2017

INSULIN ACTIONS ON HIPPOCAMPAL NEURONS

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Digital Object Identifier: https://doi.org/10.13023/ETD.2017.366

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Shaniya Maimaiti, Student
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INSULIN ACTIONS ON HIPPOCAMPAL NEURONS

_________________________________________

DISSERTATION

_________________________________________

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Shaniya Maimaiti

Lexington, Kentucky

Director: Dr. Olivier Thibault, Professor of Pharmacology and Nutritional Sciences
Lexington, Kentucky 2017

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ABSTRACT OF DISSERTATION

INSULIN ACTIONS ON HIPPOCAMPAL NEURONS

Aging is the main risk factor for cognitive decline. The hippocampus, a brain region critical for learning and memory formation, is especially vulnerable to normal and pathological age-related cognitive decline. Dysregulation of both insulin and intracellular Ca2+ signaling appear to coexist and their compromised actions may synergistically contribute to neuronal dysfunction with aging. This dissertation focused on the interaction between insulin, Ca2+ dysregulation, and cognition in hippocampal neurons by examining the contributions of insulin to Ca2+ signaling events that influence memory formation. I tested the hypothesis that insulin would increase cognition in aged animals by altering Ca2+-dependent physiological mechanisms involved in learning. The possible effects of insulin on learning and memory in young and aged rats were studied. In addition, the effects of insulin on the Ca2+-dependent afterhyperpolarization in CA1 pyramidal hippocampal neurons from young and aged animals were compared. Further, primary hippocampal cultures were used to examine the possible effects of insulin on voltage-gated Ca2+ channel activity and Ca2+-induced Ca2+-release; mechanisms known to influence the AHP.

We found that intranasal insulin improved memory in aged F344 rats. Young and aged F344 rats were treated with Humalog®, a short-acting insulin analog, or Levemir®, a long-acting insulin analog. The aged rats performed similar to young rats in the Morris Water Maze, a hippocampal dependent spatial learning and memory task. Electrophysiological recordings from CA1 hippocampal neurons revealed that insulin reduced the age-related increase in the Ca2+-dependent afterhyperpolarization, a prominent biomarker of brain aging that is associated with cognitive decline. Patch clamping recording from hippocampal cultured neurons showed that insulin reduced Ca2+ channel currents. Intracellular Ca2+ levels were also monitored using Fura-2 in response to cellular depolarization. Results indicated that a reduction in Ca2+-induced Ca2+-release from intracellular stores occurred in the presence of insulin.

These results suggest that increasing brain insulin levels in aged rats may have improved memory by reducing the AHP and intracellular Ca2+ concentrations. This study indicates a possible mechanism responsible for the beneficial effects
of intranasal insulin on cognitive function absorbed in selective Alzheimer’s patients. Thus, insulin therapy may reduce or prevent age-related compromises to Ca2+ regulatory pathways typically associated with cognitive decline.

KEY WORDS: Insulin, Aging, Cognition, Intracellular Ca2+, Ca2+ imaging, Whole-cell patch clamping
INSULIN ACTIONS ON HIPPOCAMPAL NEURONS

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08/04/2017
I dedicate this thesis to my daughter. Alina you are my pride and joy.
ACKNOWLEDGEMENTS

Foremost, I would like to thank my extraordinary mentor Professor Olivier Thibault for his patience, motivation, enthusiasm, and wide range of knowledge. He is the funniest advisor and a smart, experienced scientist. I thank him for always focusing on my best interest and working closely with me on my research projects, for giving me the guidance, insight, and skills I needed. Thank Dr. Thibault for guiding me through all the challenges I have faced in research and personal life. I learned from Dr. Thibault 1) to do good science 2) to be a good team player 3) to fix things 4) to help others 5) give back to society by becoming involved with local community services. Dr. Thibault’s excellent mentorship was the key for all my learning progress and accomplishments in graduate school.

I would also like to thank Professor Bret N. Smith. He served as my primary advisor before I started in the graduate program. Dr. Smith introduced me to neuroscience when I was undergrad, and without his encouragement, enthusiasm, and support I would not be where I am today. I will always be grateful for him for believing in me and seeing me as a potential neuroscientist.

I would also like to thank Dr. Nada M Porter, Chair of Pharmacology and Nutritional Sciences, for encouraging me and advising me during one of the most difficult times of my life. I never forgot her and I hope to be as kind, lively, enthusiastic, and energetic as she is. I am also very grateful for her direction
and support on my research projects.

I would also like to thank Dr. Robert W. Hadley for teaching me in graduate courses and serving as one of my committee members. I am grateful for his immense knowledge, guidance, and constructive feedback on my graduate training. I also very thankful for his fun jokes during our meetings which made them less intense and more enjoyable.

I would also like to thank Dr. Alexandre Martin for being my outside examiner.

I would also like to thank Dr. Rolf J. Craven, Director of Graduate Studies for the Department of Pharmacology and Nutritional Sciences, for keeping his office door open for me and keeping me on track.

I would also like to thank Dr. Donna Weber, former Director of Graduate Studies for the Department of Pharmacology and Nutritional Sciences, for providing so much support and guidance. She always goes the extra mile and beyond to help students.

I would also like to thank Veronique Thibault for being there for me and guiding me thought the hardest time in my life. Importantly, she helped me bring my daughter Alina into my life. Without her help and encouragement, I couldn’t put myself together and move forward. When nothing worked in my life and I felt lost, she guided me through the darkness and “pumped me up”. I thank her for being someone I can trust and depend on. This dissertation could not be completed without her help and support.
I would also like to thank Dr. Lawrence Brewer. I truly appreciate the mentorship and friendship he gave me all these years. Whenever things did not work out well in the lab or in my personal life, he was the first person I talk to. Because he always said right thing and knew how to pick me up. He didn’t fail me once. Importantly, whenever I had questions related to research or life, he always has the answers for me, and I will always be grateful for his help and guidance.

I would also like to thank Dr. Chris Gant for teaching me the techniques, knowledge in the field, and for helping me troubleshoot any problems I had in the lab. Also, I thank him for sharing with me his hard times as a graduate student to ease my frustrations.

I would also like to thank past and present members of the Thibault Lab (Dr. Tristano Pancani, Chris DeMoll, Katie L Anderson, Ben Rauh, Hilaree N Frazier, Sarah Sternbach, Adam O Ghoweri, and Grant A Fox) for providing me help and friendship.

I also thank department for creating a great atmosphere for research and graduate training.

Thank to Miss Kelley M Secrest and Miss Deborah J Turner, for helping me through various problems related to my training as well as their guidance and friendship throughout graduate school.

I would also like to thank my friends, who made these years so much exciting and fun, for your friendship through both good and bad times.
I would also like to thank Miss Toni and Miss Alison, for taking great care of Alina while I have completed my education.

I would also like to thank my parents for encouraging me to pursue my dream. Also, I am grateful to them for giving me everything I needed in life. I am also grateful for my in-laws for taking care of Alina while I worked and studied.

I like to thank my husband and daughter for nurturing me and pushing me hard to graduate as soon as possible. I deeply appreciate their comfort and love.
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Chapter 1 Thesis Overview and Significance

Aging, an inexorable natural process, often presents many challenges ranging from ambulatory to neuronal function. One of these challenges, age-related cognitive decline has received significant public attention because of its profound social and economic impact. A well-recognized mechanism contributing to age-related cognitive decline involves dysregulation of intracellular calcium. Although Insulin has been recognized as the key hormone regulating cellular glucose metabolism, its downstream effects on learning and memory have only recently been found. Some evidence suggests that insulin undergoes age-related changes that ultimately influence calcium regulatory mechanisms in various brain regions, including the hippocampus, an area of the brain closely related to learning and memory. Thus, the negative impact of brain aging on cognitive function may be due to impaired insulin signaling and intracellular calcium homeostasis. However, the link between insulin and intracellular calcium in the context of cognitive decline with aging is not understood. This dissertation focused on the effects of insulin on memory and its underlying neuronal mechanisms. I specifically focused on studying 1) the effects of insulin treatment on learning and memory in aged animals as well as 2) the effects of insulin on intracellular calcium regulation. Understanding the links between insulin and neuronal mechanisms involving hippocampal calcium processes may help develop therapeutic strategies to decrease complications associated with age-related cognitive decline.
1.1 Brain Aging and cognition

