oxidative stress, inflammatory cytokines and amino acids in inducing insulin resistance in the periphery, very little is known about their role in neurons.

1.2 Insulin signaling in the brain

1.2.1 *Insulin and insulin receptor levels in the brain*

Over three decades ago, Havrankova et al. (33) reported that the insulin receptor is widely distributed in the central nervous system with its highest expression in the olfactory bulb, followed by the cerebral cortex, hippocampus and the pre-optic area, hypothalamus and amygdala. The same study also reported a 25-fold higher insulin concentration in whole brain compared to plasma as determined by radioimmunoassay (33). However, other studies have reported different concentrations of insulin, which suggest that there is no general agreement about the concentration of insulin in the brain, and needs further investigation (34, 123, 124). Insulin levels reported depends on the type of sample that was measured for example either brain or cerebrospinal fluid (CSF) and neither of these areas accurately reflect the insulin concentration surrounding a neuron.

Interestingly, the brain insulin levels were 2-fold higher after 48 hr of streptozotocin treatment, which destroys the pancreatic beta cells than in untreated control rats (34). This finding suggests that not all the brain insulin came from the circulation. Insulin is produced in the brain by hippocampal neurons and can be released from the neurons following exocytosis of the synaptic vesicles in response to a membrane depolarization in a calcium-dependent manner (35). Expression levels of the insulin receptors are developmentally regulated in a rat brain (36). Brain regions like thalamus, caudate-putamen and some mesencephalic and brainstem nuclei had higher concentrations of insulin receptors during neurogenesis compared to the adult rat brain (36). Also, glia cells were found to have lower insulin receptor levels compared to neurons and these levels decreased with age (37). It was not until 1991, that Schwartz et al demonstrated that insulin crossed the blood brain barrier (BBB) by a saturable mechanism (38). Later studies showed that insulin could cross the BBB by a receptor-mediated transcytosis process (39). The brain endothelial cells that comprise the BBB and the blood cerebrospinal fluid barrier contain binding sites for insulin, (40,41). The insulin binding sites at the BBB aid in the transport of insulin across the BBB and also function as receptor sites that help activating the intracellular machinery of the barrier cell (39).

1.2.2 *Effects of insulin in the brain*

Insulin has widespread effects on the brain including its role in control of food-intake and in cognition (42). Chronic administration of insulin into the ventricular system or the hypothalamus decreases food intake and body weight in a dose-dependent manner while maintaining peripheral euglycemia (43). However, administration of insulin antibodies

increases food intake in the ventromedial areas like the prefrontal cortex, which is mainly involved in the processing of risk, fear and decision making (44,45). These findings suggest that insulin plays an important role in regulation of food intake and body weight in different regions of the brain. The role of insulin in memory and cognition has been well evidenced by some studies in animals using the Morris water maze or passive avoidance tasks that have shown to improve memory performance in rats upon administration of insulin (46,47). An increase in insulin receptor expression was observed in the hippocampal dentate gyrus and CA1 field when rodents were trained on a spatial memory task, suggesting that synthesis of insulin receptor may be augmented in these areas as a result of learning (48,49). Insulin pre-treatment improved the memory deficits in rats with hippocampal lesions, which were previously suffering from severe loss of learning and memory ability (50-52). All the above studies provide evidence for the role of insulin in improving learning and memory.

1.3 Role of insulin in Alzheimer's disease

Over the past decade, the link between impaired insulin sensitivity and Alzheimer's disease (AD) has been one of the major topics of discussion (53) This is evident with observed impaired insulin signaling and abnormal levels of insulin in the CSF and plasma of patients with AD (54,55). A defect in systemic insulin sensitivity was observed in patients with AD (46). Defective insulin receptor signaling was noted in patients with dementia of the Alzheimer type accompanied with higher levels of non-metabolized glucose in the cerebral blood suggesting that AD could be a kind of T2D in the brain

(44,53). In patients with AD the levels of insulin in the CSF are lower compared to the peripheral plasma insulin levels (55).

T2D is associated with higher risk for cognitive decline and AD (19,56). One of the most common features in both AD and T2D is insulin resistance (57). Role of insulin in improving cognitive functions is very well documented by studies relating to AD and insulin resistance (56,58). Insulin and insulin receptor levels in the brain are decreased in patients with sporadic AD (59). Memory performance is ameliorated in patients with AD upon systemic administration of insulin under the conditions of euglycemia or hyperglycemia (55,60,61). Aggregation of β-amyloid (Aβ) peptide is one of the main hallmarks of Alzheimer's disease. Studies have shown links between Aβ and insulin signaling. In fact, Aβ can inhibit brain insulin signaling, through binding of soluble Aβ to insulin receptors causing a downregulation of the plasma membrane insulin receptors leading to synaptic spine loss in hippocampal neuron cultures (62,63). This effect was reversed upon pretreatment with insulin (63). All the above studies indicate that insulin is very important for learning and memory in both humans and animals.

1.4 The Synaptic vesicle cycle

Neurotransmitter release is triggered by an action potential from a pre-synaptic nerve terminal that initiates synaptic transmission. The neurotransmitters are released into the synaptic cleft between two neurons by exocytosis of synaptic vesicles filled with neurotransmitters. Once, the neurotransmitters are released, synaptic vesicles are rapidly

reformed by endocytosis and refilled with neurotransmitters to maintain sustained synaptic transmission. An overview of the synaptic vesicle cycle is shown in **Figure 1.2.**

1.4.1 *Exocytosis*

Exocytosis involves three major steps namely: docking, priming and fusion. During docking the loaded synaptic vesicles dock near the release sites. Docking is mediated by a group of proteins that form the active zone complex: rab-3 interacting molecules, Munc-13 proteins, Bassoon and Piccolo, ELKS proteins and Liprin -α proteins by forming a scaffold at the active zone and act as the pre-synaptic spatial organizer, thereby integrating the synaptic vesicle cycle (64,65).

The next step is priming where the synaptic vesicles are prepared for fusion in response to calcium influx. Formation of SNARE complex of proteins is one of the major events during priming where the R-SNARE protein synaptobrevin from the synaptic vesicle forms a complex with Q-SNAREs SNAP-25 and syntaxin from the plasma membrane (PM) thereby making the primed synaptic vesicle ready to be exocytosed (66,67).

The synaptic vesicles now get ready to fuse quickly in response to the membrane depolarization of an action potential as shown in **Figure 1.2**. The action potential generated activates the voltage-gated Ca^{2+} channels, causing an influx of Ca^{2+} that is detected by the synaptotagmin family of proteins (68). Ca^{2+}binding to synaptotagmin causes the fusion pore to open and secretion of neurotransmitters hence leading to exocytosis of the primed synaptic vesicle (69).

1.4.2 *Endocytosis*

Immediately following exocytosis, synaptic vesicles are reformed by endocytosis. This involves the recovery of the synaptic vesicle proteins that are exocytosed to the PM followed by the reformation of the vesicles by endocytosis. The main pathway of endocytosis is the clathrin-mediated endocytosis (CME).

CME is initiated by a Ca^{2+} influx with the help of calmodulin as a sensor that provides powerful coupling of endocytosis to exocytosis thereby facilitating synaptic vesicle cycling (70). Following dispersion of the synaptic proteins to the PM by exocytosis, the proteins are re-assembled through interactions with the AP-2 adapter protein complex which facilitates the clathrin to bind with AP-2 and hence form an inward curvature of membrane in the form of a coated pit that finally grows into a spherical invagination (71). Dynamin then mediates pinching off the coated pit to form a single clathrin-coated vesicle (72). Following detachment from the plasma membrane, the vesicles are uncoated. Uncoated endocytic vesicles are then re-acidified and reform the synaptic vesicles by either direct recycling or by reaching an early endosome where the synaptic vesicles are restored from the recycling endosome and reloaded with neurotransmitters thus facilitating the synaptic vesicle cycle (73).

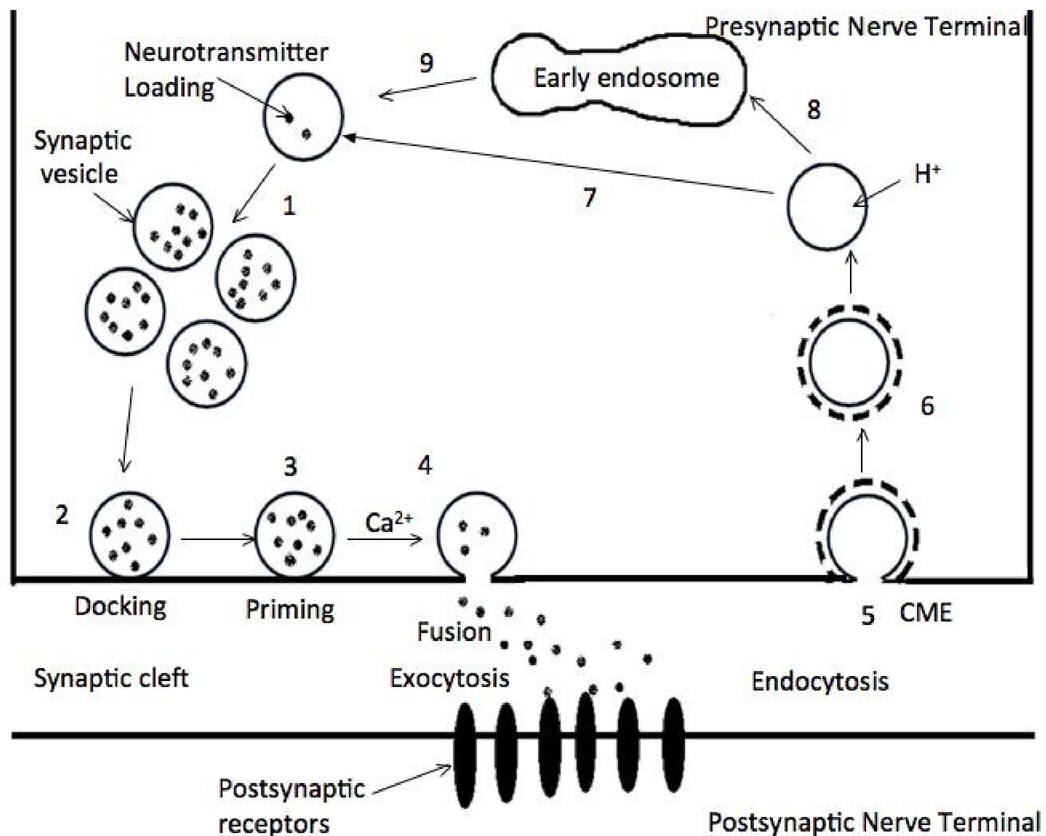

Figure 1.2 Synaptic vesicle cycle

1) Synaptic vesicles are loaded with neurotransmitters at the presynaptic nerve terminal. **2)** Loaded vesicles are then docked near the plasma membrane followed by **3)** priming which makes them competent for Ca^{2+} triggered fusion-pore opening **(4)** thereby triggering exocytosis of the synaptic vesicle**. 5)** Clathrin-mediated endocytosis retrieves the synaptic vesicles. **6)** The vesicles are uncoated and re-acidified. **7**) Re-acidified vesicles are either directly recycled from step 7 to step 1 or **8)** reach an early endosome where the synaptic vesicles are reformed and **9)** reloaded with neurotransmitters.

Adapted from (74)

1.5 Role of insulin / insulin signaling in synaptic function

Insulin and insulin-like growth factor 1 (IGF-1) support neuronal growth and survival (75). Insulin regulates synaptic plasticity by modulating activities of excitatory and inhibitory receptors such as glutamate and gamma-amino butyric acid (GABA) receptors (52). The effects of insulin on the postsynaptic side are best known. Studies have shown that an acute exposure to insulin initiated a robust potentiation of responses to N-Methyl-D-aspartic acid (NMDA) mediated by NMDA receptor in *Xenopus* oocytes expressed with NMDA receptors (76,77). An increase in tyrosine phosphorylation was reported in the NR2A and 2B subunits of NMDA receptors when rat hippocampal slices were exposed to insulin suggesting that insulin is involved in mediating NMDA receptor mediated neurotransmission (78,79). Insulin facilitates postsynaptic receptor trafficking in GABA receptors by mediating synaptic inhibition that plays an important role in neuronal functions associated with learning and memory (52,79). Insulin receptors recruit functional GABA receptor to the postsynaptic membrane thereby regulating the GABA transmission (52). Insulin is also known to facilitate endocytosis of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in the hippocampus and cortex from the neuronal synaptic membrane thereby causing a long-term depression (LTD) of the synaptic transmission in a Ca^{2+} dependent manner (80). Thus insulin/ insulin receptors play a vital role in postsynaptic receptor trafficking, which is very important for the regulation of synaptic plasticity, learning and memory.

1.6 Insulin and mitochondrial function

1.6.1 *Insulin resistance and mitochondrial dysfunction*

Insulin resistance and/or T2D is linked with decreased mitochondrial mass and function (81). Kelley et al found reduced mitochondrial size in obese subjects with insulin resistance and/or T2D (82). Magnetic resonance spectroscopy studies showed impaired insulin stimulated mitochondrial function in elderly subjects with insulin resistance (83). A couple of years later, Sparks et al. reported reduced mRNA levels of PGC1α and PGC-1 β in the muscle of healthy male subjects after three days of high fat feeding (84). Thiazolidinediones like pioglitazone and rosiglitazone have been reported to activate mitochondrial biogenesis in human adipose tissue (85). Despite these findings, it is not unambiguously clear whether the observed mitochondrial dysfunction was a primary cause of insulin resistance or a consequence of insulin resistance. Numerous studies have investigated the link between impaired mitochondrial function and insulin resistance in the skeletal muscle and liver, but very little is known about the link between insulin/insulin resistance and mitochondrial function in the brain.

1.6.2 *Mitochondrial biogenesis in the brain*

The peroxisome proliferator-activated receptor gamma (PPARγ) coactivator (PGC-1) family of inducible transcriptional co-activators is one of the key elements in mitochondrial biogenesis and promotes gene transcription involving mitochondrial metabolism and function (86). Most of the studies relating mitochondrial biogenesis are focused on skeletal muscle and liver. Little is known about mitochondrial biogenesis in the brain. In 2011, Hathorn et al reported that in a mouse model of Huntington's disease,

motor deficits were improved by nicotinamide and lead to an increase in PGC-1α and brain-derived neurotropic factor (BDNF) gene expression (87). A study published in the same year showed that mitochondrial biogenesis in the brain was increased by exercise training in mice (88). Mitochondrial biogenesis related genes, PGC-1α, Silent mating type information regulation 2 homolog (SIRT1) and Citrate synthase were increased after 8 weeks of treadmill running in mice (88). Impaired mitochondrial biogenesis, synaptic degeneration and defective axonal transport of mitochondria were observed in a mouse model (AβPP) of Alzheimer's disease which was restored to normal by a mitochondria-targeted antioxidant SS31 (89). Although, there is a correlation between decreased mitochondrial biogenesis and synaptic degeneration, the exact mechanistic links are yet to be elucidated.

1.7 Mitochondria in the synapse

Neuronal mitochondria are known to be present throughout the cell bodies and neurites with relatively high density in the synaptic terminals (90). Mitochondria regulate synaptic calcium levels and act as calcium buffers (91). Mitochondrial ATP is very important for maintaining neurotransmission since it provides energy during many steps of the synaptic vesicle cycle (92). This is evident by studies on *dynamin-related protein* (*drp1*), which mediates fusion and is important for the cellular distribution of mitochondria and hence mutations in these proteins would lead to the absence of mitochondria at the synapses in neurons (92). Mutations in *drp1* led to defects in mobilization of reserve pool vesicles due to lack of synaptic ATP (92). Further, supplementing the *drp1* synapses with ATP facilitated the mobilization of reserve pool vesicles (92). In 2003, Kann et al showed that

ATP production in mitochondria is regulated by synaptic activity indicating the role played by mitochondria in regulating synaptic strength (93).

1.8 Niemann -Pick Type C disease

Niemann- Pick Type C (NPC) disease is a rare neurodegenerative genetic disorder in humans affecting nearly 1 in 150 000 live births. It is associated with rapid neurological decline and shortens the life span to less than 20 years in many cases (94). One of the main hallmarks of this disease is cholesterol accumulation in the endosomes (95-97).

1.8.1 *Clinical symptoms*

NPC disease causes progressive neurodegeneration in humans. Some of the commonly seen symptoms in NPC disease include ataxia, dystonia, dysphagia and seizures (97). These symptoms become apparent at a young age and ultimately lead to death within a decade. NPC infants also suffer from neonatal jaundice and splenomegaly and a few of these infants ultimately die from acute liver failure/liver dysfunction (97). Another major characteristic symptom of NPC disease is the loss of Purkinje cells in the cerebellum and appearance of swollen neurites (97,98). Cholesterol accumulation in the lysosomes, gangliosides (GM2 and GM3) and other lipids characterizes the biochemical phenotype of NPC deficient cells or tissues (97,99-101).

1.8.2 *Etiology*

NPC disease is caused by mutations in the NPC1 or NPC2 genes (97,102). NPC1 is an integral membrane protein in the membrane of the late endosomes (96,103). NPC2 is a

soluble protein that was originally identified as a secretory protein in the human epididymis and was therefore originally known as HE1 (104). Genetic mutations in the NPC1 gene are more common compared to mutations in the NPC2 gene (94).

1.8.3 *Insulin signaling in NPC disease*

Altered cholesterol trafficking is the major defect observed in NPC disease (105,106). Few studies have investigated insulin signaling in NPC disease. Insulin receptors (IR) have been shown to function via lipid rafts that are cholesterol-rich membrane microdomains involved in regulating various signal transduction events and membrane trafficking (107). High plasma membrane cholesterol in the rafts has been reported to cause impaired insulin signaling (108). In 2005, Vainio et al showed that IR levels were increased and the receptor activation was decreased in NPC hepatocytes (108). They also observed an augmented association of the insulin receptor with the detergent resistant membrane (DRM) in NPC hepatocytes and plasma membrane fractions suggesting that the lipid imbalance in NPC hepatocytes may contribute to insulin resistance (108). Later in 2012, Ong et al studied the expression of various proteins in the insulin-signaling pathway in 9 week old brain of homozygous mutant BALB/c NPC^{nih} mice (109). Phosphorylation of Akt was greatly reduced at residue T308 than S473 accompanied with a lower GSK3β phosphorylation and also a reduction in the insulin receptor substrate (IRS-2) in 9 week old NPC^{nih} mouse brain (109). Although there are a few studies focusing on the insulin signaling in NPC disease, till date there is no evidence about insulin resistance in the NPC diseased brain.

1.9 Objective of the study

The main objective of this study was to investigate the effects of insulin on synapse formation and function. Firstly, we wanted to study the effects of low or high levels of insulin on synaptic vesicle exocytosis to test how much insulin is needed for the formation of functional synapses. Second, we wanted to examine the effect of neurons that were grown in the presence of factors known to cause insulin resistance in the periphery and their role in synapse formation and maturation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Neurobasal medium, B27 and fetal bovine serum (FBS) were purchased from Life Technologies (Burlington, ON, Canada). N- (3-triethylammoniumpropyl)- 4-(4-(dibutylaminostyryl) - pyridinium dibromide (FM1-43) was purchased from Biotium (SynaptoGreenTM C4, Hayward, CA, USA). Poly-D-lysine hydrobromide was purchased from Peptides International (Louisville, KY, USA). Recombinant human insulin (Catalog #A11382IJ) was purchased from Gibco (NY, USA) and the insulin from bovine pancreas was from Sigma, USA. Troglitazone was from Enzo Life Sciences, Inc. (NY, USA). 5-Aminoimidazole-4-carboxamide ribotide (AICAR) was purchased from Ascent Scientific (MA, USA). The rabbit anti-pAkt (T308), rabbit anti-pAkt (Ser473), rabbit Anti-Akt antibodies were purchased from Cell Signaling (MA, USA). Rabbit anti- Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) antibodies were from Novus Biologicals and rabbit anti- voltage dependent anion channel 1 (VDAC1) from Abcam. Synaptotagmin 1 antibodies were from Chemicon (Temecula, CA USA). Goat anti-Actin antibodies came from Santa Cruz (CA, USA) and mouse anti-tubulin from Sigma (Oakville, ON, Canada). All other chemicals were from Sigma or Life Technologies. All fluorescent images were acquired with a CCD camera (Orca; Hamamatsu, Bridgewater, NJ, USA) on a Nikon TE2000 epifluorescence microscope and excitation and emission filters from Semrock (Rochester, NY).

2.2 Cell culture and treatments

Primary hippocampal neurons were prepared from E17 embryonic Sprague–Dawley rats obtained from Jackson Laboratories (Bar Harbor, ME, USA) as described previously (110). Dissociated neurons were plated in Neurobasal medium supplemented with B27 supplement, 0.25 μM glutamate, 0.5 mM glutamine, antibiotics (Penicillin and streptomycin), and 5 % heat-inactivated FBS onto dishes that had been coated with 0.1 % (w/v) poly-D-lysine and conditioned in minimum essential medium (MEM) with 5 % FBS for 3 h. $B27^{+ins}$ refers to the normal B27 with insulin added by the manufacturer (Life Technologies) and $B27^{-ins}$ refers to B27 without insulin. This nomenclature will be followed throughout the thesis from here on. The plating density of the neurons was maintained at 41250 cells /well for a 12-well plate. After 3 hours (h), medium was replaced with the same medium without serum and with $B27^{+ins}$ or $B27^{-ins}$ added as indicated. To prevent proliferation of glia cells, 2.5 μM cytosine arabinoside was added to all cultures 6 days (DIV6) after plating. Acute insulin treatments on the neurons were done with insulin from bovine pancreas and the chronic insulin treatments were done with recombinant human insulin. Leucine, tumor necrosis factor (TNFα), Troglitazone and AICAR were added on DIV 3 at final concentrations of 1 mM, 1 nM, 5 μM and 50 μM respectively. All procedures were approved by the animal ethics committee of Dalhousie University based on the standards established by the Canadian Council of Animal Care.

2.3 Synaptic vesicle release

Synaptic vesicle release was measured using the amphiphilic dye FM1-43 as described in Hawes CM et al., 2010 (110). Glass coverslips with 14 days old neurons were transferred to a perfusion chamber and washed with perfusion buffer (HBS2/1) [1.24 molar (M) Nacl, 30 mM KCl, 50 mM D-glucose, 100 mM Hepes, 20 mM $CaCl_2$*$2H_{20}$, 10 mM $MgCl_2$*$6H_{20}$, pH 7.4, osmolarity adjusted to 260-270 mOsm]. Ten-micromolar 6,7-dinitroquinoxaline- 2,3-dione (DNQX) and 50 μM 2-amino- 5-phosphonopentanoic acid (APV) were added to the HBS2/1 buffer throughout the experiment. FM1-43 was incorporated into the synaptic vesicles by field stimulation of the neurons in the presence of 15 μM FM1-43 in HBS2/1 buffer with 900 stimuli at 10 Hz. Neurons were then washed with HBS2/1 to remove non-internalized dye, and perfused with HBS2/1 for the rest of the experiment. Following removal of the dye, fluorescence was recorded for 20 seconds (s) without stimulation (baseline) then during stimulation with 900 stimuli at 10 Hz. Images were taken every 2 s using a 40·x objective and 482/35 nanometer (nm) excitation and 583/120 nm emission filters.

2.4 Image analysis

Image acquisition and analysis was carried out using IPLab (BD Biosciences, Mississauga, ON, Canada) and Igor Pro (Wavemetrics, Portland, OR, USA) software as described previously by Hawes et al, 2010 (110) and Mailman T et al. 2011 (111). ImageJ was used to background subtract and align the time-lapse images. Using a threshold-based segmentation algorithm written for IPLab, baseline images were averaged and segmented in IPLab assuming the segments as regions of highest-above-

background fluorescence not larger than 12 pixels (111). All fluorescent punctae that were not localized on a neurite and those superimposed with cell bodies were erased and taken out of the analysis by overlaying the segments on the corresponding phase images in IPLab software. We adopted a neutral strategy to avoid introducing a bias by choosing a threshold for the extent of destaining and by excluding weakly responding synaptic boutons, segmentation was not performed on differential images of fluorescence at the beginning and the end of stimulation (110). The fluorescence over time for each synaptic bouton was calculated using the segmented image in IPLab software. These data were imported into Igor Pro software (Wavemetrics) that yields one wave per fluorescent bouton following which each wave was normalized separately to the fluorescence at the beginning of stimulation (F0 at t = 0 s) as described in Hawes et al, 2010 (110). F0 was calculated from a linear regression fit of the baseline, and can have a different value from the initial fluorescence measured at time t = 0 s. Normalized curves obtained from each coverslip of all fluorescent punctae were averaged, following which all coverslips of one experiment were averaged. Means and standard error of the means were calculated using the averages of curves from each treatment group for at least three independent experiments. The fluorescence decrease $\Delta F/F0$ after 100 or 900 stimuli (t = 10 or 90 s) was calculated from an exponential regression fit of the fluorescence for each synaptic bouton during stimulation and averaged as described previously in Mailman et al. 2011 (111). The averages from the responsive synapses were calculated by excluding all fluorescent punctae, which lost less than 11 % of their initial fluorescence during the first 20 s of stimulation using Igor (111). Finally, separate graphs were made for both responsive synapses and all synapses indicating synaptic vesicle release measurement.

The Mann–Whitney test for non-parametric samples was used to test for significant differences between distribution means and was performed on separate curves.

2.5 Cell collection and protein determination

Hippocampal neurons were lysed in PBS containing 0.1 % SDS, protease inhibitors (10 μM leupeptin, 5 μg/ml aprotinin, 1 μM pepstatin A, 0.5 mM PMSF) and phosphatase inhibitors (2 mM sodium orthovanadate, 1 mM sodium fluoride). Cell protein levels were measured using the bicinchoninic acid assay (BCA assay kit, Pierce).

2.6 SDS-PAGE and Western blotting

Following the lysis of cells, samples were mixed with sodium dodedecyl sulfate (SDS) reducing sample buffer to a final concentration of 62.5 mM Tris-HCl [pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol] (112) and boiled for 5 minutes. Equal amount of protein were resolved by SDS PAGE in running buffer [25mM Tris [pH8.3], 192 mM glycine, 0.1 % SDS] at 100 V for approximately 2 h following which proteins were transferred from the gel to a polyvinylidene fluoride membrane (GE) in transfer buffer (25 mM Tris, 250 mM glycine, 20 % methanol, 5 % isopropanol). Primary antibodies were used at the following dilutions: rabbit anti-pAkt (1:1000), mouse anti-Akt (1:1000), goat anti-Actin (1:1000), mouse anti-Tubulin (1:1000), rabbit anti–VDAC1 (1:2000), rabbit anti-PGC1α (1:1000), mouse anti-Synaptotagmin 1 (1:1000). Secondary antibodies (donkey anti-rabbit IgG, donkey anti-mouse IgG or donkey anti-goat IgG conjugated with horseradish peroxidase, Jackson Immunoresearch) were used at 1:5000. The signal was observed using enhanced chemiluminescence reagents (ECL; Amersham Bioscience,

Piscataway, NJ) or Luminata Crescendo western HRP substrate (Millipore Corporation, MA, USA) as indicated. All experiments were repeated at least 3 times and representative results are presented in the figures.

2.7 Measurement of insulin response

The rat hippocampal neurons were harvested for western blotting analysis on DIV 10 following an acute insulin treatment. The media present in the wells was first aspirated and quickly washed twice with HBS 2/1 buffer (with 25 mM glucose, no insulin). The HBS 2/1 buffer was left on the neurons for 30 min at 37 ^{0}C and then a 15 min insulin treatment was given to the neurons. Each treatment group had 2 wells with 100 nanomolar (nM) insulin and 2 wells with 0 nM insulin. After 15 min of insulin response, the media in the wells was aspirated and replaced with harvesting buffer by adding ~120 (µl) to each well of a 12 well plate following which the neurons were collected for protein determination and western blot analysis.

2.8 Preparation of brain slices

Hippocampus and cerebellum were dissected from 3 week old mouse brain following which they were immediately chopped with a tissue chopper. The chopped brain slices were placed in a dissection solution with 70 mM sucrose without insulin and incubated for 30 min at room temperature (RT). After 30 min of incubation, the slices were then transferred to a dissection solution without sucrose and incubated again for 30 min at room temperature. Meanwhile, 2 x 6 well plates were prepared with 2.5 ml dissection solution having 0 and 500 nM recombinant human insulin in the wells. Slices were

transferred into each well and incubated for 10 min at room temperature following which they are collected on ice in eppendorf tubes with homogenization buffer [10 mM K-Hepes pH 7.4, 1mM Ethylene diamine tetra acetic acid (EDTA), 1 mM ethylene glycol tetra acetic acid (EGTA), 1% Triton X 100 and 0.5 % Nonidet (NP) 40]. The slices were homogenized immediately using a small rotary homogenizer and frozen at -20 ^{0}C.

2.9 Isolation of mRNA and quantitative PCR

mRNA was collected using Trizol reagent (Invitrogen) and quantified spectrophotometrically. Complementary DNA (cDNA) templates were generated using the iScript™ cDNA Synthesis Kit (Bio-Rad) and random hexamers and oligo dT primers for real time quantitative polymerase chain reaction (qPCR). The reaction set up for the cDNA synthesis included 4 µl 5X iScript reaction mix, 1 µl iScript Reverse Transcriptase, 1 µg of RNA template and nuclease free water to a total volume of 20 µl. This reaction mix was incubated for 5 min at 25 ^{0}C, followed by a 30 min incubation at 42 ^{0}C which was further incubated for 5 min at 85 ^{0}C and finally held at 4 ^{0}C. For qPCR, a master mix was first prepared which included 7.5 µl of qPCR supermix, 0.45 µl (300 nM) forward primer, 0.45 µl (300 nM) of reverse primer and 4.1 µl of PCR water per sample which is then mixed with 2.5 µl of the cDNA sample obtained from the cDNA synthesis reaction. For amplification of cyclophilin, the thermocycler was programmed for an initial denaturation step of 3 min at 94 ^{0}C, followed by 30 temperature cycles for 30 s at 94 ^{0} C, 40 s at 55 ^{0} C and 40 s at 72 ^{0}C. For amplification of PGC1α, the thermocycler was programmed for an initial denaturation step of 3 min at 94 ^{0}C, followed

by 30 temperature cycles for 30 s at 94^{0} C, 40 s at 53.4 ^{0}C and 40 s at 72 ^{0}C.Primers used for qPCR were as listed **(Table 2.1).**

2.10 Statistical analyses

Significance was calculated by one-way analysis of variance (ANOVA) or two-way ANOVA using GraphPad Prism software unless indicated otherwise. Tukey's post hoc test was applied for multiple between-group comparisons. Significance was assumed for $p < 0.05$. Data are shown as the mean ± SEM of at least three independent experiments. Experiments were considered independent if they were conducted on different days using neurons from different litters of embryos.

Table 2.1 List of Primers used in Thesis

Primer	Primer Sequence
PGC1alpha forward primer	5'-ATTGAGAGACCGCTTTGAAG-3'
PGC1alpha Reverse primer	5'-TCGACCTGCGTAAAGTATATCC-3'
Cyclophilin forward primer	5'-TCT TCT TGC TGG TCT TGC CAT TCC-3'
Cyclophilin Reverse primer	5'-TCC AAA GAC AGC AGA AAA CTT TCG-3'

CHAPTER 3

RESULTS

3.1 Impaired synaptic vesicle release in rat hippocampal neurons grown in the absence of insulin

To investigate the role of insulin in synapse formation and function, rat hippocampal neurons were grown in medium with different levels of insulin. These neurons are commonly grown in a defined serum-free medium (Neurobasal medium) with a supplement mixture B27 to sustain growth. The original description of $B27^{+ins}$ states that it contains 4 µg/ml insulin (113). However, the composition of commercially available $B27^{+ins}$ is not revealed by the manufacturer Life technologies. To generate conditions of potentially insufficient insulin signaling, we decided to lower the insulin concentration from 4 µg/ml to lower concentrations (2, 1, 0.5, 0 µg/ml insulin), and to test under these conditions how much insulin is needed for the formation of functional synapses. At DIV 7, cultured hippocampal neurons form many synapses from pre-synaptic specializations and it is generally during the second week in culture when the synapses mature with increasing complexity of the post-synaptic specialization and spine formation (114). On DIV 14, we measured synaptic vesicle exocytosis using the fluorescent amphiphilic dye FM1-43 (111,115). FM1-43 intercalates into the neuronal plasma membrane and is incorporated into synaptic vesicles during electrical field stimulation (116). Neurons are then washed with a perfusion buffer so that FM1-43 dye is washed out of the plasma membrane and only remains in the synaptic vesicle membrane. During renewed field

stimulation in the absence of FM1-43, synaptic vesicle release leads to loss of the dye and an exponential decrease in fluorescence is indicative of synaptic vesicle exocytosis (116). The morphology of the neurons grown in the complete absence of insulin was strikingly different when compared to control neurons and neurons grown in the presence of low insulin levels. Neurons grown in the absence of insulin appeared less healthy and seemed to have a lower neurite density than control neurons grown in the presence of B27ins supplement (**Figure 3.1**). When synaptic vesicle release was measured, control neurons and neurons grown with $B27^{-ins}$ + 2, 0.5 or 1 μg/ml insulin, showed typical exponential fluorescence decrease during field stimulation with 900 stimuli at 10 Hz, whereas neurons grown in the absence of insulin released only little FM1-43 **(Figure 3.2 A)**. Both initial and total vesicle release were significantly lower in neurons grown in the absence of insulin compared to control **(Figure 3.2 C)**. The initial release represents the synaptic vesicle release after 10 s of stimulation and the total release corresponds to the synaptic vesicle release after 90 s of stimulation. Neurons grown in the presence of $B27^{-ins}$ + 0.5, 1 or 2 μg/ml insulin were not significantly different from control during both initial and total vesicle release **(Figure 3.2 C)**. As the curves shown in Figure 3.2 A represent the average of all the fluorescent punctae in the field, the low fluorescent decrease could be due to impaired vesicle release from most synapses and/or a normal release from a small percentage of synapses. To distinguish between these possibilities, we calculated the average fluorescent time lapse curve after excluding the non-responsive synapses, defined as the fluorescent punctae that lost less than 11% of their initial fluorescence during the first 20 s of stimulation (111) (**Figure 3.2 B**). This value also corresponded approximately to loss of fluorescence from bleaching. When the non-responsive synapses

were excluded, neurons grown in the absence of insulin still had a significantly lower initial and total vesicle release compared to control **(Figure 3.2 D)**, indicating that the lower defect observed in the total average was not only due to a large number of non-responsive synapses. After exclusion of non-responsive synapses, neurons grown in the presence of $B27^{-ins}$ + 0.5, 1 or 2 μg/ml insulin still were not significantly different than control during both initial and total vesicle release **(Figure 3.2 D)**. These results demonstrate that neurons require more insulin for the formation of functional synapses than is necessary to merely support growth.

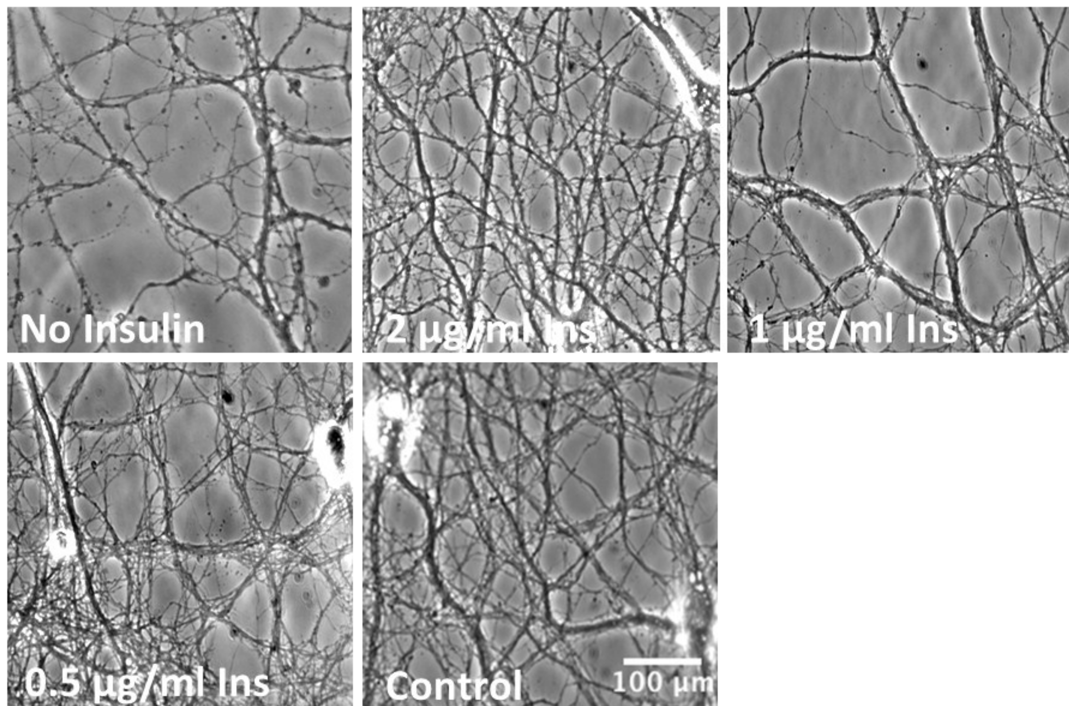

Figure 3.1 Poor growth of neurons in the absence of insulin. Phase images of DIV 14 neurons treated with $B27^{+ins}$ (control), $B27^{-ins}$ + 2, 1, 0.5, 0 µg/ml insulin, scale bar: 100 µm.