Aging leads to a function decline of many organs, including the brain. Age-related changes in the brain such as reduced insulin signaling, reduced neuronal glucose uptake, increased glucocorticoid levels, and increased intracellular calcium concentration may give rise to pathological brain aging. One of the hallmarks of brain aging is cognitive decline [1, 2]. Many factors could influence the age-related decline of cognitive function, including dysfunctions of circadian clock [3], and oxidative stress [4-9]. Growing evidence supports the concept that brain insulin and calcium may play an important role in the mechanisms behind memory decline in advanced age and Alzheimer’s disease (AD) [10-12]. At the cellular level, brain insulin deficiency and insulin resistance may be one of the fundamental changes associated with altered memory function during pathological aging [13-20]. Brain insulin signaling disruption has been closely associated with cognitive decline and AD [14, 21-25]. At the receptor level, the amount of insulin receptors and their functions are decreased in aging and AD animal models [26-28]. At the clinical level, studies suggest that memory improved in AD patients are due to changes in their insulin levels [29]. In aged animals, it has also been shown that intranasal delivery of insulin to the central nervous system (CNS) positively correlates with increased brain cognitive function [30]. The intranasal route of insulin administration increases insulin acutely in the brain without risk of peripheral hypoglycemia. Importantly, it significantly enhances the memory in both aged and AD patients [31-35].
Although intranasal insulin enhances memory in AD patients, varying outcomes arise with its clinical setting usage. Men and women with AD respond differently to intranasal insulin dosage [36]. In addition, some clinical studies reported that patients carrying at least one copy of the Apolipoprotein E4 (the strongest genetic risk factor for late onset Alzheimer’s disease) did not benefit from intranasal insulin treatment [37]. Therefore, gender and ApoE status in AD patients increase outcome variability in intranasal insulin treatment. These varying outcomes highlighted the need to understand the underlying neuronal mechanism the effects of insulin on cognitive function with aging.

1.2 Insulin

Identifying the link between the pancreas and diabetes by Von Morning and Minkowski in 1890 opened the door for the discovery of insulin as a therapy. This life-sustaining hormone saved an enormous number of lives since its discovery. In the early twentieth century, Sharpey-Schafer hypothesized that pancreas secretes a hormone that controls glucose levels [38]. In 192, this hypothesis was confirmed and a novel protocol to purify insulin was developed. In 1922, insulin therapy was successful on a type 1 diabetic patient. Since the introduction of human insulin in 1982 and insulin analogs in 1996, insulin therapy become the standard treatment options for diabetic patients [39, 40] by mimicking physiological insulin secretion in the body to regulate blood glucose levels. After almost 100 years of research, it is clear that Insulin is a natural peptide hormone secreted by the pancreatic β-cell in
response to elevated blood glucose concentration, and that it plays an indispensable role in maintaining blood glucose concentration and regulating metabolism [41]. In insulin therapy, to enhance the insulin’s effects and reduce the number of injection different routes of insulin delivery, as well as different insulin formulations were designed by combining insulin with zinc and/or basic proteins protamine [42].

1.3 Zinc

Zinc is an essential ion involved in many physiological processes ranging from cell proliferation to neuronal function. Zinc plays a critical role in regulating insulin synthesis, storage, and secretion in the pancreatic beta cells [43-45]. Zinc combined with insulin and induces proinsulin hexamerization, also co-releases with insulin from the beta cells [46]. It is well documented that zinc, pancreatic beta cell, and diabetes have a strong cause and effect relationship. Zinc is highly abundant in the pancreatic islet and zinc deficiency has been associated with diabetes [47]. High levels of zinc can be toxic to the cells, specifically to the neurons [48, 49]. Zinc present in the brain with its highest concentration in the olfactory bulb, hippocampus, hypothalamus, and cortex. Increased zinc stimulates synaptic events via AMPA receptors and improves synaptic summation [50] and influences LTP induction and memory [51].

1.4 Insulin formulations and alternative routes for insulin administration
Insulin formulations (Table 1.1), also different routes of insulin delivery (e.g., nasal, pulmonary inhaled, intravenous insulin infusion, insulin pumps) were developed [52, 53]. Currently, insulin cannot take orally because it will break down in stomach before it regulates glucose levels. Earlier research has focused on understanding the molecular mechanism through which insulin regulates glucose to uncovered insulin’s potential effects on memory enhancement. Although insulin is effective and widely used, researchers continue to study the molecular mechanisms by which insulin regulates glucose uptake in fat, muscle, and neuronal cells to obtain even more benefits from this life-sustaining hormone.
Table 1. Insulin Types by Brand Name, Generic Name, Onset, Peak, and Duration of Action.

<table>
<thead>
<tr>
<th>Type of Insulin</th>
<th>Brand Name</th>
<th>Generic Name</th>
<th>Onset</th>
<th>Peak</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid-Acting</td>
<td>_NovoLog</td>
<td>_Insulin aspart</td>
<td>15 minutes</td>
<td>30 to 90 minutes</td>
<td>3 to 5 hours</td>
</tr>
<tr>
<td></td>
<td>_Apidra</td>
<td>_Insulin glulisine</td>
<td>15 minutes</td>
<td>30 to 90 minutes</td>
<td>3 to 5 hours</td>
</tr>
<tr>
<td></td>
<td>_Humalog</td>
<td>_Insulin lispro</td>
<td>15 minutes</td>
<td>30 to 90 minutes</td>
<td>3 to 5 hours</td>
</tr>
<tr>
<td>Short-Acting</td>
<td>_Humulin R</td>
<td>_Insulin (R)</td>
<td>30 to 60 minutes</td>
<td>2 to 4 hours</td>
<td>5 to 8 hours</td>
</tr>
<tr>
<td></td>
<td>_Novolin R</td>
<td>_Insulin lispro</td>
<td>1 to 3 hours</td>
<td>8 hours</td>
<td>12 to 16 hours</td>
</tr>
<tr>
<td>Intermediate-Acting</td>
<td>_Humulin N</td>
<td>_Insulin NPH (N)</td>
<td>1 hour</td>
<td>Peakless</td>
<td>20 to 26 hours</td>
</tr>
<tr>
<td></td>
<td>_Novolin N</td>
<td>_Insulin determir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>_Novolin N</td>
<td>_Insulin glargine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long-Acting</td>
<td>_Levemir</td>
<td>_Insulin detemir</td>
<td>1 hour</td>
<td>Peakless</td>
<td>20 to 26 hours</td>
</tr>
<tr>
<td></td>
<td>_Lantus</td>
<td>_Insulin determir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-mixed NPH (Intermediate-acting) and regular (short-acting)</td>
<td>_Humulin 70/30, _Novolin 70/30</td>
<td>_70% NPH and 30% regular</td>
<td>30 to 60 minutes</td>
<td>Varies</td>
<td>10 to 16 hours</td>
</tr>
<tr>
<td></td>
<td>_Humulin 50/50</td>
<td>_50% NPH regular</td>
<td>30 to 60 minutes</td>
<td>Varies</td>
<td>10 to 16 hours</td>
</tr>
<tr>
<td>Pre-mixed insulin lispro protamine suspension (Intermediate-acting) and insulin lispro (rapid-acting)</td>
<td>_Humalog Mix 75/25</td>
<td>_75% insulin lispro protamine and 25% insulin lispro</td>
<td>10 to 15 minutes</td>
<td>Varies</td>
<td>10 to 16 hours</td>
</tr>
<tr>
<td></td>
<td>_Humalog Mix 50/50</td>
<td>_50% insulin lispro protamine and 50% insulin lispro</td>
<td>10 to 15 minutes</td>
<td>Varies</td>
<td>10 to 16 hours</td>
</tr>
<tr>
<td>Pre-mixed insulin aspart protamine suspension (Intermediate-acting) and insulin aspart (rapid-acting)</td>
<td>_NovoLog Mix 70/30</td>
<td>_70% insulin aspart protamine and 30% aspart</td>
<td>5 to 15 minutes</td>
<td>Varies</td>
<td>10 to 16 hours</td>
</tr>
</tbody>
</table>
1.5 Insulin signaling in the periphery

Insulin plays a vital role in maintaining blood glucose levels in the periphery. Insulin in the muscle and fat tissues promote glucose uptake. Insulin stimulates glucose storage, and inhibits glucose secretion in the liver [41, 54-61].