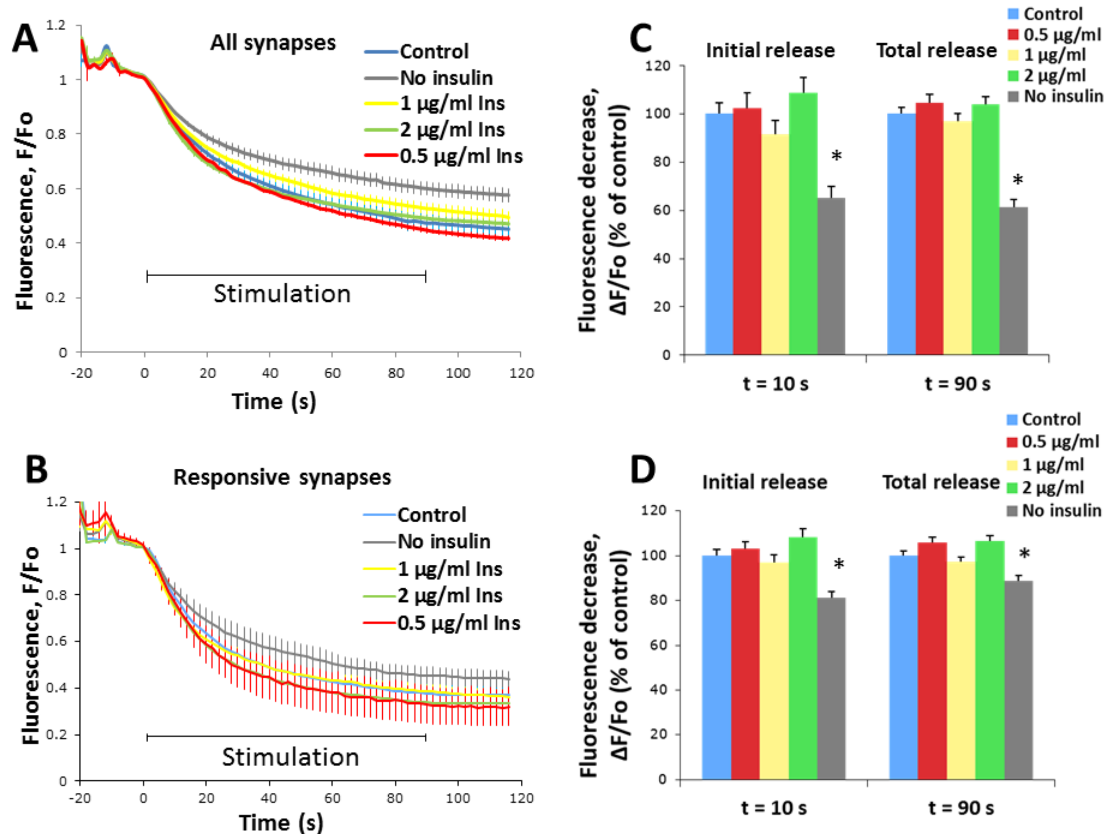

Figure 3.2 Synaptic vesicle release is impaired in neurons grown in the absence of insulin. DIV14 neurons treated for 14 days with $B27^{+ins}$ (control) and $B27^{-ins}$ + 2, 1, 0.5, 0 µg/ml insulin were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A, B). Time course of fluorescence F normalized to the fluorescence F0 at the time of stimulation. (C, D) Fluorescence decrease ΔF/F0 at 10 (initial release) and 90 s (total release), expressed as percent of control. All synapses (A ,B): all fluorescent punctae stained with FM1-43 were included in the analysis. Responsive synapses (C, D): fluorescent punctae that lost less than 11% of the initial fluorescence F0 within the first 20 s of stimulation were regarded as non-responsive and excluded from the analysis. Data are means ± SEM of three independent experiments with 1-3 curves per treatment group per experiment. *$p < 0.05$ compared with untreated control.

3.2 Decreased mitochondrial biogenesis in neurons grown in the absence of insulin

Insulin increases mitochondrial biogenesis and oxidative metabolism (117). Mitochondria are important in pre and post-synaptic specializations for energy and calcium homeostasis (118,119). To examine mitochondrial biogenesis in neurons with insufficient insulin signaling, rat hippocampal neurons were grown in neurobasal media with $B27^{+ins}$ (control) or $B27^{-ins}$ for a period of 9 days followed by immunoblotting analysis to measure PGC1α and VDAC1 levels. PGC1α levels were reduced in neurons grown in the absence of insulin (**Figure 3.3 A)**. There was also a noticeable reduction in the levels of VDAC1 protein for the neurons grown in the absence of insulin (**Figure 3.3 B**). Densitometric analysis confirmed a significant reduction in the levels of PGC1 α and VDAC1 in neurons grown in the absence of insulin compared to control **(Figure 3.3 C)** suggesting that a lack of insulin leads to impaired mitochondrial biogenesis in neurons.

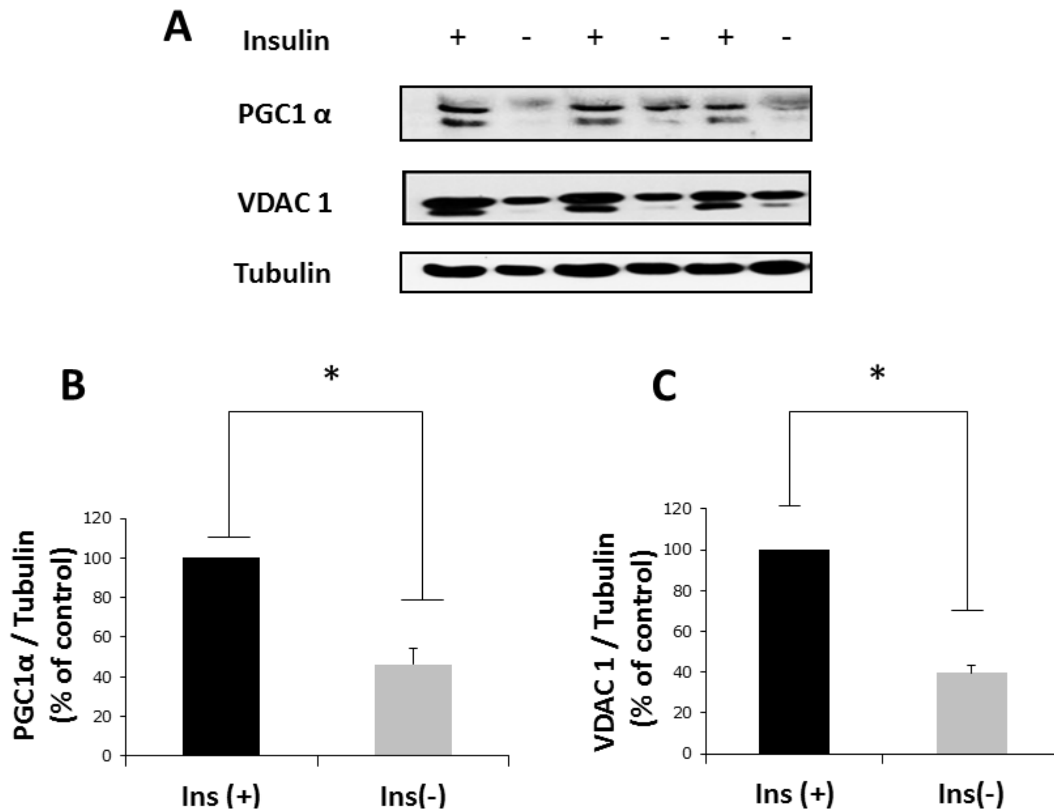

Figure 3.3 Mitochondrial biogenesis is decreased in neurons grown in the absence of insulin. Rat hippocampal neurons were grown in Neurobasal medium treated with $B27^{+ins}$ (control) or $B27^{-ins}$ for 9 days before being harvested into PBS containing 0.1 % SDS and protease and phosphatase inhibitors and immunoblotted with antibodies against PGC1α and VDAC1 (A). All lanes were loaded with $1/5^{th}$ of cells from a well per lane. Tubulin was used as a loading control. Densitometric analysis of the western blots is expressed as the ratio of PGC1 alpha relative to tubulin (B) and VDAC1 relative to tubulin (C). Data represent means ± SEM of three independent experiments showing triplicate samples of each treatment. $^*p < 0.05$ compared with untreated control.

3.3 Impaired synaptic vesicle release in rat hippocampal neurons grown in the presence of low insulin or high insulin

The previous experiments had shown that a complete absence of insulin negatively affects neuronal growth, whereas 0.5 μg/ml insulin were sufficient to support the formation of functional synapses. To test whether decreased levels of insulin can lead to synaptic defects in neurons while supporting normal growth, we supplemented B27^{-ins} with a very low amount of insulin (0.05 μg/ml or 0.1 μg/ml insulin). In addition to the low insulin treatment, we also wanted to test the effect of high insulin levels on synapse formation and vesicle release by adding 4 μg/ml insulin to B27^{-ins} media. An important point to note in this experiment was that all neurons showed good neurite growth and survival in contrast to our previous observation where the neurons grown in the absence of insulin had experienced poor growth and impaired synaptic vesicle release (**Figure 3.4).** We measured the synaptic vesicle release as before and observed a lower decrease in fluorescence in neurons grown in the presence of B27^{-ins} + 0.05 μg/ml (low insulin) (**Figure 3.5 A, D**) and B27^{-ins} + 4 μg/ml Insulin (**Figure 3.5 B, D**) both during initial and total vesicle release compared to the other treatment groups B27^{-ins} + 0.1, B27^{-ins} + 2 μg/ml insulin or control (B27^{+ins}**) (Figure 3.5 C, D)**. The impaired synaptic vesicle release in neurons grown in B27^{-ins} + 4 μg/ml insulin suggested that the addition of 4 μg/ml insulin to B27^{-ins} media led to a higher activity than present in control B27^{+ins} supplement. Even after the elimination of the non-responsive synapses, both initial and total vesicle release were significantly lower in neurons treated with very low or high insulin compared to control (**Figure 3.6 A, B, D).** In neurons treated with B27^{-ins} + 0.1 or

2 μg/ml insulin both initial and total vesicle release were comparable to control after the exclusion of the non-responsive synapses (**Figure 3.6 C, D**).

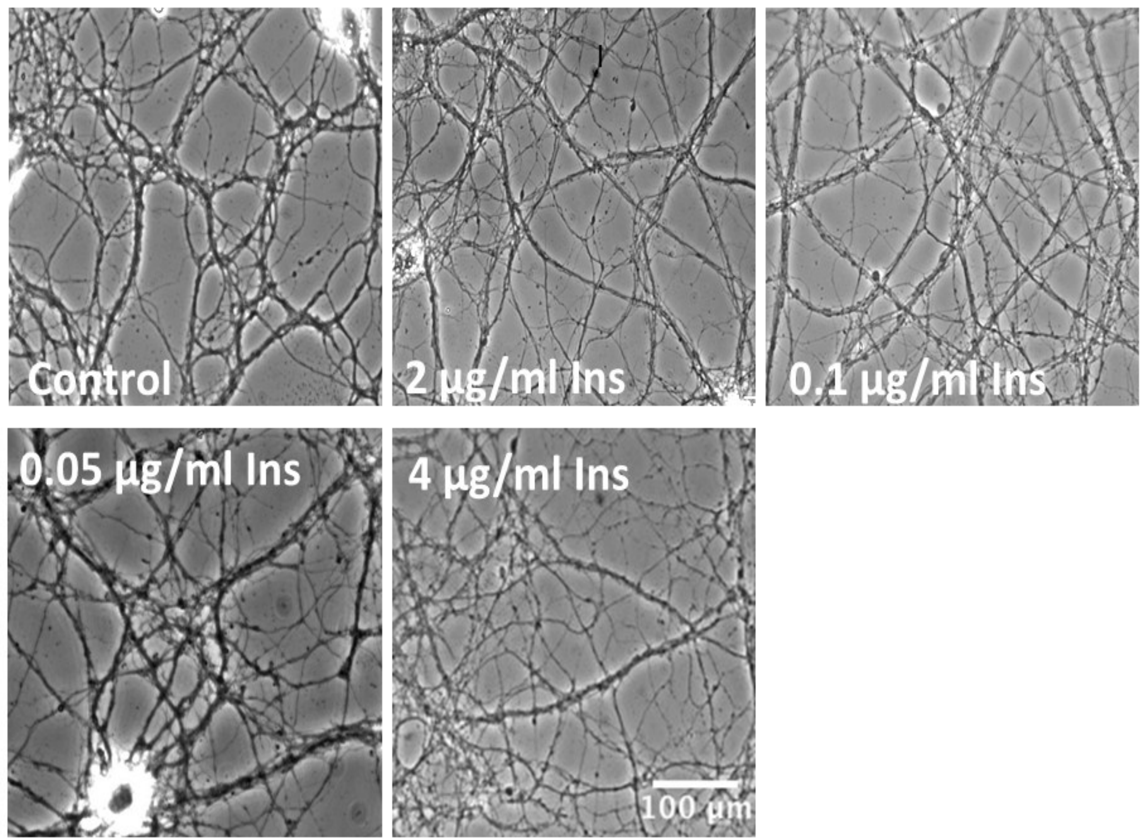

Figure 3.4 Morphology of neurons grown under different concentrations of insulin. Phase images of DIV 14 neurons grown in Neurobasal medium with $B27^{+ins}$ (control), $B27^{-ins}$ + 4, 2, 0.1, 0.05 µg/ml insulin, scale bar: 100 µm.

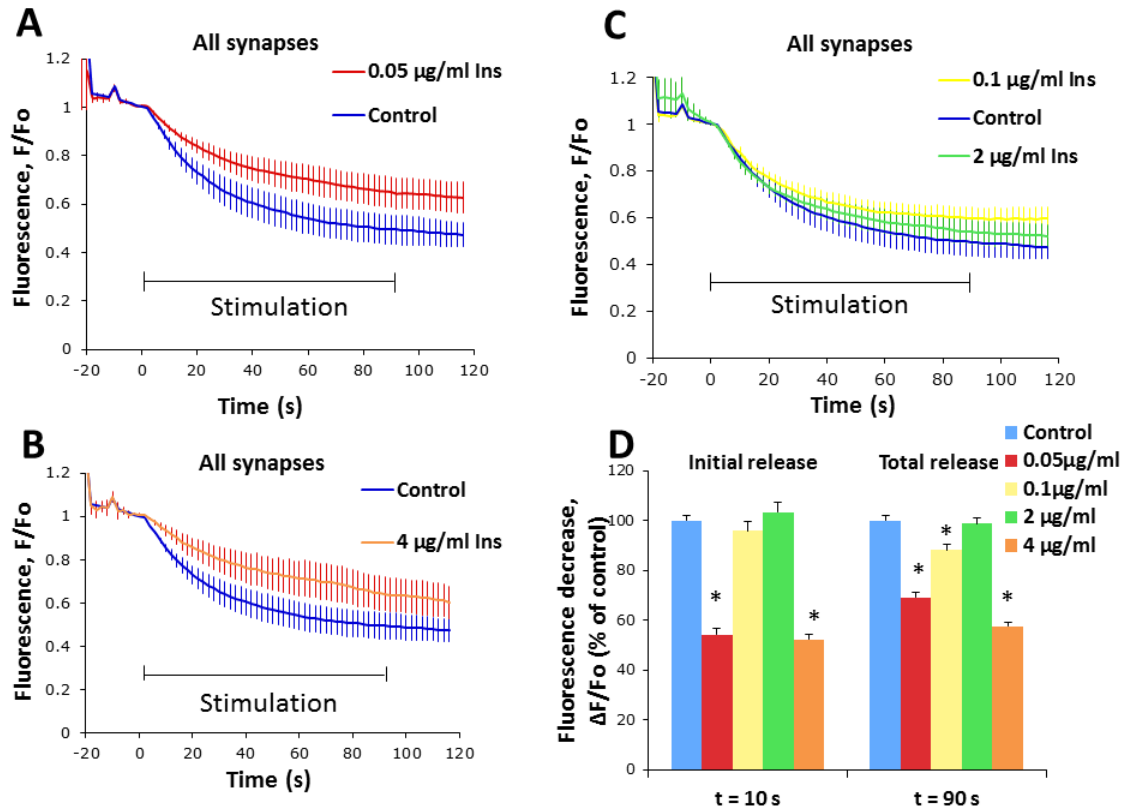

Figure 3.5 Synaptic vesicle release is impaired in neurons grown in the presence of low insulin or high insulin representing all synapses. DIV14 neurons grown in neurobasal medium with $B27^{ins}$ (control) or $B27^{-ins}$ + 4, 2, 0.1, 0.05 µg/ml insulin were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A-C). Time course of fluorescence F normalized to the fluorescence F0 at the time of stimulation. (D) Fluorescence decrease ΔF/F0 at 10 (initial release) and 90 s (total release), expressed as percent of control. All synapses (A-C): all fluorescent punctae stained with FM1-43 were included in the analysis. Data are means ± SEM of three independent experiments with 1-3 curves per treatment group per experiment. $^{*}p < 0.05$ compared with untreated control.

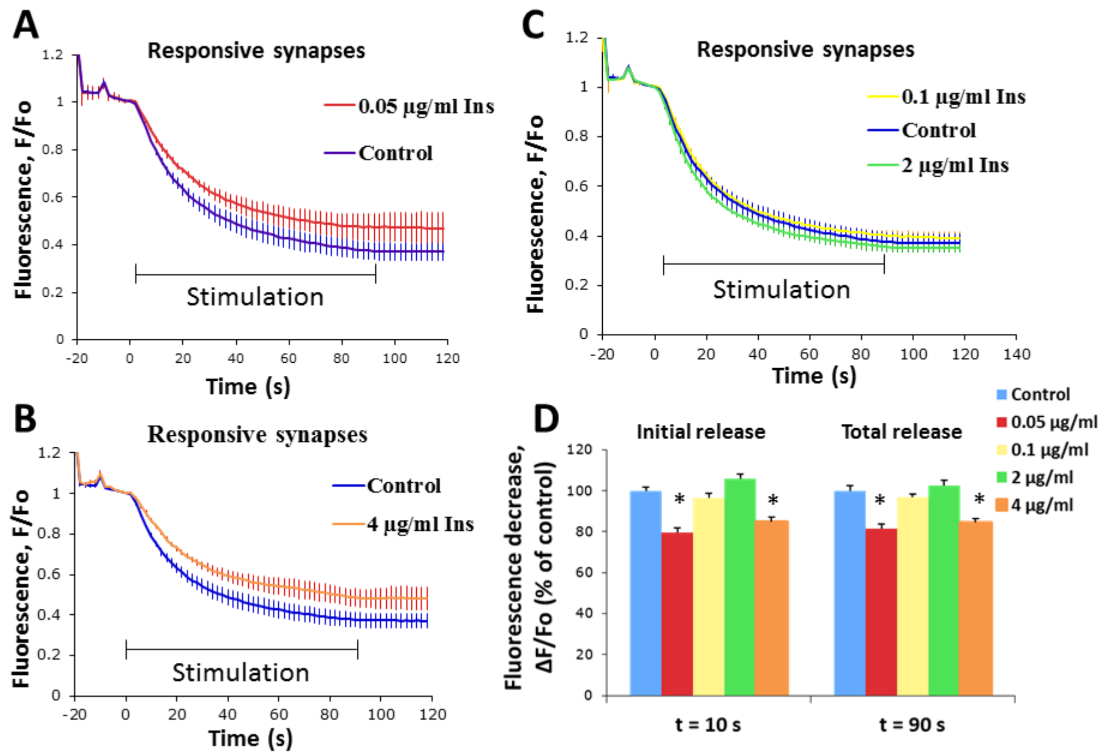

Figure 3.6 Synaptic vesicle release is impaired in neurons grown in the presence of low insulin or high insulin representing responsive synapses. DIV14 neurons grown in neurobasal medium with B27^{ins} (control) or B27^{-ins} + 4, 2, 0.1, 0.05 µg/ml insulin were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A-C). Time course of fluorescence F normalized to the fluorescence F0 at the time of stimulation. (D) Fluorescence decrease ΔF/F0 at 10 (initial release) and 90 s (total release), expressed as percent of control. Responsive synapses (j-l): fluorescent punctae that lost less than 11% of the initial fluorescence F0 within the first 20 s of stimulation were regarded as non-responsive and excluded from the analysis. Data are means ± SEM of three independent experiments with 1-3 curves per treatment group per experiment. *$p < 0.05$ compared with untreated control.