Insulin regulates glucose uptake in fat and muscle cells by inducing translocation of glucose transporters to the plasma membrane [62-68] by PI3K/AKT pathway [69]. Insulin also regulates glucose concentration via suppression of hepatic glucose production (HGP) [61, 70, 71]. Previous studies suggest that insulin regulates HGP by binding to the insulin receptor and activating serine/threonine kinases AKT [72, 73], which further inhibits Foxo1 phosphorylation [74-76]. Therefore, insulin prevents large alteration in blood glucose levels during the day by enhancing glucose metabolism in the cells and suppressing hepatic glucose production.
In healthy individuals, blood glucose levels are tightly controlled by insulin, a natural hormone produced by the pancreatic beta cells in response to high blood glucose levels. In fat tissues, insulin increases triglyceride synthesis/storage and decreases triglyceride breakdown. In the liver, insulin increases glycogen deposition and glucose use while decreasing glycogenolysis and gluconeogenesis. In muscle cells, insulin increases glucose uptake and protein synthesis while decreasing protein degradation.
Insulin promotes glucose uptake by cells through four major glucose transporter isoforms are widely distributed throughout the body. The glucose transporter 4 (Glut-4) is highly expressed in fat and muscle cells [77]; Glut-1 and Glut-2 are expressed in the endothelial cells lining the blood vessels and liver, intestine, kidney, pancreatic cells; and Glut-3 is expressed in the neurons [78]. When insulin binds to the insulin receptors on the surface of target cells, it promotes translocation of glucose transporters to the plasma membrane, which moves glucose into the cell. Once glucose move inside the cell, it delivers energy to it. Altered insulin levels, insulin receptors and/ or glucose transporters all can lead to obesity, insulin resistance, and diabetes [79-83]. Although insulin is recognized as glucose regulating hormone, it is increasingly clear now that insulin also plays a critical role in the regulation of neuroplasticity, neuromodulation, neurotrophism, neuronal growth and survival [84-92]. Therefore, insulin is important not only for regulating sugar, but also for learning and memory. To initiate insulin signaling, insulin has to bind to the insulin receptor and activates the insulin receptor tyrosine kinase.

Together with human insulin receptor characterizations, studies of insulin receptor tyrosine kinase activity have enhanced our understanding of the mechanisms of insulin action in various organs. Indeed, insulin acts on the fat, muscle, liver, and brain through the insulin receptor substrate pathways.
Figure 1.2 Insulin signaling pathways and translocation of glucose transporter (GLUT) to the plasma membrane by insulin.

To initiate insulin signaling, insulin must bind to insulin receptors on the plasma membrane. Insulin receptors contain two α subunits outside the cell and two β subunits inside the cell. α subunits have an insulin binding site, and β subunits have a tyrosine kinase phosphorylation site. Once insulin binds to insulin receptors, it triggers autophosphorylation of β subunits of insulin receptors, which leads to phosphorylation of insulin receptor substrate (IRS), which further phosphorylates PI3K and AKT. Next, downstream signaling pathways promote translocation of glucose transporters (GLUT) to the plasma membrane, which brings glucose inside the cell modified from [93].
1.6 Insulin signaling in the brain

While insulin plays an essential role in the brain, brain insulin itself is important for regulating insulin secretion from the pancreatic beta cells [94]. Important actions of insulin in the brain are now gradually becoming clear, even through the brain has been considered an insulin-independent organ for several decades. Insulin and insulin-like growth factor (IGF1-2) bind to insulin receptor and IGF-1R and initiate insulin signaling. Insulin receptor and IGF-1R co-express in the brain and share the intracellular signaling pathways; however, each of them has a unique function. This view has been further tested using neuron-specific insulin receptor and IGF-1R knockout mice. Deleting insulin receptors in the brain leads to mild obesity and insulin resistance without influencing brain size and development [95]. However, deleting IGF-1R affects brain size and development and contributes to behavior changes [96]. All the insulin receptor substrates (IRS1-4) present in the brain, and IRS2 play a vital role in the brain since their deletion results in impaired brain development [97, 98]. IRS mediate the phosphorylation of AKT/PI3K, which further activates several downstream signaling pathways [99]. Insulin downstream signaling promotes translocation of glucose transporters to the plasma membrane both in peripheral and brain tissues [62-68, 77, 100, 101]. Taken together, peripheral and brain insulin share similar signaling pathways.
The brain has been traditionally considered insulin-insensitive. However, now it is clear that normal brain functions depend on insulin, and insulin receptors are widely expressed in the brain. Insulin activates insulin signaling via binding insulin receptors and regulates various neuronal functions: it enhances learning and memory and regulates energy homeostasis, whole body metabolism, body temperature, mood, and appetite modified from [93].
This year we are celebrating the 96th anniversary of the discovery of insulin by Frederick Banting. Yet only over the past few years has interest increased in brain insulin’s role in cognition, food intake, body weight, and whole-body metabolism. Insulin in the brain has been detected at high levels in mouse embryos (E9) at early developmental stages and reduces with age [102]. Insulin receptors are located at brain synapses, and highly concentrated at the postsynaptic density [103]. Therefore, insulin may regulate synaptic function and excitatory synaptic transmission stability during neuronal development via insulin receptors. Furthermore, the insulin receptor substrate 53 (highly expressed in the postsynaptic density) also plays a role in regulating spine density in hippocampal cultured neurons [104].

1.7 Insulin receptors in the brain vs. in the periphery

Insulin receptors belong to a tyrosine kinase family and activate through insulin binding [105]. The insulin receptor is a dimeric transmembrane protein encoded by a single gene, INSR, and alternative splicing of INSR gives rise to $\alpha$ and $\beta$ subunits. The insulin receptor is composed of two extracellular $\alpha$ subunits that bind to insulin and two intracellular $\beta$ subunits with tyrosine kinase activity [106]. Insulin binds to $\alpha$ subunits of the insulin receptor, leading to autophosphorylation of $\beta$ subunits and activating the receptor, which further phosphorylates and recruits different substrate (IRS) adaptor proteins [41, 107]. Insulin and insulin receptor signaling play an important role both in the periphery and the brain and
mediate many cell functions ranging from glucose metabolism to learning and memory. Furthermore, insulin receptors are expressed in almost all tissues in the body. However, researchers have found that the anti-insulin receptor that blocked the insulin receptor in the periphery had no effect on brain insulin receptors [108]. This study strongly supports that some structural and molecular differences exist between the peripheral and brain insulin receptors. Moreover, the difference between peripheral and brain insulin receptors is primarily due to alternative mRNA splicing of the exon-11.

Brain insulin receptors lack exon-11, and therefore, brain insulin receptors have smaller subunits compared to peripheral insulin receptors [108-111]. Brain insulin receptors do not down-regulate in response to prolonged and/or a high concentration of insulin exposure [112, 113]. Brain insulin receptors reduce with aging and influence learning and memory, and learning improves insulin receptor functions [28]. These studies along with studies from the NIRKO mice model (a neuron-specific insulin receptor gene deletion [95] provide clear understanding of role of brain insulin receptor in brain function (food intake, metabolism, reproduction, cognition [114]. However, it is important to understand how insulin gets into the brain.
1.8 Insulin synthesis in the brain

1.8.1 Peripheral source of insulin

It is widely believed that insulin is synthesized and released mainly from the pancreatic beta cells in response to blood glucose levels. Insulin is initially synthesized as preproinsulin, and removal of its amino terminal single peptide generates proinsulin. Excision of C peptide within the endoplasmic reticulum gives rise to $\alpha$ (21 amino acids) and $\beta$ (30 amino acids) of insulin. C peptide is a biomarker of insulin secretion. Although various factors may influence insulin secretion, one key factor is blood glucose level. When glucose concentration rises, glucose moves into the cell by a glucose transport. Then, glucose is metabolized to generate ATP, which blocks ATP-dependent $K^+$ channels in the beta cell membrane, which results in the depolarization of the cell. Depolarization causes the Ca2+ ion channels to open, and Ca2+ ions to move in, which triggers vesicles containing insulin to move to the membrane fuse, and release the insulin by exocytosis.
Figure 1. The mechanism of insulin secretion by the pancreatic beta cell.