3.4 PPAR gamma agonist increases synaptic vesicle release in rat hippocampal neurons

The decrease in PGC1α levels observed in neurons grown in the absence of insulin suggested that the impaired synaptic function of neurons grown without or with low levels of insulin might in part have been caused by impaired mitochondrial biogenesis (92). Recent studies have shown that brain mitochondrial function and peripheral insulin resistance in high fat fed rats were improved with PPAR gamma agonists (120), and that this treatment also improved learning and memory in insulin resistant animal models (120). We therefore wanted to investigate the effects of the PPAR gamma agonist, troglitazone on synapse formation and function and test whether it normalizes the synaptic vesicle release in neurons grown in the presence of low insulin levels. Rat hippocampal neurons were grown in neurobasal media with $B27^{-ins}$ + 2 μg/ml insulin or $B27^{-ins}$ + 0.05 μg/ml insulin (low insulin) with or without the addition of troglitazone (5 μM), and synaptic vesicle release was measured on DIV 14 as before. We observed that synaptic vesicle release was improved in neurons grown in the presence of low insulin + troglitazone compared to neurons grown in low insulin alone **(Figure 3.7 A)**. After exclusion of non-responsive synapses, the synaptic vesicle release was still impaired in neurons treated with low insulin compared to low insulin + troglitazone or $B27^{-ins}$ + 2 μg/ml insulin (**Figure 3.7 B**) but the significance cannot be determined as there were only two independent experiments. These results support the hypothesis that impaired mitochondrial function contributed to the impaired vesicle release in neurons grown with insufficient insulin.

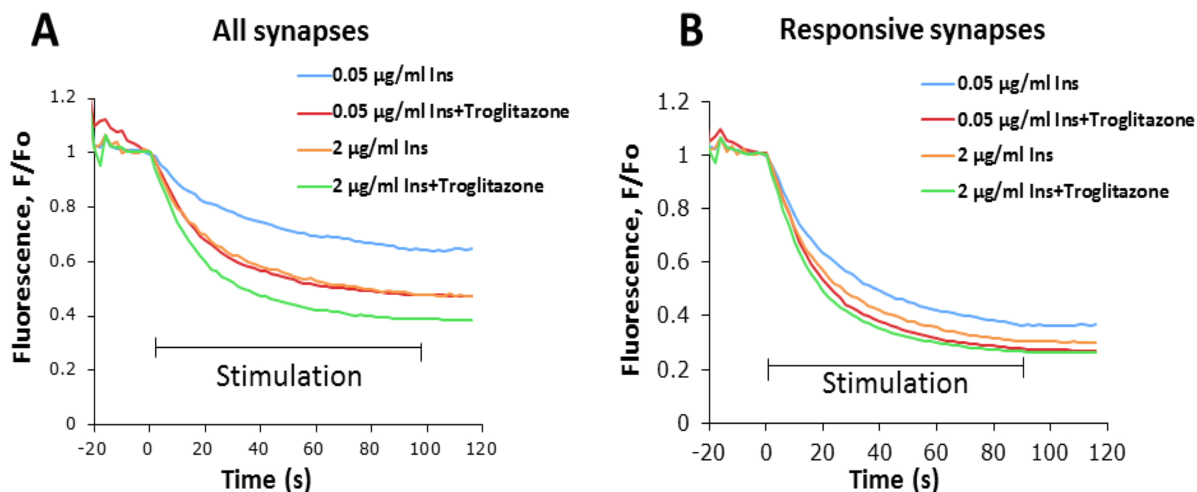

Figure 3.7 Synaptic vesicle release is improved in neurons with the addition of a PPAR gamma agonist grown in the presence of low insulin. DIV14 neurons treated with B27^{-ins} +2 µg/ml insulin, B27^{-ins} +2 µg/ml insulin + troglitazone (5 µM), low insulin or low insulin + troglitazone (5 µM) were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A, B). All synapses (A): all fluorescent punctae stained with FM1-43 were included in the analysis. Responsive synapses (B): fluorescent punctae that lost less than 11% of the initial fluorescence F0 within the first 20 s of stimulation were regarded as non-responsive and excluded from the analysis. Data represent average of 2 independent experiments with 1-3 curves per treatment group per experiment.

3.5 Chronic PPAR gamma agonist treatment has no effect on PGC1 alpha mRNA and protein levels in neurons

The results from the previous section showed that the PPAR gamma agonist, troglitazone improved the synaptic vesicle release in neurons grown in the presence of low insulin. Also, PPAR gamma agonist has been shown to improve mitochondrial biogenesis by increasing the PGC1α levels in the adipocytes of high fat fed mice (121). Therefore, we determined the mRNA levels of PGC1α in neurons grown in the presence of $B27^{-ins}$ + 0.05 or 2 μg/ml insulin with or without the addition of troglitazone (5 μM). Surprisingly, qPCR analysis of PGC1α mRNA levels did not show any significant differences between these treatment groups **(Figure 3.8)**, possibly due to a difference between acute effects reported in the literature (122) and our chronic treatment. We also measured the PGC1α and VDAC1 protein levels in neurons with similar culture conditions as above by immunoblotting. Similar to findings in neurons grown in the absence of insulin, PGC1α levels were significantly lower in neurons cultured with $B27^{-ins}$ + 0.05 μg/ml insulin compared to neurons cultured with $B27^{-ins}$ + 2 μg/ml insulin, supporting the hypothesis that differences in the PGC1α levels and decreased mitochondrial biogenesis may have contributed to the impaired synaptic vesicle release. However, chronic troglitazone treatment had no effect on PGC1α protein levels **(Figure 3.9 A, B).** VDAC1 levels remained unchanged in all the treatments suggesting that low insulin and $B27^{-ins}$ + 2 μg/ml insulin had no effect on the total mitochondrial content **(Figure 3.9 A, C)**.

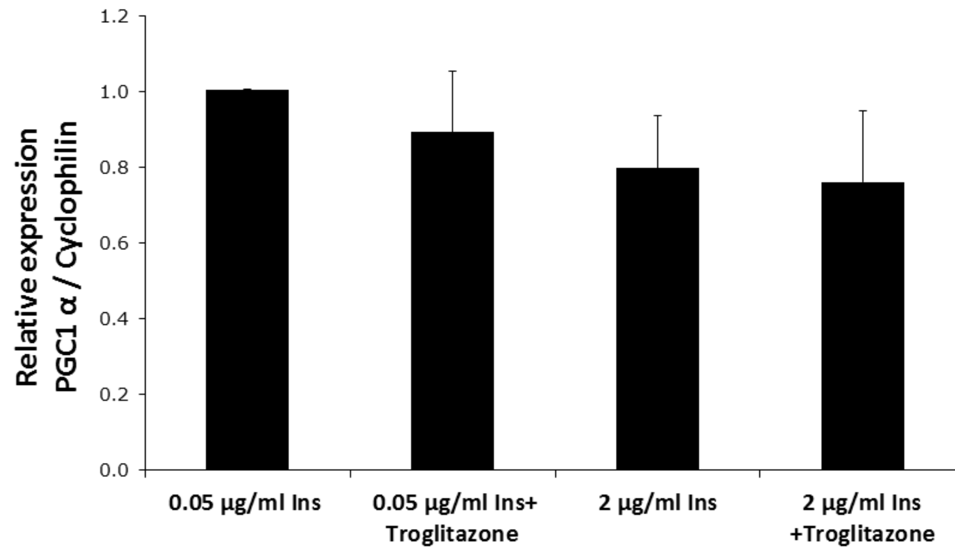

Figure 3.8 PGC1α mRNA levels were unaltered even after the addition of PPAR gamma agonist in neurons. qPCR analysis of mRNA for PGC1α in rat hippocampal neurons grown in Neurobasal medium with $B27^{-ins}$ + 0.05 µg/ml insulin, $B27^{-ins}$ + 0.05 µg/ml insulin + troglitazone (5 µM), $B27^{-ins}$ + 2 µg/ml insulin or $B27^{-ins}$ + 2 µg/ml insulin + troglitazone (5 µM) for 9 days. Data is expressed as PGC1α mRNA expression relative to cyclophilin mRNA expression by the ΔΔ CT method with cyclophilin as the housekeeping gene. Data are means ± SEM of three independent experiments in triplicate.

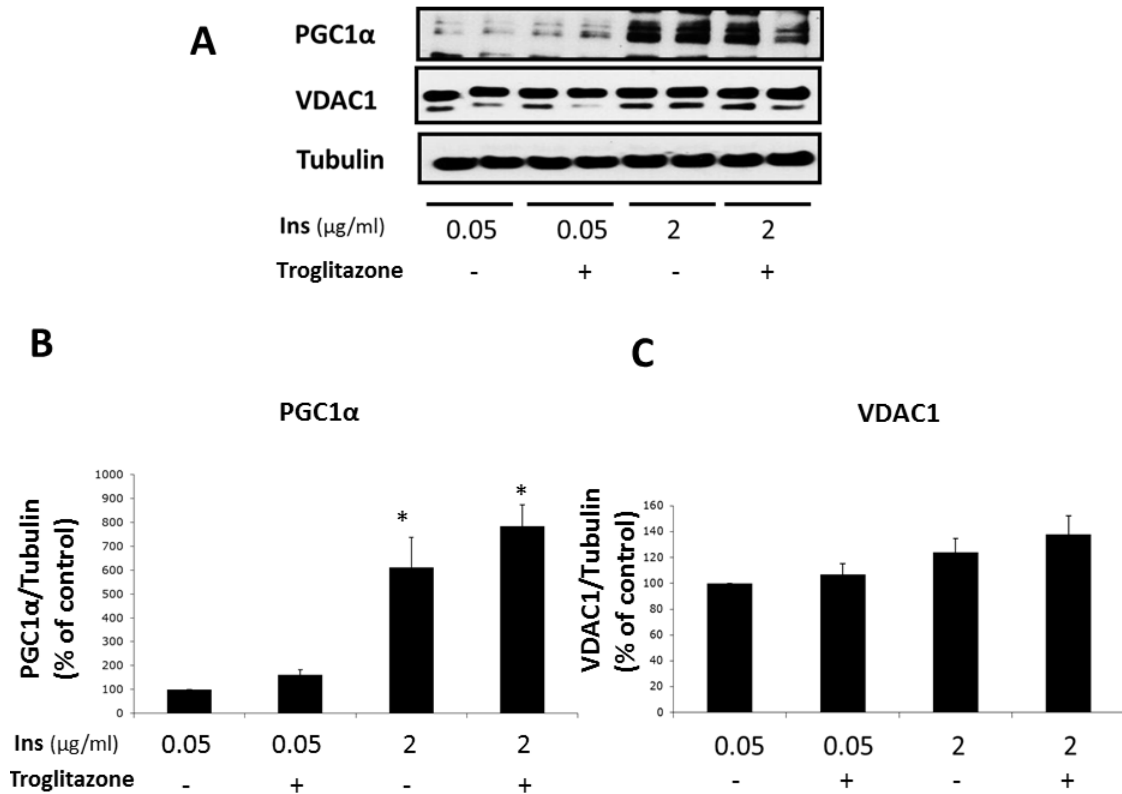

Figure 3.9 Decreased mitochondrial biogenesis in neurons grown in the presence of low insulin. Rat hippocampal neurons were grown in Neurobasal medium with $B27^{-ins}$ + 0.05 µg/ml insulin, $B27^{-ins}$ + 0.05 µg/ml insulin + troglitazone (5 µM), $B27^{-ins}$ + 2 µg/ml insulin or $B27^{-ins}$ + 2 µg/ml insulin + troglitazone (5 µM) for 9 days before being harvested into PBS containing 0.1 % SDS and protease and phosphatase inhibitors and immunoblotted with antibodies against PGC1α and VDAC1 (A). All lanes were loaded with 1/5th of cells from a well per lane. Tubulin was used as a loading control. Densitometric analysis of the western blots is expressed as the ratio of PGC1 alpha relative to tubulin (B) and VDAC1 relative to tubulin (C). Data represent means ± SEM of three independent experiments showing duplicate samples of each treatment. *$p < 0.05$ compared with untreated control.

3.6 Acute insulin exposure leads to activation of Akt in a dose responsive manner in rat hippocampal neurons

Actual levels of insulin in the brain are unknown, since contradicting results have been reported in the literature (34,123,124). One hypothesis would be that type 2 diabetes leads to decreased levels of insulin due to insulin resistance of the blood brain barrier endothelial cells and decreased transport of plasma insulin into the brain. It is also possible that hyperinsulinemia in the plasma, at least in early stages of type 2 diabetes leads to hyperinsulinemia also in the brain. Even in the presence of high levels of insulin, the insulin signal received by the neurons could become insufficient if insulin resistance develops. Therefore, we investigated under what conditions neurons may become insulin resistant and what the consequences would be. The findings in neurons grown in the presence of relatively high (4 μg/ml) insulin suggested that hyperinsulinemia may cause insulin resistance in neurons. Other factors that are known to cause insulin resistance in the periphery include high amino acids and inflammatory cytokines (26,27). Although there are a lot of studies describing insulin resistance in the periphery, very little is known about insulin resistance in neurons. We therefore wanted to investigate which factors could induce insulin resistance in neurons. To measure the neuronal response to insulin, neurons were deprived of insulin for 30 min followed by acute exposure to insulin for 15 min before harvesting. The insulin response was measured by determining the levels of Akt phosphorylation by immunoblotting. We first measured a dose response curve using acute insulin concentrations of 0, 10, 100 or 200 nM in rat hippocampal neurons grown in neurobasal media treated with $B27^{+ins}$ (control) or $B27^{+ins}$ + 4 μg/ml Insulin (high insulin) for 9 days. Western blot analysis indicated that the neurons grown in the presence of

B27^{+ins} (control) showed a dose responsive increase in Akt phosphorylation (**Figure 3.10 A**). Neurons grown in the presence of high levels of insulin showed only a non-significant trend to increasing Akt phosphorylation, suggesting that these neurons were less insulin sensitive than control neurons. Densitometric analysis confirmed that neurons grown in the presence of B27^{+ins} (control) exhibited a significant increase in the phosphorylation of Akt with 100 or 200 nM acute insulin stimulation whereas neurons treated with high insulin did not show a statistically significant increase in Akt phosphorylation (**Figure 3.10 B**).

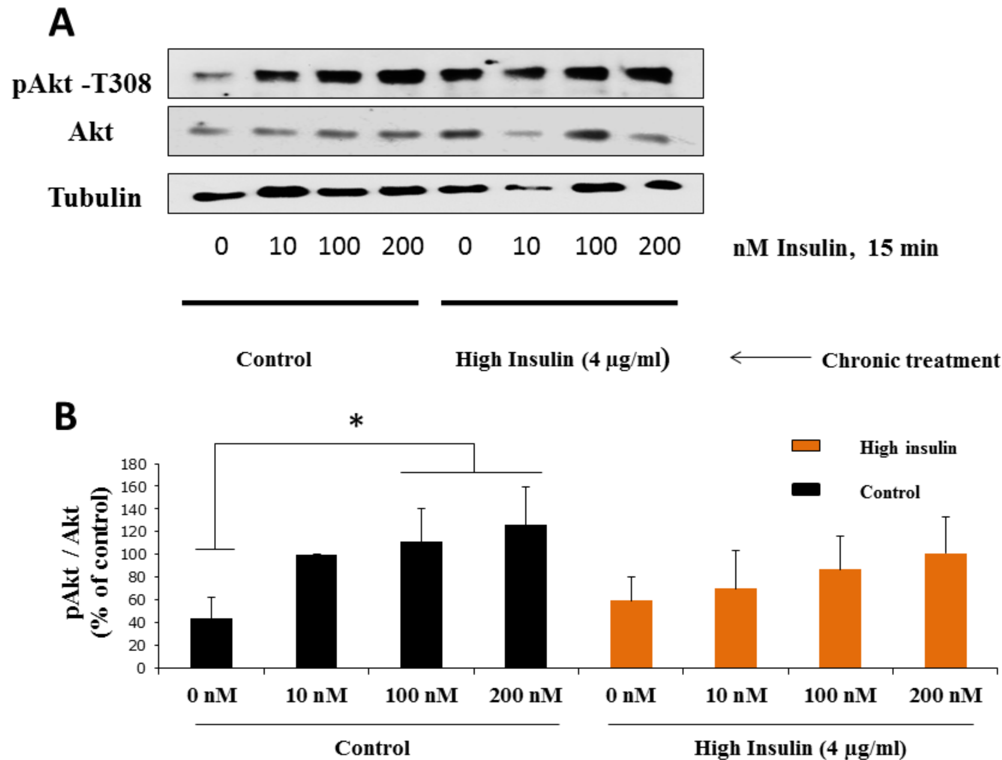

Figure 3.10 Acute Insulin stimulation leads to the activation of Akt in a dose responsive manner in rat hippocampal neurons. Rat hippocampal neurons were treated with B27^{+ins} (control) or B27^{+ins} + 4 µg/ml insulin (high insulin) for 9 days and then stimulated with 0, 10, 100 & 200 nM insulin for 15 min (A). Cell lysates were prepared in PBS containing 0.1 % SDS and protease and phosphatase inhibitors and separated by SDS PAGE and transferred to PVDF membranes. Immunoblot membranes were probed sequentially with antibodies against phosphorylated Akt (pAkt), total Akt and tubulin (A). All immunoblot lanes were loaded with 1/5th of cells from a well per lane. Densitometric analysis of the western blot is expressed as the ratio of Akt phosphorylation to total Akt per percent control (10 nM insulin) (B). Data are means ± SEM of three independent experiments in duplicate. *$p < 0.05$ compared with untreated control.

3.7 Treatment with leucine or TNFα downregulates the ability of rat hippocampal neurons to respond to acute insulin stimulation and leads to a high basal Akt phosphorylation

Although studies have shown that treatment with leucine or TNFα induces insulin resistance in skeletal muscle and adipose tissues, little is known about their effects on neurons (26,27). Hence, we investigated the effect of chronic leucine and TNFα exposure on neurons grown in $B27^{+ins}$ with 1 mM leucine or 1 nM TNFα for 9 days. Then insulin responsiveness was measured as before with a 30 min insulin deprivation and 15 min exposure to 100 nM insulin (**Figure 3.11 A**). As before, control neurons showed a significant increase in pAkt/Akt following exposure to insulin. In contrast, neurons treated with leucine, high insulin, high insulin + leucine, or TNFα did not show a significant response to insulin, even though we did observe a non-significant trend to higher pAkt/Akt levels. Densitometric analysis of Western blots confirmed that only the control samples resulted in a statistically significant increase in acute insulin stimulated Akt phosphorylation (**Figure 3.11 B**). Also, an increase in basal Akt phosphorylation was noted in neurons treated with leucine, high insulin or high insulin + leucine following an acute insulin treatment (**Figure 3.11 B**). These results suggest that the chronic treatment with leucine, TNFα or high insulin made the neurons less insulin sensitive.

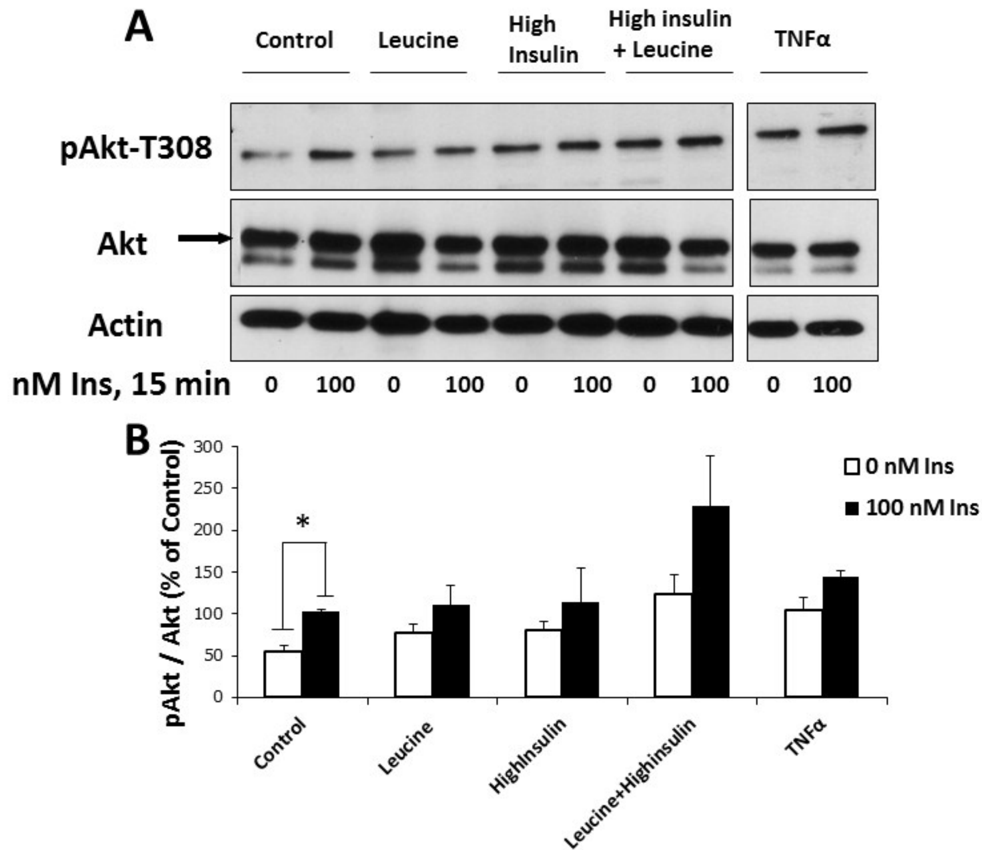

Figure 3.11 Neurons are less insulin sensitive upon treatment with leucine, high insulin or TNFα. Rat hippocampal neurons grown in Neurobasal medium with $B27^{+ins}$ were treated with leucine (1 mM), high insulin + leucine, high insulin or TNFα (1 nM) for 9 days and then stimulated with 100 nM insulin for 15 min followed by western blotting (A). Densitometric analysis of the western blot is expressed as the ratio of pAkt to total Akt per percent control (B). Cell lysates were prepared in PBS containing 0.1 % SDS and protease and phosphatase inhibitors and separated by SDS PAGE and transferred to PVDF membranes. Immunoblot membranes were probed sequentially with antibodies against phosphorylated Akt (pAkt), total Akt and actin. All immunoblot lanes were loaded with $1/5^{th}$ of cells from a well per lane. Data are means ± SEM of three independent experiments in duplicate. . *$p < 0.05$ compared with untreated control.

3.8 Synaptic vesicle release is impaired in neurons treated with leucine, TNFα or low antioxidants

Neurons grown in the presence of leucine or TNFα appeared less insulin sensitive than control neurons (**Figure 3.11**), indicating defects in the insulin-signaling pathway and a decreased insulin signal. Since insufficient insulin signaling due to low levels of insulin caused synaptic defects, we next investigated whether insulin resistance caused by leucine, TNFα or low antioxidant treatments also had any effects on synapse formation and vesicle release. On DIV 14, synaptic vesicle release was measured with the FM1-43 dye in rat hippocampal neurons grown in Neurobasal medium with B27^{+ins} treated with leucine, high insulin + leucine, lower antioxidants, lower antioxidants + high insulin or TNFα for 11 days. The morphology and growth of the neurons in all treatment groups was comparable with healthy neurites except for the treatment with leucine where the neurons were less dense compared to control and other treatment groups (**Figure 3.12**). Both initial release and total vesicle release were significantly lower in neurons treated with leucine, high insulin + leucine (**Figure 3.13 A, C**), low antioxidants, low antioxidants + high insulin or TNFα (**Figure 3.13 B, D**) compared to control (B27^{+ins}). When the non-responsive synapses were excluded, we observed that responsive synapses in neurons treated with leucine still showed a significantly lower total vesicle release but no significant differences in the initial vesicle release compared to control whereas those treated with high insulin + leucine showed a significantly lower initial and total vesicle release compared to control (**Figure 3.14 A, C**). Neurons grown under conditions of increased oxidative stress due to low levels of antioxidants also showed a significantly reduced initial and total vesicle release compared to control whereas those treated with

low antioxidants+ high insulin or TNFα showed no significant changes in either initial or total vesicle release compared to control after the exclusion of non-responsive synapses (**Figure 3.14 B, D**). These results demonstrate that there may be defects in synaptic vesicle release in neurons treated with factors known to cause peripheral insulin resistance in type II diabetes.

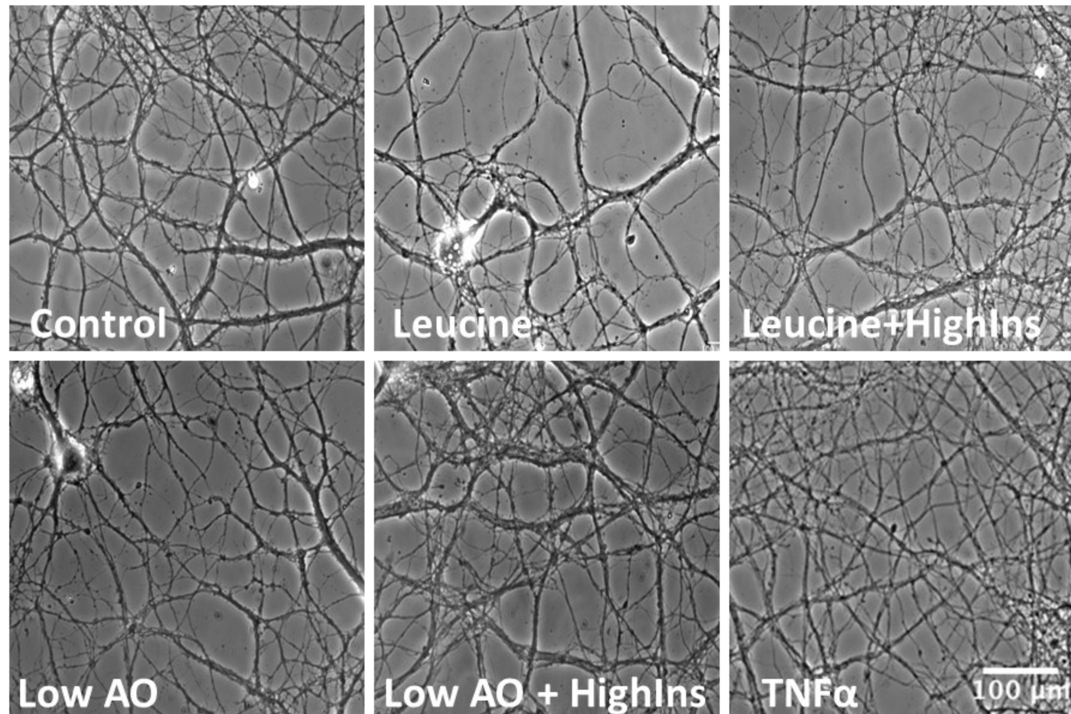

Figure 3.12 Morphology of neurons grown in the presence of leucine, leucine + high insulin, low antioxidants, low antioxidants + high insulin or TNFα. Phase images of DIV 14 neurons growing in Neurobasal medium with $B27^{+ins}$ treated with leucine, high insulin + leucine, low antioxidants, low antioxidants + high insulin or TNFα for 11 days, scale bar: 100 μm.

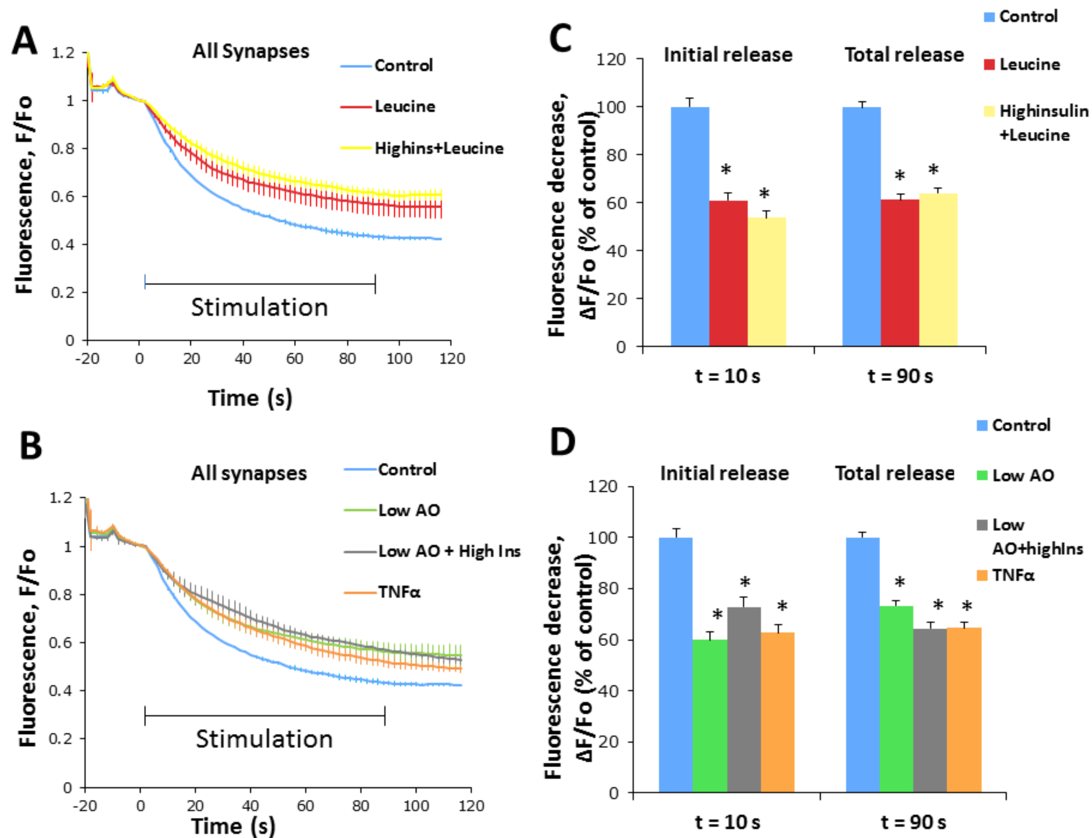

Figure 3.13 Impaired synaptic vesicle release in neurons treated with leucine, TNFα or low antioxidants representing all synapses. DIV14 neurons grown in Neurobasal medium with B27^{+ins} treated with leucine, high insulin + leucine, low antioxidants, low antioxidants + high insulin or TNFα for 11 days were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A, B). Time course of fluorescence F normalized to the fluorescence F0 at the time of stimulation. (C, D) Fluorescence decrease ΔF/F0 at 10 (initial release) and 90 s (total release), expressed as percent of control. All synapses (A, B): all fluorescent punctae stained with FM1-43 were included in the analysis. Data are means ± SEM of three independent experiments in triplicate. *p < 0.05 compared with untreated control.

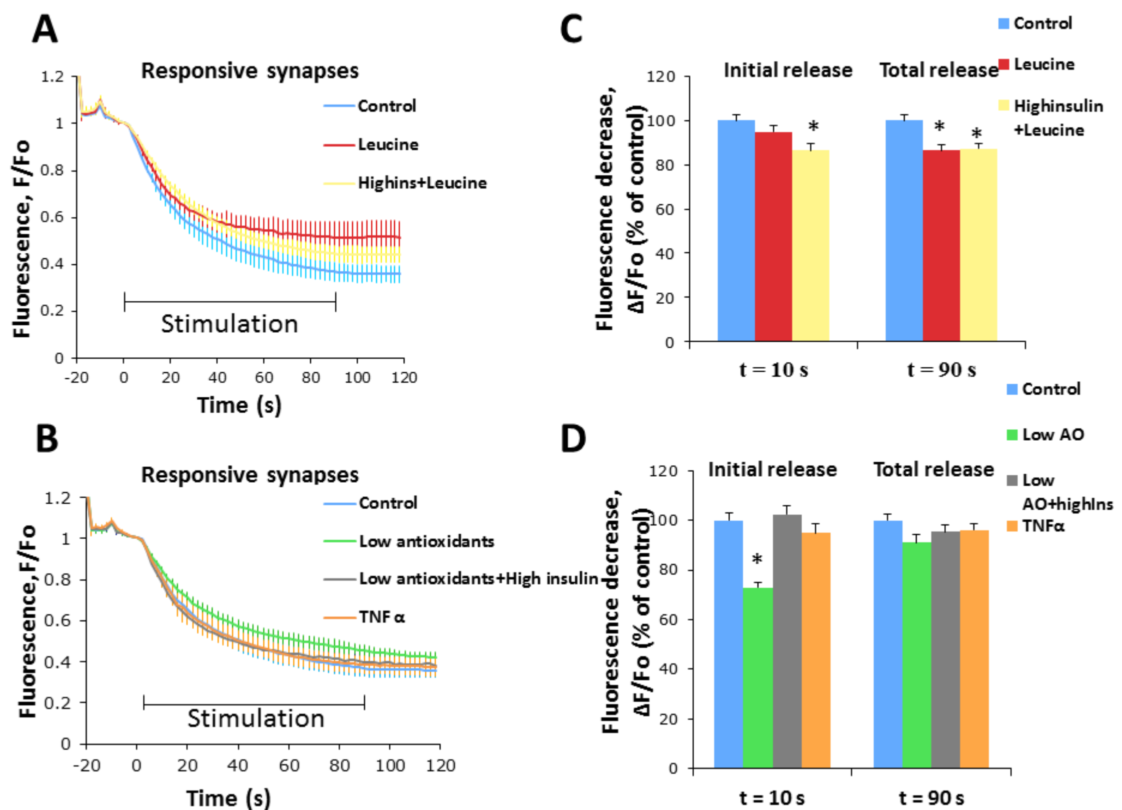

Figure 3.14 Impaired synaptic vesicle release in neurons treated with leucine, TNFα or low antioxidants representing responsive synapses. DIV14 neurons grown in Neurobasal medium with $B27^{+ins}$ treated with leucine, high insulin + leucine, low antioxidants, low antioxidants + high insulin or TNFα for 11 days were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A, B). Time course of fluorescence F normalized to the fluorescence F0 at the time of stimulation. (C, D) Fluorescence decrease ΔF/F0 at 10 (initial release) and 90 s (total release), expressed as percent of control. Responsive synapses (C, D): fluorescent punctae that lost less than 11% of the initial fluorescence F0 within the first 20 s of stimulation were regarded as non-responsive and excluded from the analysis. Data are means ± SEM of three independent experiments in triplicate. *$p < 0.05$ compared with untreated control.

3.9 Decreased Synaptotagmin 1 in rat hippocampal neurons treated with high insulin and leucine

Synaptotagmin 1 is a regulator of neurotransmitter release and alterations in its levels have been reported to cause abnormal neurotransmitter release and lower synaptic vesicle exocytosis (125). VDAC1 is the main ion and metabolite transporter in the mitochondrial outer membrane and often used as a marker for mitochondria. Moreover, increased VDAC1 levels have been found in patients with AD (126). As shown earlier, neurons treated with leucine or TNFα were less insulin sensitive compared to control neurons. Hence, we wanted to investigate the effects of these treatment groups on neurotransmitter release and mitochondrial protein VDAC1 to test if there were any changes in its levels. Rat hippocampal neurons were grown in Neurobasal medium with $B27^{+ins}$ (control) and treated with leucine (1 mM), high insulin (4 μg/ml) + leucine, TNFα (1 nM), low antioxidants or low antioxidants + high insulin for 9 days and then subjected to immunoblot analysis of synaptotagmin 1 and VDAC1. We observed that treatment with leucine + high insulin reduced the levels of the synaptotagmin 1 compared to control whereas the VDAC1 levels remained unchanged in all the treatment groups **(Figure 3.15 A)**. This was further confirmed by densitometric analysis of the Western blots which showed significantly reduced levels of synaptotagmin 1 in neurons treated with leucine + high insulin compared to control **(Figure 3.15 B)** but the VDAC1 levels remained unchanged in all the treatment groups **(Figure 3.15 C)**. Differences in Synaptotagmin 1 may have contributed to the defects in synaptic function as observed previously in neurons treated with leucine and high insulin. However, none of the above mentioned

treatments induced changes in the levels of VDAC1 suggesting that these treatments did not have an effect on the outer mitochondrial membrane levels.

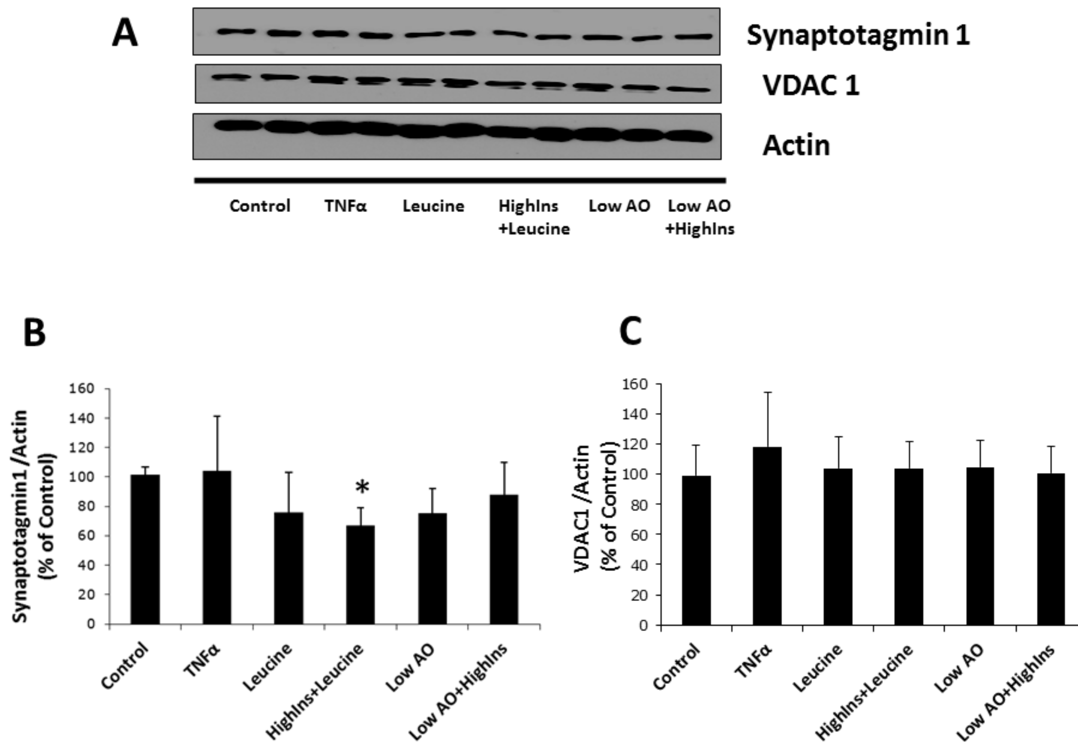

Figure 3.15 Decreased synaptotagmin 1 levels in neurons treated with high insulin and leucine but no changes in the levels of VDAC1 in neurons treated with leucine, TNFα or low antioxidants. Rat hippocampal neurons growing in neurobasal $B27^{+ins}$ were treated with leucine (1 mM), high insulin + leucine, low antioxidants, low antioxidants +high insulin or TNFα (1 nM) for 9 days (A). Cell lysates were prepared in PBS containing 0.1 % SDS and protease and phosphatase inhibitors and immunoblotted with antibodies against synaptotagmin 1 and VDAC1. Anti-actin was used as a loading control. All immunoblot lanes were loaded with $1/5^{th}$ of cells from a well per lane. Densitometric analysis of the western blots is expressed as the ratio of synaptotagmin 1 to actin per percent control (B) or VDAC1 to actin per percent control (C). Data are means ± SEM of three independent experiments in duplicate. $^*p < 0.05$ compared with untreated control.