1.8.2 Central source of insulin

Previous studies show that insulin produced by the pancreas could enter the brain by crossing the blood brain barrier to maintain energy balance [107, 115-120]. Furthermore, insulin permeability changes during aging and in patients with diabetes and AD [121, 122]. Based on different experimental evidence, the brain also produces its own supply of insulin [10]. In 1983, Dorn et al. confirmed the hypothesis that part of the brain insulin is produced in the central nervous system (CNS). Results from the study of human cadaver brains demonstrated that insulin and C-peptide were present in most nerve cells [123]. Further, C-peptide and insulin receptors in the brain decreased with aging [124]. Unlike mammals, in drosophila and C.elegans, neurons are the primary cells to
produce insulin [99]. Together with animal studies and observations from hippocampal cultured neurons studies, it is clear that insulin can be synthesized locally in the brain and released from the neurons in response to depolarization [125, 126]. While brain insulin and insulin receptor signaling undergo changes with aging, insulin resistance may contribute to pathological brain aging [127-133].

Insulin also is valuable for treating a wide variety of non-diabetic diseases, such as schizophrenia, anxiety, nervousness, cancer, and cognitive function. While insulin in the brain enhances learning and memory, insulin resistance is linked to Alzheimer’s disease and other neurodegenerative disorders [134-136]. Currently, increasing insulin signaling in the brain is one of the best treatment options to improve memory in aged and AD populations [137].

1.9 Metabolism and the brain

The brain is a complex organ and needs lots of energy to run properly [138]. Glucose is the main substrate that satisfies brain high-energy demands. The brain (rich with neurons) weighs only 2% of body weight, but neurons use 25 percent of all the glucose energy in the body while its energy store capacity is small. Hence, the brain requires a constant glucose supply from the blood, and glucose is transported into the brain via the glucose transporters through the blood brain barrier. Although the brain is an energy-intensive organ, it has traditionally been considered insulin-insensitive. However, recently this view has
been challenged, and now it is clear that normal brain function depends on insulin. Moreover, brain functions such as learning and memory are positively correlated with effective glucose metabolism in the brain. Dysregulation of brain glucose metabolism can lead to loss of energy for brain functions, which further influences the neurotransmitters, neuronal communication, and cognitive function. Therefore, the traditional role of Insulin in the brain is regulating glucose metabolism and enhancing neuronal survival. It is also known that insulin and insulin receptors in the brain reduce with aging [124]. Additionally, the link between metabolic disturbances and neurodegenerative diseases have been well documented. Researchers have suggested that Alzheimer’s disease is positively correlated with obesity and diabetes [16, 139-145]. Previous studies suggest that type 2 diabetes increases the risk of developing AD [146-149]. Also, some studies suggest that AD is a brain- specific form of diabetes (type 3 diabetes) [150-155]. Furthermore, clinical studies suggest that AD patients present abnormal brain glucose metabolism [26, 156]. Normal brain glucose metabolism is vital for brain function and controlled by brain insulin/insulin receptor signaling pathways [157].

Insulin in the brain is the key to many neurological disorders. In fact, brain insulin regulates both periphery and central glucose metabolism, learning, and memory [87, 107, 158, 159]. Insulin exerts its effects though insulin receptors, which are widely distributed in various regions of the brain [107, 160-162]. More specifically, insulin receptors are highly expressed in the hippocampus [107,
163], a brain region that is critical in synaptic plasticity, learning, memory, and cognition. Brain insulin and insulin receptor levels decrease with aging and AD, which is linked to age-related memory impairment [152, 164-166]. Insulin receptors are also expressed in the hypothalamus, the widely studied region of insulin action in the brain. Insulin circulates at the level proportionate to body fat mass, inhibits food intake through its actions on the hypothalamic neurons [167, 168], and regulates glucose metabolism [169] and energy expenditure [170] both in the periphery and the brain [171]. The highest concentration of insulin and insulin receptors are detected in the olfactory bulb [172, 173], and insulin regulates olfactory functions, which are important for food intake and appetite [174]. Insulin receptors are also present in the cerebral cortex and cerebellum [28] and plays an important role for neuronal survival [158]. Furthermore, Kahn et al created neuron-specific insulin receptor knockout mice (NIRKO) to study insulin actions in the brain. The study results suggest that depletion of insulin receptors in neurons lead to obesity and dysregulation of a reproduction hormone [95]. Insulin has diverse effects in the brain. Insulin may promote better nerve cell communication, but the disruption of brain insulin signaling may lead to metabolic dysregulation and cognitive impairment [87, 134, 175]. Insulin has also been viewed as the potential missing link between memory and intracellular Ca2+ dysregulation with aging. Insulin regulation of glucose homeostasis is an important modulator of neuronal plasticity. Also, maintenance of intracellular Ca2+ homeostasis is vital for normal synaptic plasticity as well as cell excitability.
1.10 Intracellular calcium levels

Calcium (Ca2+) is an essential ion that rules many aspects of cellular life, including cellular excitability, exocytosis, apoptosis, toxicity, and transcription, and mediates many cell functions, including muscle contraction, chemical sense, cell proliferation, fertilization, immune response, learning and memory. For this reason, intracellular Ca2+ levels are precisely regulated in young healthy individuals. However, failure of this regulation occurs with aging and AD. The first proposal of a “Ca2+ hypothesis of brain aging” by Khachaturian in 1984 provides a simple cellular mechanism that alters Ca2+ homeostasis underlying many age-related changes. After two more decades of studies, the relationship between dysregulation of intracellular Ca2+ and pathological brain aging has become clear and is now the key therapeutic target to treat many neurodegenerative diseases.

Thanks to the excellent work of Sydney Ringer, who in 1883 discovered the importance of Ca2+ signaling in the survival of fish, heart, and muscle contraction. After 50 years, in the early 1940s, Lewis Victor Heilbrun confirmed the theory that Ca2+ is a universal intracellular messenger able to regulate all cellular reactions and that Ca2+ also involves neurotransmitter release into nerve terminals [176, 177]. After several decades of research, it is clear that in the brain, Ca2+ plays a critical role for synaptic activity, neuronal development, neuronal survival, and cognition [178-180]. Discovery of patch clamping and Ca2+ imaging techniques advanced our understanding of pathology associated
with dysfunctions of Ca2+ signaling cascades [181]. At rest, intracellular Ca2+ concentration is very low (~100 nM free) and extracellular Ca2+ concentration (~2 mM) is high, about a 20,000–fold concentration gradient. Intracellular [Ca2+] is maintained within a specific limit by the different Ca2+ handling mechanisms [182]. However, brain aging contributes to the loss of intracellular Ca2+ homeostasis [183, 184]. In healthy individuals, intracellular Ca2+ homeostasis is tightly regulated and plays a vital role in neuronal survival and function. In contrast, intracellular Ca2+ levels increase with aging, which contributes to memory decline and other neurodegenerative disorders such as AD [180, 185, 186]. Specifically, an age-related increase in hippocampus Ca2+ levels has been much investigated during the last several decades because Ca2+-dependent after hyperpolarization is larger (AHP) in aged animals and links to cognitive impairment with aging and AD [180, 182, 185-192]. In addition, high intracellular Ca2+ levels and larger Ca2+ dependent AHP are seen in the hippocampus of diabetic rats [193, 194].

It appears that the increase in hippocampal intracellular Ca2+ levels are mainly mediated through voltage gated Ca2+ channels and N-methyl-D-aspartate (NMDA) receptors on the plasma membrane, and intracellular Ca2+ release via ryanodine receptors on the endoplasmic reticulum [195].

1.10.1 Voltage-gated Ca2+ channels

Voltage-gated Ca2+ channels (VGCCs) are found in the membrane of neurons,
muscles, glial cells (excitable and non-excitatory cells), and are permeable to Ca2+ ions [196]. VGCCs are voltage-sensitive and mediate the fastest Ca2+ influx in response to membrane depolarization, thus, changing intracellular Ca2+ levels greatly within milliseconds [197-201]. As a result, Ca2+ enters the cell through voltage-gated Ca2+ channels and serves as intracellular second messengers to control a wide variety of cell functions. In cardiac, smooth, and skeletal muscles, activation of VGCCs mediates contraction by increasing intracellular Ca2+ levels and by further activating ryanodine dependent Ca2+-induced Ca2+-release in the sarcoplasmic reticulum [202-206]. In the endocrine cells, VGCCs activation influences the hormone secretion [207]. In the neurons, Ca2+-mediated process via VGCCs include synaptic transmission, neurotransmitter release, and gene transcription; it also activates calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) which are important for neuronal survival, learning and memory [198, 208-217]. While Ca2+ channels serve as a key pathway for entry of Ca2+ into the neuron and initiate many physiological events, inappropriate expression or dysfunction of VGCCs can result in pathophysiological changes leading to neuronal Ca2+ dysregulation and toxicity in the brain, which have been linked to neurodegenerative diseases [218-221]. Furthermore, VGCCs density increases with aging, and this contributes to cognitive decline with aging and AD [184, 222-224]. Therefore, voltage-gated Ca2+ channels are important therapeutic targets for age-related cognitive decline and other conditions.
1.10.2 Structure and classification of voltage-gated Ca2+ channels

The five-polypeptide subunit that forms voltage-gated Ca2+ channels is a central pore-forming α1 subunit, dimer of α2 and δ subunits and intracellular β subunit, and transmembrane γ subunit. There are ten Cavα1 subunits that share four homologous domains, each with six membrane-span helices (S1-S6). S4 (Positively charged and hydrophobic) is the key controller for voltage-dependent activation [197, 198, 208, 225-227] and P loop motif (negatively charged) is located between S5 and S6. Together they form a pore that is permeable to Ca2+ and barium [197, 228].