3.10 AICAR rescues synaptic vesicle release and prevented the defect caused by leucine

Based on the previous results, neurons supplemented with leucine-induced defects in synaptic vesicle release. Leucine increases mTOR activation and downregulates AMPK activity (27). To determine whether reduced AMPK activity might be one of the causes for impaired synaptic vesicle release in neurons treated with leucine, we supplemented leucine-treated neurons with an AMPK activator, AICAR and tested the effects on synaptic vesicle release using FM1-43 dye. There was a significant improvement in both initial and total vesicle release in DIV 14 neurons grown in Neurobasal medium with $B27^{+ins}$ treated with leucine and AICAR for 11 days compared to the neurons treated with leucine alone (**Figure 3.16 A, C)**. Even after the elimination of non-responsive synapses, both initial and total vesicle release were significantly improved in neurons treated with AICAR in addition to leucine compared to those treated with leucine alone, even though release was still lower than in control neurons (**Figure 3.16 B, D**). Thus, AICAR prevented the defect in synaptic function caused by leucine. These results demonstrate that AMPK activation plays a major role in improving the synaptic defects caused by leucine.

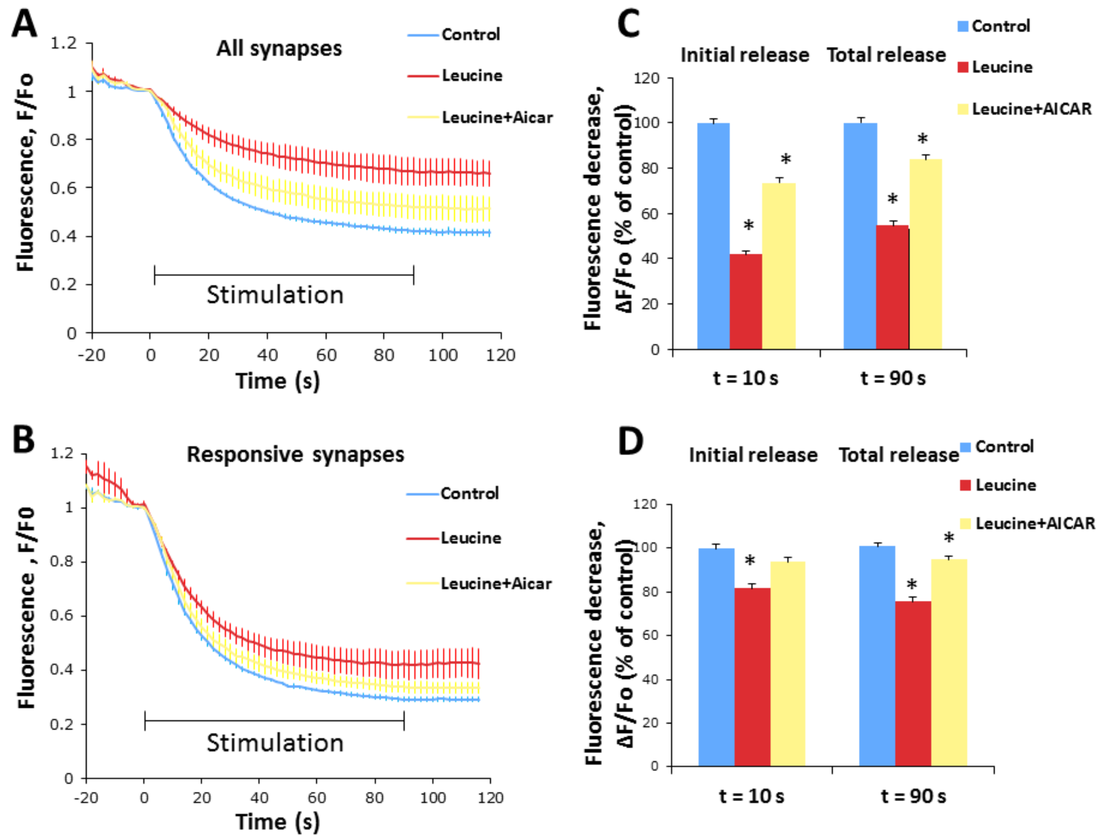

Figure 3.16 AICAR improves synaptic vesicle release in neurons treated with leucine. DIV 14 neurons grown in Neurobasal medium with B27^{+ins} treated with or without leucine or leucine + AICAR for 11 days were loaded with FM1-43 by field stimulation with 900 stimuli at 10 Hz. Fluorescence was recorded every 2 s during stimulation with 900 stimuli at 10 Hz with a baseline of 22 s prior to stimulation (A, B). All synapses (A, C): all synaptic boutons stained with FM1-43 were included in the analysis. Responsive synapses (B, D): synaptic boutons that lost less than 11% of the initial fluorescence F0 within the first 20 s of stimulation were regarded as non-responsive and excluded from the analysis. Data are means ± SEM of three independent experiments with 1-3 curves per treatment group per experiment. *p < 0.05 compared with untreated control.

3.11 NPC1 deficient neurons show a trend towards decreased insulin sensitivity in the hippocampus and cerebral cortex

Niemann -Pick type C disease is a rare neurodegenerative disease that is characterized by the accumulation of cholesterol in the late endosomes (105). Defects in insulin signaling have been previously reported in NPC diseased mouse brain and hepatocytes (108,109). Vainio et al reported increased cholesterol in the plasma membrane fractions of NPC hepatocytes suggesting that this could alter the properties of lipid rafts and lead to impaired insulin signaling (108). We therefore wanted to use NPC1 deficient and wild type neurons as a model of insulin resistance, and test whether the increased cholesterol in the plasma membrane could lead to impaired insulin signaling. Therefore, we investigated the effect of short-term insulin treatment on both wild type NPC1 and NPC1- deficient brain slices and checked whether they were insulin resistant. Brain slices prepared from 3 week old wild type and NPC1-deficient mouse hippocampus and cerebral cortex were deprived of insulin for 30 min followed by acute exposure to insulin for 15 min before harvesting. Western blotting analysis showed an increasing trend towards acute insulin stimulation (500 nM Insulin) for 15 min in wild type NPC1 neurons in both cerebral cortex and hippocampus compared to NPC1 deficient neurons, which rarely exhibited any response to acute insulin treatment in both the brain regions **(Figure 3.17 A, B)**. Also, a high basal Akt phosphorylation was observed in NPC1 deficient neurons after the short-term insulin treatment (**Figure 3.17 A, B).** Moreover, the results were highly variable and hence we could not draw a final conclusion with these experiments.

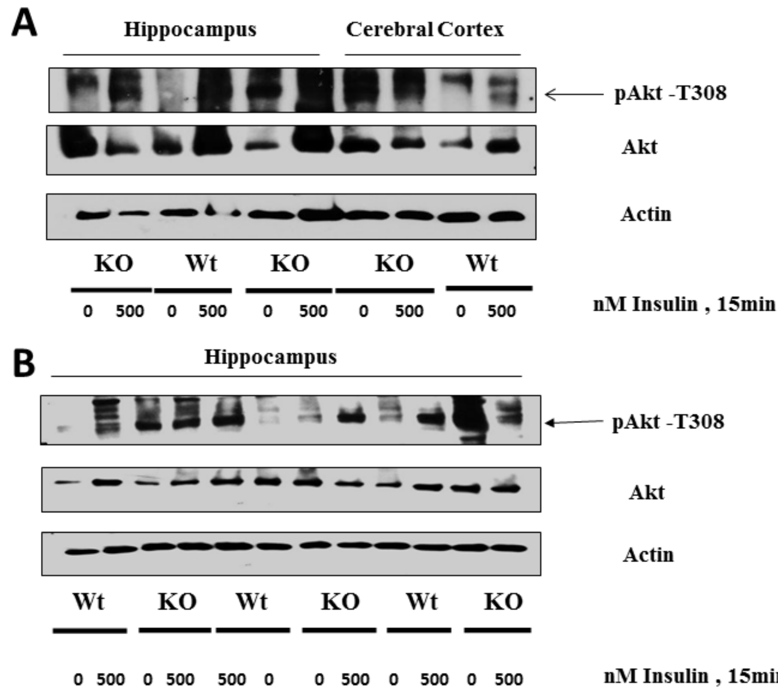

Figure 3.17 Insulin response in wild type NPC1 and NPC1- deficient neurons

3 week old hippocampal and cerebral cortical brain slices were prepared from both wild type NPC1 and NPC1 deficient neurons followed by a short-term acute insulin treatment of 500 nM insulin for 15 min (A, B). Cell lysates were prepared in PBS containing 0.1 % SDS and protease and phosphatase inhibitors and immunoblotted with antibodies against phosphorylated Akt (pAkt). The blots were stripped and reprobed with antibodies against total Akt. Anti-actin blot demonstrates the equal loading of the proteins. All lanes were loaded with 15 μg of protein. Data represent two independent experiments in duplicate.

CHAPTER 4

DISCUSSION

4.1 Neurons require an optimum amount of insulin for normal synaptic function

Although the role of insulin in learning and memory is well known, there still remain unanswered questions about the effects of insulin in pre-synaptic nerve terminal involving synapse formation, maturation and vesicle release. Our overall goal was to investigate the effects of insulin on synaptic function and vesicle release. Firstly, we wanted to explore the effects of altered levels of insulin on synapse formation and function in cultured hippocampal neurons. Our studies showed that the synaptic vesicle exocytosis was drastically impaired in neurons grown in the absence of insulin compared to neurons grown in neurobasal medium with $B27^{+ins}$ **(Figure 3.2).** In contrast, neurons grown in the presence of 0.5, 1 or 2 µg/ml insulin had normal synaptic vesicle release. Also, the morphology of the neurons grown in the absence of insulin was less healthy and had poor growth compared to control and other treatment groups (**Figure 3.1)**. When insulin was added at concentrations below 0.1 µg/ml, it was sufficient to support normal growth but synaptic function was still impaired. This indicated that hippocampal neurons required some amount of insulin to support neuronal growth and survival.

Our studies with high levels of insulin showed an impaired synaptic vesicle release in hippocampal neurons (**Figure 3.5 and 3.6)**. We hypothesize that the high insulin treatment might be contributing to insulin resistance in these neurons. A study reported

that exposure to high insulin downregulates the ability of adult sensory neurons to form neurites thereby affecting their survival (127). Also chronic exposure of 20 nM insulin for 24 h has been reported to cause insulin resistance in cultured dorsal ganglion neurons (15). Insufficient levels of insulin accompanied with hypoglycemia were previously reported to cause neuronal damage in the form of seizures in the cortex of a nine-week-old mouse brain (128). This finding was in line with our studies where we saw an impaired synaptic function in neurons grown under low levels of insulin. Together, these results suggested that hippocampal neurons require an optimum concentration of insulin for normal synaptic function.

4.2 Decreased mitochondrial biogenesis in neurons

Insulin regulates mitochondrial function and mitochondrial metabolism (129). Mitochondria provide the major source of ATP and regulate synaptic stimulation, formation and activity (92). The role of mitochondria in synaptic transmission was characterized in studies on *drp1* deficient motor neurons that lacked the synaptic mitochondria and were unable to mobilize the reserve pool vesicles (92). This study indicated that synaptic mitochondria are very important for mobilization of reserve pool vesicles, as they are the main source of ATP generation during synaptic transmission (92). In 2008, Turner et al reported that mitochondrial DNA and mitochondrial genes were reduced in the muscle of insulin resistant subjects indicating mitochondrial dysfunction in these subjects (86). Decreased mitochondrial biogenesis has also been reported in patients with AD providing a link to some of the previously reported literature

stating the possible role of insulin resistance in AD patients (19,56,130). Asmann Y, et al showed that mitochondrial transcription factor A (TFAM) mRNA levels were decreased in the muscle of non-diabetic individuals with response to 7h of low levels of insulin indicating that low levels of insulin input causes a decrease in mitochondrial function (131). As mitochondria are involved in regulating synaptic transmission, we tested the effect of low insulin input on mitochondrial biogenesis in neurons. Neurons grown in the absence of insulin or presence of low insulin had low levels of PGC1α, indicating defective mitochondrial biogenesis (**Figure 3.3)**.

PPAR gamma agonists increase insulin sensitivity via regulation of glucose and lipid homeostasis by forming heterodimers with retinoid X receptors (RXR) (132). PPAR gamma agonists corrected the defects in brain mitochondrial biogenesis and neuronal insulin resistance in rats fed a high fat diet for 12 weeks and restored normal synaptic function (120). This study also observed a reduction in the brain mitochondrial swelling and mitochondrial ROS production upon chronic treatment of rosiglitazone to rats fed on a high fat diet (120). We observed a similar effect after the addition of a PPAR gamma agonist, troglitazone to neurons grown in the presence of low insulin that showed an improved synaptic vesicle release compared to neurons grown in low insulin alone (**Figure 3.7).** With the growing evidence of mitochondrial and synaptic defects in insulin resistant subjects, the role played by PPAR gamma agonists becomes very important for providing normal mitochondrial function and synapse formation.

As discussed earlier, PPAR gamma agonists are known to increase mitochondrial biogenesis and have been used as a therapeutic target for treating type II diabetes (133). In 2010, Yanfei Wang et al reported that 10 μM troglitazone treatment for 24 hr caused no changes in the PGC1α mRNA levels in human lung adenocarcinoma A549 cells (134). However 50 μM troglitazone treatment for 24 hrs downregulated the mRNA expression of PGC1α suggesting that troglitazone acts in a dose response manner in these cells (134). In our study we observed that long-term treatment of troglitazone (5 μM) to neurons treated with low insulin for 9 days caused no changes in the expression levels of PGC1α (**Figure 3.8)**. Similarly, chronic treatment of PPAR gamma agonists to neurons grown in the presence of low insulin did not cause any significant increase in the PGC1α protein levels compared to those treated with low insulin alone suggesting that PGC1α protein levels are not regulated by PPAR gamma agonists. This was surprising as neurons treated with troglitazone showed an improved synaptic vesicle exocytosis in neurons treated with low insulin but failed to increase the PGC1α levels in the same culture conditions suggesting that it affected PGC1α on another level or it acted independent of PGC1α. Also, chronic treatment of PPAR gamma agonist might not be able to increase PGC1α mRNA or protein levels. The dosage of troglitazone also plays a very important role in the protein or mRNA expression of PGC1α. These results indicate that PPAR gamma agonists are involved in improving the synaptic function in neurons but fail to improve the mitochondrial biogenesis under the same culture conditions.

4.3 Insulin resistance may contribute to impaired synaptic vesicle release in neurons

Insulin is critical for neuronal survival and development (135). Examining the effects of high insulin on synapse formation and function, suggested that these neurons might be mimicking hyperinsulinemia under chronic insulin treatment supporting the assumption that insulin resistance could be contributing to the impaired synaptic vesicle release in these neurons. Hyperinsulinemia has been previously reported to cause insulin resistance in dorsal root ganglion (DRG) neurons following a chronic insulin exposure of 20 nM for 24 hrs (15). Also a high basal Akt phosphorylation was reported in these neurons upon chronic insulin exposure (15). We observed a very similar effect in our culture system where chronic insulin exposure of 4 μg/ml insulin for 9 days resulted in a reduced insulin stimulated Akt phosphorylation suggesting that the neurons were less insulin sensitive upon chronic insulin treatment (**Figure 3.10)**. A high basal Akt phosphorylation was also observed in these neurons upon chronic insulin exposure (**Figure 3.10)**. Akt phosphorylation mainly depends on the kinase and phosphatase activities, which could mean that the phosphatases were less active upon exposure to chronic insulin leading to a high basal level Akt phosphorylation in these neurons (136,137). Hui-Yu Liu, et al reported high basal Akt phosphorylation (Ser 473) in the liver of mice fed on a high fat diet causing insulin resistance in these mice (137). However, the exact reason behind high basal Akt phosphorylation in these mice was not addressed in that study (137). The observed insulin resistance in these mice was also associated with decreased mitochondrial production and increased oxidative stress suggesting that there could be a possible mitochondrial defect associated with these mice (137).

Apart from hyperinsulinemia, there are other factors that have been reported to cause insulin resistance in the periphery, including TNFα and leucine (26,27,57). TNFα knockout mice responded to an exogenous dose of insulin much more efficiently than TNFα wild-type mice suggesting that TNFα may be involved in inducing insulin resistance (138). Insulin receptor signaling and phosphorylation of serine residues on IRS-1 have been identified as potential targets for TNFα induced insulin resistance (138).

Leucine activates the mTOR/S6K1 pathway, which in turn decreases tyrosine phosphorylation and increases serine IRS1 phosphorylation thereby leading to insulin resistance (139). Chronic leucine treatment led to an increase in the mTOR and S6K1 signaling causing insulin resistance in primary mouse hepatocytes (140). The same study also reported that leucine deprivation caused a reduction in the mTOR and S6K1 signaling leading to AMPK upregulation and insulin sensitivity in primary mouse hepatocytes suggesting that activation of mTOR signaling led to insulin resistance (140). We observed that chronic treatment of hippocampal neurons with TNFα or leucine for 9 days resulted in a reduced response to acute insulin stimulation (100 nM) and led to high levels of basal Akt phosphorylation (**Figure 3.11).** The decreased response to insulin in neurons supplemented with leucine could be due to activation of the mTOR signaling and/or downregulation of AMPK activity as suggested in the leucine-induced insulin resistance model (**Figure 4.1**). The exact reason behind the reduced insulin sensitivity in neurons treated with leucine or TNFα is not entirely known.

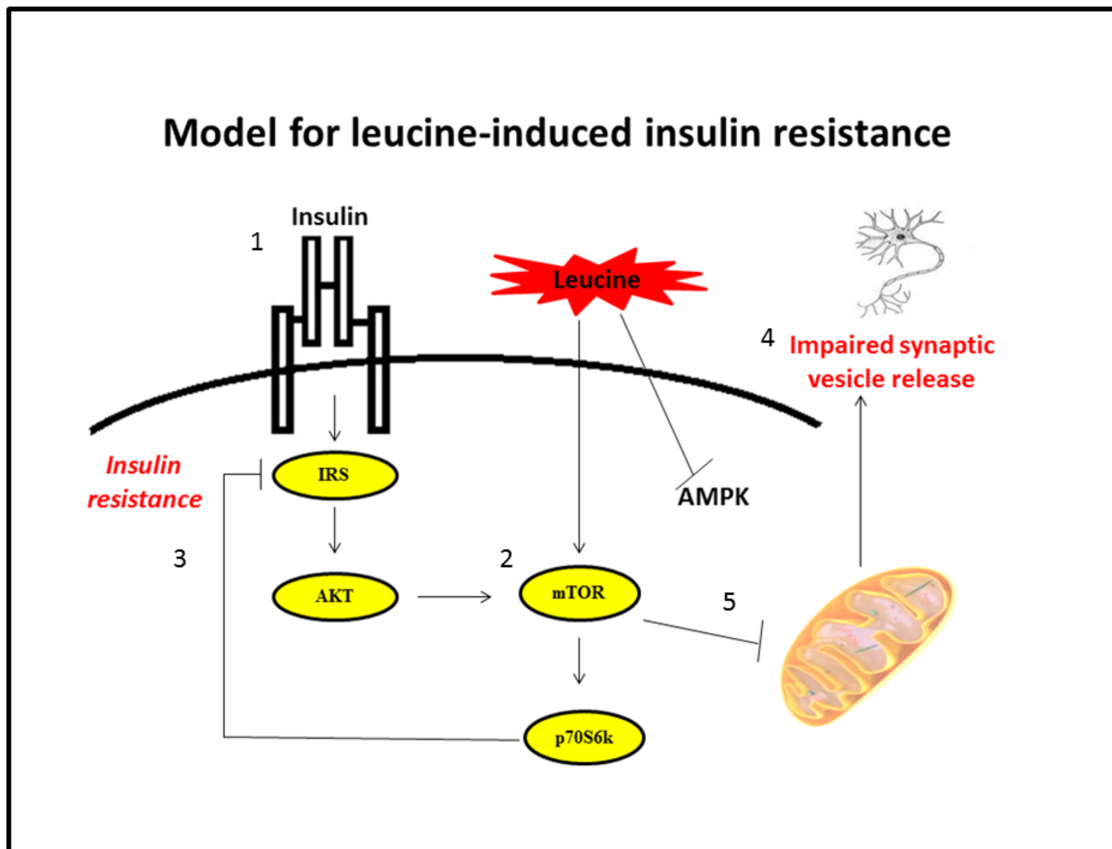

Figure 4.1 Leucine-induced insulin resistance

1) Insulin binds to its receptor and phosphorylates IRS, which in turn leads to the activation of other signaling molecules and Akt. 2) Akt phosphorylates mTOR, which is also activated by leucine. 3) This leads to the phosphorylation of S6K1 that causes an increased serine phosphorylation of IRS through a negative feedback mechanism leading to insulin resistance. 4) Supplementation with leucine leads to an impaired synaptic vesicle release in neurons. 5) mTOR activation could also contribute to a mitochondrial defect ultimately leading to impaired synaptic vesicle release in neurons.

Synaptotagmin 1 regulates Ca^{2+} dependent neurotransmitter release and any alteration in its function affects synaptic transmission (69). We observed that neurons grown in the presence of high insulin + leucine had reduced levels of synaptotagmin 1 (**Figure 3.11)** suggesting that this decrease in synaptotagmin 1 may have contributed to the observed defect in neurotransmitter release as synaptotagmin 1 protein mainly acts a calcium sensor for neurotransmitter release (141).

VDAC1, an outer mitochondrial membrane protein functions as a gatekeeper for the exchange of ions and molecules between the mitochondria and rest of the cell (142). Alterations in mitochondrial function in AD patients are linked with abnormally high levels of VDAC1 protein (126). However, in our study we observed no changes in the levels of VDAC1 in neurons treated with TNFα, leucine, high insulin and leucine, low antioxidants or low antioxidants + high insulin (**Figure 3.11)** suggesting that these treatment groups had no effect on the total mitochondrial content.

Next, we wanted to test the hypothesis that insulin resistance contributes to impaired synaptic vesicle release in neurons. We tested this hypothesis by measuring the synaptic vesicle release in neurons grown in the presence of leucine, leucine + high insulin, low antioxidants, low antioxidants+ high insulin or TNFα and observed that synaptic vesicle release was significantly impaired in these neurons compared to control (**Figure 3.14 and 3.15).** These findings supported the assumption that insulin resistance may contribute to the impaired synaptic vesicle release observed in neurons supplemented with leucine, low antioxidants or TNFα.

4.4 Role of mitochondria in synaptic transmission

Mitochondria are very important for maintaining normal synaptic transmission, as they are the primary source of energy generation for many steps in the synaptic vesicle cycle (92). Verstreken et al examined the importance of synaptic mitochondria in studies relating to mutations in *drp1* neuromuscular junctions, which lack synaptic mitochondria by measuring effects of these mutants on reserve pool vesicle mobilization (92). It was found that there was a defect in the mobilization of reserve pool vesicles during prolonged stimulation in the *drp1* deficient motor neurons due to the lack of synaptic ATP from the mitochondria (92). This effect was later restored by the supply of exogenous ATP suggesting that synaptic mitochondria are very important for the mobilization of reserve pool vesicles (92). In our studies, neurons supplemented with leucine, leucine + high insulin, low antioxidants or TNFα exhibited a significantly lower initial and total synaptic vesicle release compared to control. After the exclusion of the non-responsive synapses, the initial vesicle release in neurons treated with leucine was not significantly different compared to control whereas the total vesicle release was still significantly lower than control suggesting that leucine treatment caused defects in total vesicle release (**Figure 3.15)**. According to the study reported by Verstreken et al, the total release during prolonged stimulation requires the mobilization of reserve pool vesicles (92). This could mean that leucine inhibits the mobilization of reserve pool vesicles during prolonged stimulation. This suggests that there may be a defect with synaptic mitochondria or a bio-energetic mitochondrial dysfunction in neurons upon treatment with leucine or leucine + high insulin. A study published by Amaral, et al in 2010 observed mitochondrial dysfunction with the supplementation of leucine in a rat

brain (143). The addition of leucine decreased the oxygen consumption in the mitochondria indicating a defect in mitochondrial respiration (143). Thus, we hypothesized that addition of leucine to neurons may induce a defect in the mitochondria in our culture system thereby affecting synaptic vesicle release. Future work could be focused on investigating the link between mitochondrial dysfunction and leucine induced impaired synaptic vesicle release.

4.5 Role of AICAR in modulating the defects in synaptic vesicle release caused by leucine

AICAR has been shown to prevent insulin resistance and increase mTOR signaling in muscle cells caused by leucine via upregulation of AMPK activity and phosphorylation of TSC2 (27). AICAR improves neurite outgrowth in PC-12 cells in the presence of glial conditioned medium (144). The same study also reported that AICAR blocked the reactive oxygen species generation in glia cells induced by Aβ or sphingomyelinase (144). We found that defects in synaptic vesicle release caused by leucine in neurons were improved by chronic treatment with AICAR (**Figure 3.16)**. Supplementation with AICAR could also play a role in partially rescuing the mitochondrial defect in neurons caused by leucine by inhibiting mTOR activation as described in **Figure 4.2**. Hence, AICAR could be used as a potential target for improving synaptic defects in neurons and also serve as a neuroprotective agent. Future work could be focused upon the investigation of mTOR signaling pathway in neurons cultured in the presence of high leucine that would give enough insight about mTOR signaling in neurons and their link with leucine

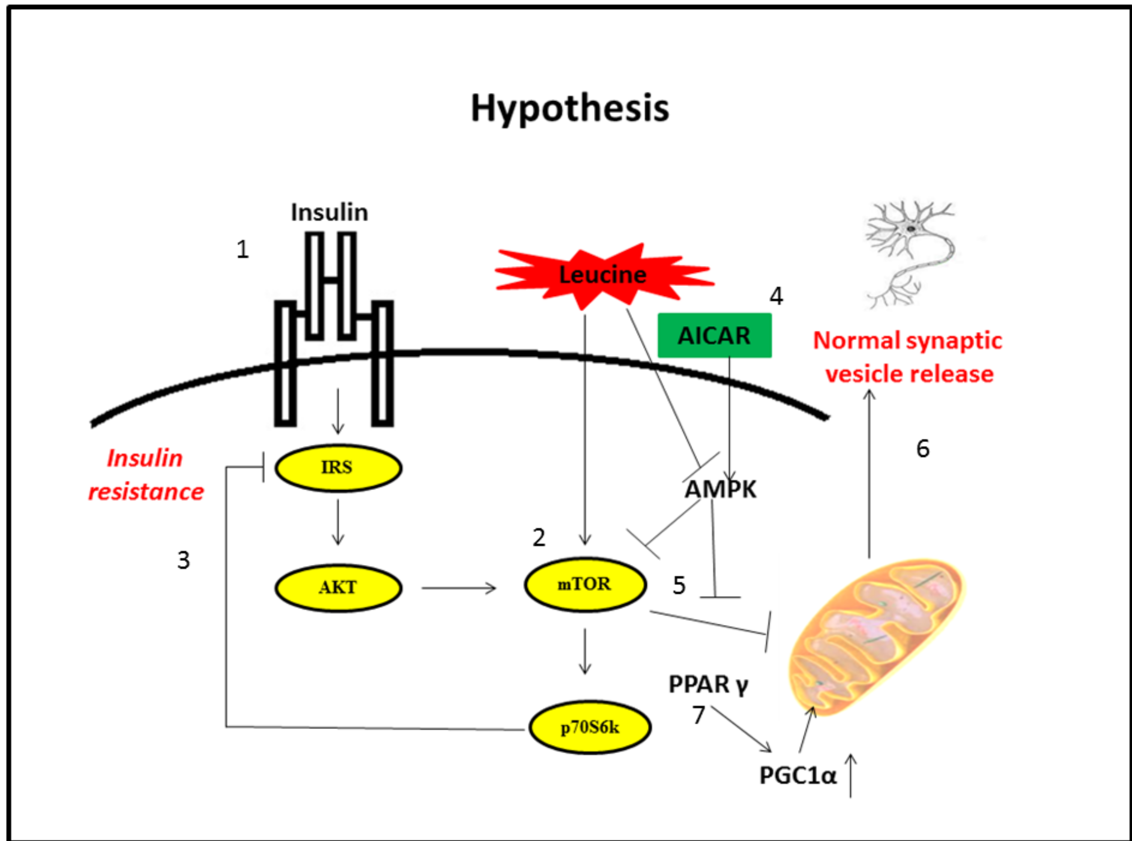

Figure 4.2 Hypothesis

1) Insulin binds to its receptor and phosphorylates IRS, which in turn leads to the activation of other signaling molecules and Akt. 2) Akt phosphorylates mTOR, which is also activated by leucine. 3) This leads to the phosphorylation of S6K1 that causes an increased serine phosphorylation of IRS through a negative feedback mechanism leading to insulin resistance. 4) Supplementation with AICAR increases the AMPK activity and downregulates mTOR. 5) This in turn prevents the possible mitochondrial defect caused by mTOR activation, 6) thereby leading to a normal synaptic vesicle release in neurons supplemented with AICAR. 7) PPAR gamma agonists increase mitochondrial function by regulating the PGC1α levels and may also contribute to the improvement of synaptic vesicle release in neurons.

4.6 Conclusion

Overall, the results presented here demonstrate that neurons require an optimum amount of insulin for the development and maturation of fully functional synapses as shown in our synaptic vesicle release measurement studies with different concentrations of insulin. Further, high levels of insulin in culture may contribute to insulin resistance in hippocampal neurons. These studies highlight the potential link between T2D and cognitive decline where insulin resistance has been observed to be the common theme in both T2D and cognitive decline. The decreased mitochondrial biogenesis in neurons grown in the absence or low levels of insulin suggests that neurons require an optimum amount of insulin for normal mitochondrial function. In addition, there seems to be a defect in the downstream insulin-signaling pathway when neurons were supplemented with leucine, TNFα, high insulin or low antioxidants making them less insulin sensitive and exhibiting a high basal Akt phosphorylation. The impaired synaptic vesicle exocytosis observed in neurons grown in the presence of leucine was improved by AICAR, which is a very novel approach to improve the synaptic vesicle exocytosis but the exact mechanism behind this still need to be investigated. To summarize, the above studies indicate that there is a strong connection between mitochondrial function, insulin resistance and synapse formation, which forms the basis of future work so as to investigate this link with more attention.

REFERENCES

1. Chang, L., Chiang, S.H., and Saltiel, A.R. (2004) Insulin signaling and the regulation of glucose transport. *Mol.Med.* **10,** 65-71

2. Saltiel, A.R., and Kahn, C.R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature.* **414,** 799-806

3. Lizcano, J.M. (2002) The insulin signalling pathway. *Current biology.* **12,** 236

4. Pessin, J.E., and Saltiel, A.R. (2000) Signaling pathways in insulin action: molecular targets of insulin resistance. *J.Clin.Invest.* **106,** 165-170

5. Bouché, C., Serdy, S., Kahn, C.R., and Goldfine, A.B. (2004) The cellular fate of glucose and its relevance in type 2 diabetes. *Endocr.Rev.* **25,** 807-830

6. DeFronzo, R.A. (2010) Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia.* **53,** 1270

7. Prikoszovich, T., Winzer, C., Schmid, A.I., Szendroedi, J., Chmelik, M., Pacini, G., Krššák, M., Moser, E., Funahashi, T., and Waldhäusl, W. (2011) Body and liver fat mass rather than muscle mitochondrial function determine glucose metabolism in women with a history of gestational diabetes mellitus. *Diabetes Care.* **34,** 430

8. Savage, D.B., Petersen, K.F., and Shulman, G.I. (2007) Disordered lipid metabolism and the pathogenesis of insulin resistance. *Physiol.Rev.* **87,** 507-520

9. Sykiotis, G.P., and Papavassiliou, A.G. (2001) Serine phosphorylation of insulin receptor substrate-1: a novel target for the reversal of insulin resistance. *Mol.Endocrinol.* **15,** 1864-1869

10. Dietze, D., Koenen, M., Rohrig, K., Horikoshi, H., Hauner, H., and Eckel, J. (2002) Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes.* **51,** 2369-2376

11. Aguirre, V. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J.Biol.Chem.* **277,** 1531

12. Hotamisligil, G.S. (1996) IRS-1-Mediated Inhibition of Insulin Receptor Tyrosine Kinase Activity in TNFα-and Obesity-Induced Insulin Resistance. *Science.* 665

13. Hay, N. (2011) Akt isoforms and glucose homeostasis–the leptin connection. *Trends in endocrinology and metabolism.* **22,** 66

14. Sesti, G. (2006) Pathophysiology of insulin resistance. *Best Practice & Research Clinical Endocrinology & Metabolism.* **20,** 665-679

15. Kim, B., McLean, L.L., Philip, S.S., and Feldman, E.L. (2011) Hyperinsulinemia induces insulin resistance in dorsal root ganglion neurons. *Endocrinology.* **152,** 3638-3647

16. Grote, C.W., Morris, J.K., Ryals, J.M., Geiger, P.C., and Wright, D.E. (2011) Insulin receptor substrate 2 expression and involvement in neuronal insulin resistance in diabetic neuropathy. *Exp.Diabetes Res.* **2011,** 212571

17. Bomfim, T.R., Forny-Germano, L., Sathler, L.B., Brito-Moreira, J., Houzel, J.C., Decker, H., Silverman, M.A., Kazi, H., Melo, H.M., McClean, P.L., Holscher, C., Arnold, S.E., Talbot, K., Klein, W.L., Munoz, D.P., Ferreira, S.T., and De Felice, F.G. (2012) An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Abeta oligomers. *J.Clin.Invest.* **122,** 1339-1353

18. Kraegen, E., Clark, P., Jenkins, A., Daley, E., Chisholm, D., and Storlien, L. (1991) Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes.* **40,** 1397

19. Talbot, K., Wang, H., Kazi, H., Han, L., Bakshi, K., Stucky, A., Fuino, R., Kawaguchi, K., Samoyedny, A., Wilson, R., Arvanitakis, Z., Schneider, J., Wolf, B., Bennett, D., Trojanowski, J., and Arnold, S. (2012) Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J.Clin.Invest.* **122,** 1316-1338

20. Griffin, M.E. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes.* **48,** 1270

21. Arner, P. (2002) Insulin resistance in type 2 diabetes: role of fatty acids. *Diabetes Metab.Res.Rev.* **18 Suppl 2,** S5-9

22. Boden, G. (2011) Obesity, insulin resistance and free fatty acids. *Curr.Opin.Endocrinol.Diabetes Obes.* **18,** 139-143

23. Shanik, M.H., Xu, Y., Skrha, J., Dankner, R., Zick, Y., and Roth, J. (2008) Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse?. *Diabetes Care.* **31 Suppl 2,** S262-8

24. Wei, Y., Sowers, J.R., Nistala, R., Gong, H., Uptergrove, G.M., Clark, S.E., Morris, E.M., Szary, N., Manrique, C., and Stump, C.S. (2006) Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells. *J.Biol.Chem.* **281,** 35137-35146

25. Ohta, Y., Kinugawa, S., Matsushima, S., Ono, T., Sobirin, M.A., Inoue, N., Yokota, T., Hirabayashi, K., and Tsutsui, H. (2011) Oxidative stress impairs insulin signal in skeletal muscle and causes insulin resistance in postinfarct heart failure. *Am.J.Physiol.Heart Circ.Physiol.* **300,** H1637-44

26. Liu, L.S. (1998) Tumor necrosis factor-alpha acutely inhibits insulin signaling in human adipocytes: implication of the p80 tumor necrosis factor receptor. *Diabetes.* **47,** 515

27. Saha, A.K., Xu, X.J., Lawson, E., Deoliveira, R., Brandon, A.E., Kraegen, E.W., and Ruderman, N.B. (2010) Downregulation of AMPK accompanies leucine- and glucose-induced increases in protein synthesis and insulin resistance in rat skeletal muscle. *Diabetes.* **59,** 2426-2434

28. Niu, Y.M., Yuan, H., Zhang, N., and Fu, L. (2010) The relationship between mTor/S6K1 signaling pathway and insulin resistance and the study of aerobic exercise on this pathway. *Zhongguo Ying Yong Sheng Li Xue Za Zhi.* **26,** 399-403

29. Lee, D.F., Kuo, H.P., Chen, C.T., Wei, Y., Chou, C.K., Hung, J.Y., Yen, C.J., and Hung, M.C. (2008) IKKbeta suppression of TSC1 function links the mTOR pathway with insulin resistance. *Int.J.Mol.Med.* **22,** 633-638

30. Balage, M., Dupont, J., Mothe-Satney, I., Tesseraud, S., Mosoni, L., and Dardevet, D. (2011) Leucine supplementation in rats induced a delay in muscle IR/PI3K signaling pathway associated with overall impaired glucose tolerance. *J.Nutr.Biochem.* **22,** 219-226

31. Crandall, E.A. (1983) Effect of experimental diabetes on the levels of aromatic and branched-chain amino acids in rat blood and brain. *Diabetes.* **32,** 222

32. Suhre, K., Meisinger, C., Doring, A., Altmaier, E., Belcredi, P., Gieger, C., Chang, D., Milburn, M.V., Gall, W.E., Weinberger, K.M., Mewes, H.W., Hrabe de Angelis, M., Wichmann, H.E., Kronenberg, F., Adamski, J., and Illig, T. (2010) Metabolic footprint of diabetes: a multiplatform metabolomics study in an epidemiological setting. *PLoS One.* **5,** e13953

33. Havrankova, J., Roth, J., and BROWNSTEIN, M. (1978) Insulin receptors are widely distributed in the central nervous system of the rat. *Nature,* **272***,* 827-829

34. Havrankova, J., Roth, J., and Brownstein, M.J. (1979) Concentrations of insulin and of insulin receptors in the brain are independent of peripheral insulin levels: studies of obese and streptozotocin-treated rodents. *J.Clin.Invest.* **64,** 636

35. Wei, L.T., Matsumoto, H., and Rhoads, D.E. (1990) Release of immunoreactive insulin from rat brain synaptosomes under depolarizing conditions. *J.Neurochem.* **54,** 1661-1665

36. Kar, S., Chabot, J.G., and Quirion, R. (1993) Quantitative autoradiographic localization of [125I]insulin-like growth factor I, [125I]insulin-like growth factor II, and [125I]insulin receptor binding sites in developing and adult rat brain. *J.Comp.Neurol.* **333,** 375-397

37. Unger, J. (1989) Distribution of insulin receptor-like immunoreactivity in the rat forebrain. *Neuroscience.* **31,** 143