Figure 1.5 Voltage-gated Ca2+ channels structure.

High voltage activated (HVA) channels (P, Q, N, L, R-type) contain Caα1, Caβ, Caγ, and Caα2δ subunits. Low voltage activated channels (T-type) contain Caα1 subunit modified from [220].
The Cavα1 subunit has three main families (Cav1, Cav2, Cav3) [226, 229]. The Cav1 family includes four members (Cav1.1, Cav1.2, Cav1.3, Cav1.4) and all four members encode neuronal L-type Ca2+ channels and Cav1.1 encodes skeletal muscle Ca2+ channels [230-233]. Further, Cav1.1 knockout mice cannot contract their diaphragms and die at birth [234]. Mice that lack Cav1.2 die in utero because the heart muscle cannot contract [235]. Cav1.2 is also important for neuronal function and mutations in this subunit links to bipolar disorder, Schizophrenia, and depression [236-239]. Cav1.3 null mice are deaf due to loss of Ca2+ current [240] and loss of normal auditory brain stem development [241]. Mice deficient in Cav1.4 are blind due to loss of normal function in the outer retina [242, 243].

The Cav2 family has three members (Cav2.1, Cav2.2, Cav2.2) and gives rise to P/Q-type [244], N-type, and R-type Ca2+ channels [233]. The Cav3 family has three members (Cav3.1, Cav3.2, Cav3.3) that encode T-type channels [245]. Mice with a lack of Cav2.1, Cav3.1, and Cav3.2 display a range of dysfunctions (seizures, slow heart rate, compromised coronary, epilepsies) [246-250].

The Cavβ subunit has four members (Cavβ1- Cavβ4) and links to Cavα1 domain I-II [251, 252]. The Cavα2δ subunit has four different members (Cavα2δ1–Cavα2δ4) and is anchored to extracellular plasma membrane via glycophosphatidylinositol (GPI) [253-255]. Earlier studies suggest that the Cavα2δ subunit plays an important role in regulating synaptic targeting and
release probability of voltage-gated Ca2+ channels in neurons [256, 257].

Table 2. Types of voltage gated Ca2+ channels (VGCCs) activation and inactivation profiles modified from [258].

<table>
<thead>
<tr>
<th>Native current</th>
<th>α1-Subunit subtypes</th>
<th>Activation profile</th>
<th>Inactivation profile</th>
<th>Ca^{2+}-dependent inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/Q-type</td>
<td>α1A (Ca_{2.1})</td>
<td>High voltage</td>
<td>Moderate/slow</td>
<td>Yes/no</td>
</tr>
<tr>
<td>N-type</td>
<td>α1B (Ca_{2.2})</td>
<td>High voltage</td>
<td>Fast</td>
<td>Yes/no</td>
</tr>
<tr>
<td>L-type</td>
<td>α1C (Ca_{1.2})</td>
<td>High voltage</td>
<td>Moderate/slow</td>
<td>Yes</td>
</tr>
<tr>
<td>RO-type</td>
<td>α1D (Ca_{1.3})</td>
<td>High voltage</td>
<td>Moderate/slow</td>
<td>Yes</td>
</tr>
<tr>
<td>T-type</td>
<td>α1E (Ca_{1.4})</td>
<td>High voltage</td>
<td>Fast/moderate</td>
<td>No</td>
</tr>
<tr>
<td>R-type</td>
<td>α1F (Ca_{1.1})</td>
<td>High voltage</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>T-type</td>
<td>α1G (Ca_{3.1})</td>
<td>Low voltage</td>
<td>Very fast</td>
<td>No</td>
</tr>
<tr>
<td>T-type</td>
<td>α1H (Ca_{3.2})</td>
<td>Low voltage</td>
<td>Very fast</td>
<td>No</td>
</tr>
<tr>
<td>T-type</td>
<td>α1I (Ca_{3.3})</td>
<td>Low voltage</td>
<td>Very fast</td>
<td>No</td>
</tr>
</tbody>
</table>

There are several members of the VGCCs in the brain due to different genes that encode ten α1 subunits of Ca2+ channel, including L-type, N-type, P/Q-type, R-type, and T-type channels [214, 259]. Based on physiological and pharmacological properties, they are categorized as high voltage (HVA) and low voltage (LVA). HVA channels (L-type, P/Q-type, N-type) formed through a combination of Cavα1, Cavβ, and Cavα2δ subunits activate in response to strong and large depolarization. On the other hand, Low voltage-activated (T-type) channels only contain the Cavα1 subunit and open in response to weak depolarization [226, 260-263]. The R-type is intermediate voltage activated channels. In neurons, both HVA and LVA channels can control neuronal firing.
Activation of voltage-gated Ca\textsuperscript{2+} channels results in Ca\textsuperscript{2+} influx and mediates neurotransmitter release, neuronal firing, hormone secretion, muscle contraction, and gene expression. However, prolonged activation of Ca\textsuperscript{2+} channels contributes to elevation of intracellular Ca\textsuperscript{2+} levels, which is cytotoxic [221]. Therefore, there are two types of Ca\textsuperscript{2+} channel inactivation, voltage- and/or Ca\textsuperscript{2+}-dependent inactivation, to limit intracellular Ca\textsuperscript{2+} overload. All voltage-gated Ca\textsuperscript{2+} channels can be inactivated by voltage-dependent manner [269]. The underlying mechanism responsible for voltage-dependent Ca\textsuperscript{2+} channel inactivation is different from voltage-gated K\textsuperscript{+} and Na\textsuperscript{+} channels ("ball and chain" and "Hinged-lid") mechanisms [270]. Many studies suggest that prolonged depolarization triggers structural changes in four S6 of the \(\alpha1\) subunits, and further exposes docking site for the domain I-II linker. Thus, it modulates inactivation of voltage-gated Ca\textsuperscript{2+} channels [269, 271-273]. Other subunits of Ca\textsuperscript{2+} channels also influence the kinetics and rate of inactivation.

Voltage-gated Ca\textsuperscript{2+} channels inactivate not only in response to voltage, but also to the influx of Ca\textsuperscript{2+} [274]. Ca\textsuperscript{2+}-dependent inactivation becomes important when studying the effects of drugs on Ca\textsuperscript{2+} channel currents. A rise in intracellular Ca\textsuperscript{2+} triggers binding of Ca\textsuperscript{2+} to the channel and initiates inactivation [275] and Calmodulin plays a vital role in Ca\textsuperscript{2+}-dependent
inactivation [276]. Therefore, barium was used as a charge carrier when obtaining Ca2+ channel currents while studying insulin effects on VGCCs.

1.10.4 Ryanodine receptors

Ryanodine receptors (RyRs), intracellular Ca2+ release channels, are located in the endoplasmic (ER) reticulum membrane [136, 277], and mediate rapid intracellular Ca2+ release into the cytosol, which allows the amplification of intracellular Ca2+ concentration. RyRs are widely expressed in almost all tissues (muscle, myocardium, and neurons) and are involved in a variety of key Ca2+ signaling events [278], ranging from excitation-contraction coupling in muscle cells to learning and memory in nerve cells. There are three major RyRs isoforms (RyR1-3) and they are named after plant alkaloid ryanodine [279]. Ryanodine is a natural agonist at low concentration, which allows efflux of Ca2+ from ER and increases intracellular Ca2+ levels. At high concentrations (~20 uM), Ryanodine is an antagonist, which inhibits the Ca2+ flow out of the ER.