38. Schwartz, M.W. (1991) Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. *J.Clin.Invest.* **88,** 1272

39. Banks, W.A. (2012) Insulin in the brain: There and back again. *Pharmacology therapeutics.*

40. Frank, H.J.L. (1981) A direct in vitro demonstration of insulin binding to isolated brain microvessels. *Diabetes.* **30,** 757

41. Frank, H.J.L. (1985) Enhanced insulin binding to blood-brain barrier in vivo and to brain microvessels in vitro in newborn rabbits. *Diabetes.* **34,** 728

42. DEBONS, A.F. (1970) Direct action of insulin on the hypothalamic satiety center. *Am.J.Physiol.* **219,** 938

43. Brief, D.J., and Davis, J.D. (1984) Reduction of food intake and body weight by chronic intraventricular insulin infusion. *Brain Res.Bull.* **12,** 571-575

44. Stockhorst, U., de Fries, D., Steingrueber, H.J., and Scherbaum, W.A. (2004) Insulin and the CNS: effects on food intake, memory, and endocrine parameters and the role of intranasal insulin administration in humans. *Physiol.Behav.* **83,** 47-54

45. Gerozissis, K. (2003) Brain insulin: regulation, mechanisms of action and functions. *Cell.Mol.Neurobiol.* **23,** 1-25

46. Park, C.R. (2001) Cognitive effects of insulin in the central nervous system. *Neurosci.Biobehav.Rev.* **25,** 311-323

47. Park, C.R., Seeley, R.J., Craft, S., and Woods, S.C. (2000) Intracerebroventricular insulin enhances memory in a passive-avoidance task. *Physiol.Behav.* **68,** 509-514

48. Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M.J., and Alkon, D.L. (1999) Brain insulin receptors and spatial memory. *J.Biol.Chem.* **274,** 34893

49. Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M.J., and Alkon, D.L. (1999) Brain insulin receptors and spatial memory. Correlated changes in gene

expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. *J.Biol.Chem.* **274,** 34893-34902

50. Huppert, F.A. (1979) Normal and abnormal forgetting in organic amnesia: effect of locus of lesion. *Cortex.* **15,** 385

51. de Castro, J.M. (1976) Insulin pretreatment facilitates recovery after dorsal hippocampal lesions. *Physiology behavior.* **16,** 517

52. Zhao, W.Q. (2004) Insulin and the insulin receptor in experimental models of learning and memory. *Eur.J.Pharmacol.* **490,** 71

53. Hoyer, S. (1998) Is sporadic Alzheimer disease the brain type of non-insulin dependent diabetes mellitus? A challenging hypothesis. *J.Neural Transm.* **105,** 415

54. Neumann, K.F., Rojo, L., Navarrete, L.P., Farias, G., Reyes, P., and Maccioni, R.B. (2008) Insulin resistance and Alzheimer's disease: molecular links & clinical implications. *Curr.Alzheimer Res.* **5,** 438-447

55. Craft, S., Peskind, E., Schwartz, M.W., Schellenberg, G.D., Raskind, M., and Porte, D.,Jr (1998) Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology.* **50,** 164-168

56. Ohara, T., Doi, Y., Ninomiya, T., Hirakawa, Y., Hata, J., Iwaki, T., Kanba, S., and Kiyohara, Y. (2011) Glucose tolerance status and risk of dementia in the community: the Hisayama study. *Neurology.* **77,** 1126-1134

57. Goldstein, B.J. (2002) Insulin resistance as the core defect in type 2 diabetes mellitus. *Am.J.Cardiol.* **90,** 3G-10G

58. Hoyer, S. (2002) The aging brain. Changes in the neuronal insulin/insulin receptor signal transduction cascade trigger late-onset sporadic Alzheimer disease (SAD). A mini-review. *J.Neural Transm.* **109,** 991-1002

59. Hoyer, S. (1998) Is sporadic Alzheimer disease the brain type of non-insulin dependent diabetes mellitus? A challenging hypothesis. *J.Neural Transm.* **105,** 415-422

60. Craft, S. (1999) Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose. *Arch.Gen.Psychiatry.* **56,** 1135

61. Zhao, W.Q. (2004) Insulin and the insulin receptor in experimental models of learning and memory. *Eur.J.Pharmacol.* **490,** 71

62. Cholerton, B., Baker, L., and Craft, S. (2011) Insulin resistance and pathological brain ageing. *Diabetic Med.*

63. De Felice, F.G., Vieira, M.N.N., Bomfim, T.R., Decker, H., Velasco, P.T., Lambert, M.P., Viola, K.L., Zhao, W.Q., Ferreira, S.T., and Klein, W.L. (2009) Protection of synapses against Alzheimer's-linked toxins: Insulin signaling prevents the pathogenic binding of Aβ oligomers. *Proceedings of the National Academy of Sciences.* **106,** 1971

64. Schoch, S. (2006) Molecular organization of the presynaptic active zone. *Cell Tissue Res.* **326,** 379

65. Abraham, C. (2011) Synaptogyrin-dependent modulation of synaptic neurotransmission in Caenorhabditis elegans. *Neuroscience.* **190,** 75

66. Sutton, R.B. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature.* **395,** 347

67. Becherer, U. (2006) Vesicle pools, docking, priming, and release. *Cell Tissue Res.* **326,** 393

68. Südhof, T.C. (2004) The synaptic vesicle cycle. *Annu.Rev.Neurosci.* **27,** 509

69. Nishiki, T. (2004) Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. *The Journal of neuroscience.* **24,** 6127

70. Wu, X.S. (2009) Ca2 and calmodulin initiate all forms of endocytosis during depolarization at a nerve terminal. *Nat.Neurosci.* **12,** 1003

71. Jung, N. (2007) Clathrin Mediated Endocytosis at Synapses. *Traffic.* **8,** 1129

72. Royle, S.J. (2004) Endocytosis at the synaptic terminal. *J.Physiol.(Lond.).* **553,** 345

73. Sudhof, T.C. (2004) The synaptic vesicle cycle. *Annu.Rev.Neurosci.* **27,** 509

74. Fernandez-Chacon, R., and Sudhof, T.C. (1999) Genetics of synaptic vesicle function: toward the complete functional anatomy of an organelle. *Annu.Rev.Physiol.* **61,** 753-776

75. Brywe, K.G. (2005) IGFI neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3β?. *Eur.J.Neurosci.* **21,** 1489

76. Liu, L. (1995) Insulin potentiates N-methyl-d-aspartate receptor activity in Xenopus oocytes and rat hippocampus. *Neurosci.Lett.* **192,** 5

77. Liao, G.Y. (2002) Insulin modulation of cloned mouse NMDA receptor currents in Xenopus oocytes. *J.Neurochem.* **73,** 1510

78. Christie, J.M. (2001) Insulin causes a transient tyrosine phosphorylation of NR2A and NR2B NMDA receptor subunits in rat hippocampus. *J.Neurochem.* **72,** 1523

79. Paulsen, O., and Moser, E.I. (1998) A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. *Trends Neurosci.* **21,** 273-278

80. Huang, C.C., Lee, C.C., and Hsu, K.S. (2010) The role of insulin receptor signaling in synaptic plasticity and cognitive function. *Chang Gung Med.J.* **33,** 115-125

81. Morino, K. (2006) Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. *Diabetes.* **55,** S9

82. Kelley, D.E., He, J., Menshikova, E.V., and Ritov, V.B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes.* **51,** 2944-2950

83. Petersen, K.F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D.L., DiPietro, L., Cline, G.W., and Shulman, G.I. (2003) Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science.* **300,** 1140

84. Sparks, L.M., Xie, H., Koza, R.A., Mynatt, R., Hulver, M.W., Bray, G.A., and Smith, S.R. (2005) A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes.* **54,** 1926

85. Bogacka, I. (2005) Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. *Diabetes.* **54,** 1392

86. Turner, N. (2008) Is mitochondrial dysfunction a cause of insulin resistance?. *Trends in endocrinology and metabolism.* **19,** 324

87. Hathorn, T., Snyder-Keller, A., and Messer, A. (2011) Nicotinamide improves motor deficits and upregulates PGC-1 [alpha] and BDNF gene expression in a mouse model of Huntington's disease. *Neurobiol.Dis.* **41,** 43-50

88. Steiner, J.L., Murphy, E.A., McClellan, J.L., Carmichael, M.D., and Davis, J.M. (2011) Exercise training increases mitochondrial biogenesis in the brain. *J.Appl.Physiol.* **111,** 1066-1071

89. Calkins, M.J. (2011) Impaired mitochondrial biogenesis, defective axonal transport of mitochondria, abnormal mitochondrial dynamics and synaptic degeneration in a mouse model of Alzheimer's disease. *Hum.Mol.Genet.* **20,** 4515

90. Nguyen, P.V., Marin, L., and Atwood, H.L. (1997) Synaptic physiology and mitochondrial function in crayfish tonic and phasic motor neurons. *J.Neurophysiol.* **78,** 281-294

91. Ly, C.V., and Verstreken, P. (2006) Mitochondria at the synapse. *Neuroscientist.* **12,** 291-299

92. Verstreken, P., Ly, C.V., Venken, K.J., Koh, T.W., Zhou, Y., and Bellen, H.J. (2005) Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. *Neuron.* **47,** 365-378

93. Kann, O., Kovacs, R., and Heinemann, U. (2003) Metabotropic receptor-mediated Ca2+ signaling elevates mitochondrial Ca2+ and stimulates oxidative metabolism in hippocampal slice cultures. *J.Neurophysiol.* **90,** 613-621

94. Garver, W.S. (2007) Characterization of liver disease and lipid metabolism in the Niemann-Pick C1 mouse. *J.Cell.Biochem.* **101,** 498

95. Liscum, L. (1987) Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. *J.Biol.Chem.* **262,** 17002

96. Karten, B. (2009) Mechanisms and consequences of impaired lipid trafficking in Niemann–Pick type C1-deficient mammalian cells. *Biochimica and biophysica acta.Molecular and cell biology of lipids.* **1791,** 659

97. Vance, J.E. (2006) Lipid imbalance in the neurological disorder, Niemann-Pick C disease. *FEBS Lett.* **580,** 5518

98. Higashi, Y. (1993) Cerebellar degeneration in the Niemann-Pick type C mouse. *Acta Neuropathol.* **85,** 175

99. Sokol, J. (1988) Type C Niemann-Pick disease. Lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol. *J.Biol.Chem.* **263,** 3411

100. Watanabe, Y. (1998) Increased levels of GM 2 ganglioside in fibroblasts from a patient with juvenile Niemann–Pick disease type C. *Brain development.* **20,** 95

101. te Vruchte, D. (2004) Accumulation of glycosphingolipids in Niemann-Pick C disease disrupts endosomal transport. *J.Biol.Chem.* **279,** 26167

102. Millat, G. (1999) Niemann-Pick C1 disease: the I1061T substitution is a frequent mutant allele in patients of Western European descent and correlates with a classic juvenile phenotype. *Am.J.Hum.Genet.* **65,** 1321

103. Higgins, M.E. (1999) Niemann–Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol.Genet.Metab.* **68,** 1

104. Kirchhoff, C. (1996) Molecular cloning and characterization of HE1, a major secretory protein of the human epididymis. *Biol.Reprod.* **54,** 847

105. Chang, T.Y., Reid, P.C., Sugii, S., Ohgami, N., Cruz, J.C., and Chang, C.C. (2005) Niemann-Pick type C disease and intracellular cholesterol trafficking. *J.Biol.Chem.* **280,** 20917-20920

106. Porter, F.D. (2010) Cholesterol oxidation products are sensitive and specific blood-based biomarkers for Niemann-Pick C1 disease. *Science translational medicine.* **2,** 56ra81

107. Bickel, P.E. (2002) Lipid rafts and insulin signaling. *Am.J.Physiol.Endocrinol.Metab.* **282,** E1-E10

108. VAINIO, S. (2005) Defective insulin receptor activation and altered lipid rafts in niemann-pick type c disease hepatocytes. *Biochem.J.* **391,** 465

109. Ong, Q.R. (2012) Impaired insulin signaling in an animal model of Niemann-Pick Type C disease. *Biochem.Biophys.Res.Commun.*

110. Hawes, C.M. (2010) Pre-synaptic defects of NPC1-deficient hippocampal neurons are not directly related to plasma membrane cholesterol. *J.Neurochem.* **114,** 311

111. Mailman, T., Hariharan, M., and Karten, B. (2011) Inhibition of neuronal cholesterol biosynthesis with lovastatin leads to impaired synaptic vesicle release even in the presence of lipoproteins or geranylgeraniol. *J.Neurochem.* **119,** 1002-1015

112. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227,** 680-685

113. Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J.Neurosci.Res.* **35,** 567-576

114. Grabrucker, A. (2009) Synaptogenesis of hippocampal neurons in primary cell culture. *Cell Tissue Res.* **338,** 333

115. Harata, N. (2001) Visualizing recycling synaptic vesicles in hippocampal neurons by FM 1-43 photoconversion. *Proc.Natl.Acad.Sci.U.S.A.* **98,** 12748

116. Gaffield, M.A., and Betz, W.J. (2006) Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. *Nat.Protoc.* **1,** 2916-2921

117. Samocha-Bonet, D., Campbell, L.V., Mori, T.A., Croft, K.D., Greenfield, J.R., Turner, N., and Heilbronn, L.K. (2012) Overfeeding reduces insulin sensitivity and increases oxidative stress, without altering markers of mitochondrial content and function in humans. *PLoS One.* **7,** e36320

118. Sheng, M. (2007) The postsynaptic architecture of excitatory synapses: a more quantitative view. *Annu.Rev.Biochem.* **76,** 823

119. Mahad, D.J. (2009) Mitochondrial changes within axons in multiple sclerosis. *Brain.* **132,** 1161

120. Pipatpiboon, N. (2012) PPARγ agonist improves neuronal insulin receptor function in hippocampus and brain mitochondria function in rats with insulin resistance induced by long term high-fat diets. *Endocrinology.* **153,** 329

121. Rong, J.X. (2007) Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet–fed mice and improved by rosiglitazone. *Diabetes.* **56,** 1751

122. Rong, J.X., Klein, J.L., Qiu, Y., Xie, M., Johnson, J.H., Waters, K.M., Zhang, V., Kashatus, J.A., Remlinger, K.S., Bing, N., Crosby, R.M., Jackson, T.K., Witherspoon, S.M., Moore, J.T., Ryan, T.E., Neill, S.D., and Strum, J.C. (2011) Rosiglitazone Induces Mitochondrial Biogenesis in Differentiated Murine 3T3-L1 and C3H/10T1/2 Adipocytes. *PPAR Res.* **2011,** 179454

123. Baskin, D.G., Figlewicz, D.P., Woods, S.C., Porte, D.,Jr, and Dorsa, D.M. (1987) Insulin in the brain. *Annu.Rev.Physiol.* **49,** 335-347

124. Yalow, R.S., and Eng, J. (1983) Insulin in the central nervous system. *Adv.Metab.Disord.* **10,** 341-354

125. Nishiki, T., and Augustine, G.J. (2004) Synaptotagmin I synchronizes transmitter release in mouse hippocampal neurons. *J.Neurosci.* **24,** 6127-6132

126. Manczak, M. (2012) Abnormal interaction of VDAC1 with amyloid beta and phosphorylated tau causes mitochondrial dysfunction in Alzheimer's disease. *Hum.Mol.Genet.*

127. Singh, B., Xu, Y., McLaughlin, T., Singh, V., Martinez, J.A., Krishnan, A., and Zochodne, D.W. (2012) Resistance to trophic neurite outgrowth of sensory neurons exposed to insulin. *J.Neurochem.* **121,** 263-276

128. Bree, A.J., Puente, E.C., Daphna-Iken, D., and Fisher, S.J. (2009) Diabetes increases brain damage caused by severe hypoglycemia. *Am.J.Physiol.Endocrinol.Metab.* **297,** E194-201

129. Liu, S., Okada, T., Assmann, A., Soto, J., Liew, C.W., Bugger, H., Shirihai, O.S., Abel, E.D., and Kulkarni, R.N. (2009) Insulin signaling regulates mitochondrial function in pancreatic beta-cells. *PLoS One.* **4,** e7983

130. Sheng, B., Wang, X., Su, B., Lee, H.G., Casadesus, G., Perry, G., and Zhu, X. (2012) Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. *J.Neurochem.* **120,** 419-429

131. Asmann, Y.W., Stump, C.S., Short, K.R., Coenen-Schimke, J.M., Guo, Z., Bigelow, M.L., and Nair, K.S. (2006) Skeletal muscle mitochondrial functions, mitochondrial DNA copy numbers, and gene transcript profiles in type 2 diabetic and nondiabetic subjects at equal levels of low or high insulin and euglycemia. *Diabetes.* **55,** 3309-3319

132. Landreth, G., Jiang, Q., Mandrekar, S., and Heneka, M. (2008) PPARgamma agonists as therapeutics for the treatment of Alzheimer's disease. *Neurotherapeutics.* **5,** 481-489

133. Spiegelman, B.M. (1998) PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes.* **47,** 507

134. Wang, Y., Fang, F., and Wong, C.W. (2010) Troglitazone is an estrogen-related receptor alpha and gamma inverse agonist. *Biochem.Pharmacol.* **80,** 80-85

135. Feldman, E.L., Sullivan, K.A., Kim, B., and Russell, J.W. (1997) Insulin-like growth factors regulate neuronal differentiation and survival. *Neurobiol.Dis.* **4,** 201-214

136. Yung, H.W., and Tolkovsky, A.M. (2003) Erasure of kinase phosphorylation in astrocytes during oxygen-glucose deprivation is controlled by ATP levels and activation of phosphatases. *J.Neurochem.* **86,** 1281-1288

137. Liu, H.Y., Hong, T., Wen, G.B., Han, J., Zuo, D., Liu, Z., and Cao, W. (2009) Increased basal level of Akt-dependent insulin signaling may be responsible for the development of insulin resistance. *Am.J.Physiol.Endocrinol.Metab.* **297,** E898-906

138. Hotamisligil, G.S. (1999) Mechanisms of TNF-alpha-induced insulin resistance. *Exp.Clin.Endocrinol.Diabetes.* **107,** 119-125

139. Tremblay, F., and Marette, A. (2001) Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J.Biol.Chem.* **276,** 38052-38060

140. Xiao, F., Huang, Z., Li, H., Yu, J., Wang, C., Chen, S., Meng, Q., Cheng, Y., Gao, X., Li, J., Liu, Y., and Guo, F. (2011) Leucine deprivation increases hepatic insulin sensitivity via GCN2/mTOR/S6K1 and AMPK pathways. *Diabetes.* **60,** 746-756

141. Xu, J., Pang, Z.P., Shin, O.H., and Sudhof, T.C. (2009) Synaptotagmin-1 functions as a Ca2+ sensor for spontaneous release. *Nat.Neurosci.* **12,** 759-766

142. Shoshan-Barmatz, V., De Pinto, V., Zweckstetter, M., Raviv, Z., Keinan, N., and Arbel, N. (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol.Aspects Med.* **31,** 227-285

143. Amaral, A.U., Leipnitz, G., Fernandes, C.G., Seminotti, B., Schuck, P.F., and Wajner, M. (2010) Alpha-ketoisocaproic acid and leucine provoke mitochondrial bioenergetic dysfunction in rat brain. *Brain Res.* **1324,** 75-84

144. Ayasolla, K.R., Singh, A.K., and Singh, I. (2005) 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR) attenuates the expression of LPS- and Abeta peptide-induced inflammatory mediators in astroglia. *J.Neuroinflammation.* **2,** 21