RyRs are the largest ion channels and the highest-conductance intracellular Ca2+ channel, with a molecular mass that is greater than megadalton [280]. RyRs are regulated by the Ca2+ ion and VGCCs [281]. Ca2+ influx through voltage-gated Ca2+ channels triggers second messenger signals that open the RyRs channels and give rise to large Ca2+ release from internal Ca2+ stores (endoplasmic reticulum) [282, 283]. Thus, Ca2+ activation of the RyRs on the endoplasmic reticulum amplifies the intracellular [Ca2+] through Ca2+-induce Ca2+-release (CICR) process [284]. Additionally, various ions, small molecules,
and proteins may also regulate the RyRs function, including protein kinase A (PKA), FK506 binding proteins, and calmodulin (CaM) [285-287]. In skeletal and cardiac muscle, RyR1 and RyR2 play a critical role in excitation and contraction coupling [288, 289]. High intracellular Ca2+ concentration induces muscle contraction and Ca2+ ATPase dependent Ca2+ uptake by sarcoplasmic reticulum results in relaxation [281, 290]. In neurons, RyRs are crucial for signal transduction and influence hormone secretion, synaptic plasticity, and learning [291-293].

![Figure 1.6 Ryanodine receptors and cognitive function with aging](image_url)

*Ryanodine receptors (RYRs) on the endoplasmic reticulum (ER) undergo functional changes with aging. Furthermore, the RYRs mediated increase in Ca2+-induced Ca2+-release (CICR) is one of the major sources of Ca2+ dysregulation with aging. Moreover, amplified CICR influences the size of AHP and cognitive function. As a result, dysfunction of RYRs leads to an increase in*
intracellular Ca2+ via CICR, which further contributes to larger AHP, Ca2+ dysregulation, and memory decline.

1.10.5 Ryanodine receptor isoforms and expression

Ryanodine receptor isoforms are RyR1, RyR2, and RyR3 and are expressed in various tissues. RyR1 is the most intensely studied type and its main function is to trigger Ca2+ efflux from the sarcoplasmic reticulum membrane of skeletal muscle. RyR1 is highly expressed in the skeletal muscle [294, 295]. Also, it is widely distributed in cardiac muscle, smooth muscle, stomach, kidney, thymus, cerebellum, purkinje cells, adrenal glands, ovaries, testis, and B-lymphocytes at low levels [294, 296-299]. Dysfunction of the RyR1 gene is associated with life-threatening muscle diseases such as malignant hyperthermia [300] and multiminicore disease [301].

RyR2 is dominantly expressed in the cardiac muscles [277]. Different concentrations of RyR2 are also detected in purkinje cells of the cerebellum, cerebral cortex, stomach, kidney, adrenal glands, ovaries, thymus, and lungs [302-305]. Mutation in the RyR2 gene may cause arrhythmogenic right ventricular dysplasia type 2 and Ventricular arrhythmias by changing Ca2+ homeostasis [306-308].

RyR3 is present in the brain and mainly found in the hypothalamus, corpus striatum, and hippocampal neurons [296, 304, 309]. Different studies suggest
that inappropriate expression or dysfunction of RyR3 links to Alzheimer’s disease [136, 186].

1.10.6 Structure of ryanodine receptors

All three isoforms (RyR1, RyR2, RyR3) share similar structure. The structure of RyR1 (Skeletal muscle ryanodine receptors) is widely studied and it includes four identical subunits and is divided into two regions: a transmembrane (TM) region and a cytoplasmic (CY) region [294, 295]. The CY region plays a critical role in regulating RyR1 channel gating properties via interacting with intracellular messengers. Dysregulation of the CY region of RyR1 changes its gating and links to many diseases [310]. RyR2 is dominantly expressed in the heart and brain and is activated by Ca2+ influx through plasma voltage-gated Ca2+ channels in response to depolarization. Peng et al. used electron microscopy to map the structure of open and closed states of RyR2 from porcine hearts. The results suggest that RyR2 has different cytoplasmic domain compared to RyR1 due to rotation of the central domain, which influence the RyR2 channel gating [311].

1.11 Hippocampus

Hippocampus is critical for learning and memory [312] and is highly vulnerable to damage and loss during aging and AD [111, 191, 313-315]. The hippocampus, located in the medial temporal lobe, belongs to the limbic family,
one on each side of the brain. The human hippocampus has a curved shape resembling a seahorse; it appears as a ‘C’ shape in hippocampal cultured neurons. In addition to the hippocampus, the cerebellum, amygdala, and other systems are also involved in learning and memory.

The hippocampus is responsible for the formation and consolidation of short-term memory and long-term memory, as well as spatial navigation. The H.M study is the starting point for understanding the importance of the hippocampus in memory. H.M is a famous patient whose case provided an understanding of how our brain works. After the neurosurgeon removed H.M’s hippocampus, patient was unable to form new memories, and, as a result, he forgot daily events as fast as they occurred [316]. The H.M case study continued for five decades until his death, and the study results shed light on the relationship between cognitive and neuronal organization of memory [317]. Since H.M became amnesic after bilateral hippocampus lesion, the removal of hippocampus is responsible for his memory impairment; moreover, magnetic resonance imaging study further confirmed that lesion of the hippocampus can result in global and enduring amnesia [318]. This study, along with cumulative animal studies, made it clear that the hippocampus plays a critical role in the formation of immediate memory, long-term memory, and memory retention [319]. In addition, earlier studies suggest that the hippocampus volume decreased with aging [320]. Furthermore, in neurodegenerative diseases such as Alzheimer’s disease, the hippocampus is one of the first brain regions to become damaged.
Also, an increase in hippocampal neurogenesis is one of the hallmarks of early AD [321, 322].

On the other hand, in healthy individuals, high levels of insulin receptors have been seen in CA1, a hippocampal region that is important in learning and memory [159, 323]. The interruption of insulin production and insulin receptor activity may cause cognitive decline [28]. Also, neuron loss in the CA1 region of the hippocampus differentiates the healthy individual from AD patients [313, 324-326]. Therefore, losing and/or damaging the hippocampal neurons causes significant cognitive problems.

1.12 Insulin actions on neurons

Insulin has wide ranging effects in neurons of various brain regions, including regulating neuronal activity and synaptic function. Insulin and insulin receptors are present in the fetal central and peripheral nervous system, where it promotes axonal growth and brain development in the hippocampal cultured neurons [327]. Insulin can be produced and released from the hippocampal cultured neurons in response to depolarization [125, 126]. Insulin inhibits norepinephrine uptake by the hippocampal cultured neurons [328]. In the hypothalamus, insulin reduces the activity of POMC, AgRP, and steroidogenic factor 1 (SF-1) neurons through KATP channel activation [329, 330]. In the hippocampus, insulin increases insertion of NMDA receptors into the cell membrane [89] via tyrosine phosphorylation of NR2A and NR2B subunits [85].
Thus, insulin may promote long-term potentiation, which is important for learning and memory. Insulin-dependent internalization of the AMPA receptor [88, 331, 332] is important for long-term depression, which is another important mechanism for consolidation of navigational memory in the hippocampus. Insulin also regulates inhibitory synapse by increasing cell surface GABA<sub>A</sub> receptor expression [91, 92, 333]. In addition, insulin enhances synaptic plasticity by increasing the expression of dendritic postsynaptic density scaffolding protein (PSD-95), which is important for the formation of the postsynaptic junction [334] and Ca<sup>2+</sup> homeostasis in hippocampal neurons (Maimaiti et al., 2017). Insulin stimulates the translocation of glucose transporter-4 (Glut-4) to hippocampal plasma membranes [100]. Glut-4 is important for glucose uptake and utilization in the brain. As a result, insulin regulates neuronal metabolism and energy needed for learning and memory via Glut-4. In addition, insulin also regulates visual system function by controlling synapse density [84]. Insulin modulates L-type Ca<sup>2+</sup> current and intracellular Ca<sup>2+</sup> levels in rod photoreceptors [335].

1.13 Thesis significance

Hippocampus-dependent memory loss is one of the hallmarks of brain aging and Alzheimer’s disease (AD). This growing epidemic includes an estimated 5.3 million people in the United States, with the economic burden on healthcare estimated at 259 billion dollars (http://www.alz.org/facts/). Most importantly, the day-to-day lives of those affected by AD and their families must be considered
as we investigate new therapeutic targets and new approaches for the treatment of this devastating disease. Indeed, AD therapeutic options are very limited in their ability to improve the lives of those impacted by this disease. Therefore, innovative and effective therapeutic strategies are necessary to address the needs of this patient population.

In recent years, the study of insulin as a cognitive enhancer has gained momentum. Insulin and its receptors have been detected in the hippocampus, one of the major brain regions that is damaged and/or lost in Alzheimer’s disease [155, 336]. In addition, insulin regulates energy for proper brain function and survival [158], and insulin signaling has been shown to be deficient in postmortem brain tissue of AD patients [337, 338]. Thus, increasing insulin signaling in the brain appears to be a valid therapeutic target for improving cognitive function in aging and/or AD. Earlier clinical trials have shown that intranasal insulin treatment significantly improves memory in AD patients [31-35]. Therefore, based on these clinical studies as well as animal studies, intranasal insulin administration appears to be one of the best methods to treat cognitive decline due to its minimal effect on systemic glucose levels and noted improvements in memory processes. However, there have been only a few studies focusing on identifying the neuronal molecular mechanisms underlying these effects. My dissertation is designed to identify the potential mechanisms responsible for insulin-mediated memory improvement as those seen in human and animal studies. Specifically, I focus on identifying whether insulin can offset
cognitive decline with aging through a mechanism dependent on reestablishing Ca2+ homeostasis.

Intracellular Ca2+ signaling may be a key neuronal molecular regulator of hippocampal-dependent memory. Intracellular Ca2+ levels increase associates with memory decline in aging and AD [156, 314, 339, 340]. Thus, we examined the relationship between insulin, memory and intracellular Ca2+ levels to better understand the mechanism(s) underlying insulin action on hippocampal neurons. This information will enable to us to better understand how insulin therapy may affect patients and may also shine light on the underlying neuronal mechanisms.

The following manuscript has been published in the Journals of Gerontology: Series A: J Gerontology A Bio Sci Med Sci. 2016 Jan;71(1):30-9. Epub 2015 Feb 6. As an initial step, we explored the role of intranasal insulin in improving hippocampal-dependent memory in experimental aged animals. Additionally, we delineated insulin’s effects on Ca2+-dependent after hyperpolarization in the CA1 region of the dorsal hippocampus using hippocampal slices recordings in young and aged rats. Because brain aging is the greatest risk factor for AD, studies into how insulin may alter biomarkers of brain aging could improve current therapeutic strategies for memory loss with aging and AD.
Chapter 2 Intranasal insulin improves age-related cognitive deficits and reverses electrophysiological correlates of brain aging

Authors: Shaniya Maimaiti, Katie L Anderson, Chris DeMoll, Lawrence L Brewer, Ben Rauh, Chris J Gant, Erick Blalock, Nada M. Porter, Olivier Thibault

Synopsis

Peripheral insulin resistance is a key component of metabolic syndrome associated with obesity, dyslipidemia, hypertension, and Type 2 diabetes. While the impact of insulin resistance is well recognized in the periphery, it is also becoming apparent in the brain. Recent studies suggest that insulin resistance may be a factor in brain aging and Alzheimer’s disease (AD) whereby intranasal insulin therapy, which delivers insulin to the brain, improves cognition and memory in AD patients. Here, we tested a clinically-relevant delivery method to determine the impact of two forms of insulin, short-acting insulin lispro (Humalog®) or long-acting insulin detemir (Levemir®), on cognitive functions in aged F344 rats. We also explored insulin effects on the Ca2+-dependent hippocampal afterhyperpolarization (AHP), a well-characterized neurophysiological marker of aging which is increased in the aged, memory impaired animal. Low-dose intranasal insulin improved memory recall in aged animals such that their performance was similar to that seen in younger animals. Further, because ex vivo insulin also reduced the AHP, our results suggest that the AHP may be a novel cellular target of insulin in the brain, and improved
cognitive performance following intranasal insulin therapy may be the result of insulin actions on the AHP.

2.1 Introduction

Results from preclinical settings argue that peripheral insulin resistance, a component of type 2 diabetes and/ or the metabolic syndrome, may have a negative impact on cognitive function. Emerging studies report that in diabetic models, insulin responses are attenuated in the brain, possibly indicating the presence of insulin resistance in this tissue. Clinical and preclinical studies show that insulin and insulin-like growth factor-1 (IGF-1) receptors, their message and their function, are often reduced in the hippocampus during normal and pathological aging [27, 111, 124, 150, 341]. Similarly, in animal models of aging and Alzheimer’s disease (AD), brain insulin receptor signaling is reduced, often in the presence of peripheral metabolic dysregulation [28, 342-348]. Importantly, several studies provide evidence that brain insulin therapy can greatly reduce cognitive decline with age and/ or AD, and even improve memory in young adults [reviewed in 33].

In recent studies examining the effects of insulin on cognition, insulin has been administered via the intranasal route to bypass the blood brain barrier. Notable, Born and colleagues have shown that intranasal insulin is capable of entering the human brain rapidly [349]. However, the underlying mechanism(s) leading to cognitive improvement are not yet clear. Potential molecular/ cellular targets
affected by insulin in the brain and capable of offsetting cognitive decline include changes in neuronal firing [350], Ca2+-dependent K⁺ channels [351], GABA currents [352] and tau and Aβ metabolism [353-355]. Insulin also alters synaptic structures [356, 357], modifies synaptic plasticity [88, 358, 359] and increases delivery of NMDA receptors to the plasma membrane [89]. Similar effects have been observed in response to IGF-1 replacement, showing increased AMPA-mediated excitatory postsynaptic potentials [360, 361], and increased NMDA receptor trafficking [362], effects that appear capable of offsetting age-dependent cognitive decline [363]. Further, MRI data in men and women has provided evidence that intranasal insulin can have an impact on brain blood flow [364], and on whole-body insulin sensitivity via regulation of hypothalamic activity [365, 366]. Whether this later effect is purely mediated by central insulin effects or reflects permeation of small insulin amounts into the bloodstream is currently being investigated [367-369].

Another important candidate mechanism that may underlie the effects of intranasal insulin pertains to Ca2+ homeostasis. Ca2+ dysregulation is prominent in models of diabetes [370]. In the dorsal root ganglion and the hippocampus of animals with STZ-induced diabetes [193, 371], broader Ca2+ action potentials, larger Ca2+-dependent afterhyperpolarizations (AHPs), and aberrant intracellular Ca2+ release are seen [193, 372, 373]. Impaired memory and long-term potentiation maintenance is also present [374]. Clinical data also supports a role for Ca2+ dysregulation in peripheral insulin resistance as evidenced by the
presence of abnormal and sustained adipocyte Ca2+ elevations in hyperinsulinemic, obese subjects [375]. Similar evidence for an increase in Ca2+ levels in skin fibroblasts from Type 2 diabetics has also been reported [376]. In recent work, we have shown that the Ca2+-dependent AHP, which increases in amplitude and duration with aging [377-379], is sensitive to insulin (ex vivo), and that this sensitivity is reduced by a high fat diet [380].

While some studies in humans demonstrate the efficacy of intranasal insulin as a cognitive enhancer, studies in animal models are required to understand the potential mechanisms underlying the beneficial effects of intranasal insulin. However, only one prior study has measured the impact of intranasal insulin on brain function in the awake animal, focusing on olfactory discrimination in the young mouse [381]. Because the greatest risk factor for AD is aging, research into how central nervous system insulin signaling affects brain aging may improve understanding of the biological conditions. Further, because of the sustained growth in the aging population, implementing strategies to reduce the impact of brain aging will likely benefit the aging population, and by extension, may reduce the incidence of age-related neurodegenerative diseases that share cellular mechanisms with brain aging (e.g., AD).

We, therefore, assessed learning and memory performance in aged rats in response to two different insulin formulations (short-acting insulin Humalog® and long-acting insulin Levemir®), and quantified Ca2+ homeostasis. Insulin is
available primarily in shorter- and longer-acting forms, and intranasal longer-
acting insulin formulations (e.g., Levemir®) may potentially be superior to
shorter-acting ones (e.g., Humalog®) especially in APOE-ε4 carriers [382]. In
addition, because insulin is often formulated with zinc, to more specifically
address the impact of insulin, we studied a zinc-free insulin formulation (Apidra®
), as well as zinc alone on the AHP. Overall, our goals were to test whether
intranasal insulin could reduce cognitive decline in aged animals, as well as
redress the accompanying neuronal Ca2+ dysregulation seen in the
hippocampus of aged animals.

2.2 Methods

**Animals:** Young male F344 (3 months old), aged male F344 (21 months old),
and young male Sprague-Dawley rats (2-6 months old) were used for this study.
Young F344 animals were not treated with insulin, and were used to help gauge
the impact of intranasal insulin on memory function in aged F344. All animals
were fed an 18% protein rodent diet (Harlan Teklad, diet #2018; Madison, WI).

**In vivo insulin delivery and doses:** Levemir® doses were approximately
0.0715, 0.143, or 0.286 IU/day/rat delivered in two 5 uL applications applied to
the tip of the right naris using a P10 Eppendorf pipette (see supplemental figure
1) [adapted from 383]. Humalog® dose was chosen to deliver 0.0715 IU/day/rat
for behavioral characterization. An intranasal 0.286 IU/rat dose was also
administered once to aged animals (n = 3) to monitor the impact on peripheral
glucose (supplemental Table 1). Each application was separated by 1 min, during which the animal was held supine and immobile in a DecapiCone® (Braintree Scientific Inc., Braintree, MA). Each animal was acclimated to this procedure for 4 days prior to initiation of the Morris water maze protocol. All animal groups were exposed to the DecapiCone® for the same duration, and no signs of stress from the bagging procedure were noted. The insulin doses were chosen to approximate several clinically-relevant doses used in past studies ranging from 10 IU/day to 40 IU/day and were adjusted by brain weights. Assuming a 70 year old human brain weighs 1360 g [384], 10, 20 and 40 IU/day are equivalent to 0.0074, 0.0147, and 0.0294 IU/day/g of brain, respectively. Assuming a 21 month old F344 brain weighs 2.06 g [385], our equivalent doses were 0.035, 0.069 and 0.138 IU/day/g of brain. It should be noted that these values may underestimate final insulin concentration given the greater olfactory epithelium surface in the nasal passages of the rat (50%), compared to man (8%) [386]. Insulin solutions were made fresh weekly from 100 U/mL vials (Humalog®, insulin lispro, Lilly; Levemir®, insulin detemir, Novo Nordisk) and diluted into sterile 0.9% saline.

**Blood glucose levels:** To ensure that the intranasal delivery did not result in reduced peripheral glucose levels, and to test whether stress from the acute restraint procedures (DecapiCone®) could elevate blood glucose levels, we measured blood glucose (FreeStyle Lite glucometer; Abbott Laboratories, Abbott Park, IL) from dorsal tail veins in a subset of aged animals either exposed with
intranasal Levemir® (n = 5 from each treatment group) or Humalog® (n = 3), both before and approximately 1 hour after dosing (see supplemental Table 1).

**Animal behavior treatment groups and Morris water maze (MWM) procedures:** Two cohorts of F344 animals were used for behavioral characterization (supplemental Fig. 1B). One cohort of animals (n = 50) was used to test the impact of long-acting insulin Levemir® at 3 different doses (low, medium and high; n = 10 per group) compared to an intranasal saline young group (3 months; n = 10) and an intranasal saline aged group (21 months old; n = 10). A second cohort of aged animals (n = 20) was used to test the impact of short-acting insulin Humalog® against saline (n = 10 per group). For analysis, behavioral data from the two aged saline control groups in both cohorts were combined because memory performance on probe day was similar (path length to goal, p = 0.89 and cumulative search error, p = 0.913). One aged animal in the saline group died during acclimation prior to initiation of behavioral testing. The remaining 69 animals were trained on the MWM. Animals were considered visually impaired or unable to learn the task if they could not find the platform on all 3 of the trials on the cue day (visual platform) and at least one of the remaining 3 training days. Based on these conditions, 2 aged Humalog® animals and 3 aged low-dose Levemir® animals were removed from the analysis. Behavioral results are therefore presented on 64 animals, 10 young saline, 19 aged saline, 8 aged low-dose Humalog® (10 IU/day), 7 aged low-dose Levemir® (10 IU/day), 10 aged medium-dose Levemir® (20 IU/day), and 10 aged high-
dose Levemir® (40 IU/day). On cue day, no significant differences were found across any of the groups on measures of path length (1-way ANOVA, p>0.05) and swim speed across insulin dose (1-way ANOVA, post-hoc p>0.05), indicating motivation and motor activity were likely similar.

Insulin or saline treatment in both cohorts was initiated 4 days prior to the initiation of training (sup. Fig. 1B) and was delivered 1-3 hours before testing on the MWM. The MWM protocol has been published elsewhere [380, 387], but briefly, water temperature was maintained at 26-27°C, and black liquid tempera paint was used to make the water opaque and hide the submerged escape platform (~1.5 cm below water surface). A Videomex-V acquisition system and water maze analysis software (version 4.64, Columbus Instruments, Columbus, OH, USA) were used to track and measure animal movements. Animals were allowed 60 s to find the platform, after which they were guided to it. Each animal stayed on the platform for 30 s before returning to a heated holding chamber for about 2 min. On cue day, a white cup hanging above the partially submerged platform helped orient the animals. Animals were subjected to 3 trials per day (semi-random drop location for each trial) for cue and training days. Twenty-four hours after the last training day, a probe trial was initiated with the platform removed (60 s max swim time). Swim speed was derived from the distance travelled over time on the last trial of the third training day and on the first trial of the cue day. On all cue and training days the inter-trial interval was approximately 150 s. All behavioral experiments were conducted with the
experimenters blind to the treatment groups using color coding of the insulin and saline vials.

**Electrophysiology:** Hippocampal slices were used across two electrophysiological setups, one monitoring extracellular field potentials and, the other measuring intracellular potentials (e.g., AHP). All data were acquired using pClamp 8.0 (Molecular Devices MDS, Toronto, Canada) through a Digidata 1320A A/D converter (Molecular Devices). Potentials (e.g., amplitude and duration of AHPs, field EPSPs) were quantified off-line using Clampfit (Molecular Devices). Nineteen of the twenty behaviorally characterized aged animals (second cohort used to test the impact of short-acting insulin Humalog® against saline) were used 2-5 weeks after the last day of behavioral characterization to determine the impact of single intranasal insulin dose in vivo. From those, a total of 30 slices used for extracellular field potential recordings (Fig. 2D and 2E) and 13 cells used for intracellular recordings (Fig. 2B and 2C) were used for statistical analyses and are presented using an n of 1 per cell/slice. In data presented in figure 3, 16 Sprague-Dawley animals (2-6 months old) were used to monitor the impact of zinc and Humalog® ex-vivo both extracellularly and intracellularly (30 slices and 23 cells, respectively). Work presented in figure 4 (intracellular physiology only) is compiled from a total of 17 F344 male rats split into young (n = 8; 3 months) and aged (n = 9; 22 months). This last group of animals yielded recordings from 6 cells for young ACSF, 8 cells from young Apidra®, 6 cells for aged ACSF and 5 cells for aged Apidra®.
Figure 2.1 Morris water maze spatial learning and memory task.

(A) Path length to submerged goal platform on the initial 3 days of training showed improved performance across all groups tested (RM ANOVA p<0.0001), however, no group effect was seen. On probe day, with platform removed, animals receiving low doses of insulin (Humalog® or Levemir®) showed reduced path lengths to goal (B) and improved memory performance based on reductions in cumulative search error (C). Analysis of swim speed measured on the third training day shows aged F344 animals are slower (note no effect of insulin dose on speed (D)). Means ± SEM are shown. Asterisks indicate significant differences from the young saline group at p<0.05; pound sign indicates a trend (p=0.059) in comparison to the young saline group.
Figure 2.2 Impact of in vivo intranasal Humalog® on intracellular and extracellular physiology.

(A) Representative intracellular recording of the AHP following a series of 4 action potentials (truncated to emphasize the AHP). The mAHP (B) and sAHP (C) amplitudes recorded 3-7 hours after intranasal Humalog® in F344 rats were not significantly different. EPSP amplitudes (D) and I/O slopes (E) when recorded extracellularly 3-7 hours after intranasal Humalog® also were not significantly altered. Means ± SEM are shown.
Figure 2.3 Impact of ex vivo Humalog® and zinc on the AHP.

(A) Representative voltage trace of the AHP recorded in a cell from an ACSF treated slice (black) and a Humalog® treated slice (1 nM, grey). Data are derived from young Sprague-Dawley animals. Traces are truncated to emphasize the AHP. Significant reductions in the sAHP amplitude (C) and duration (D) were seen in response to Humalog® as well as zinc. Note that zinc and low concentration Humalog® seem to work more robustly on the sAHP (only a trend was noted for the mAHP (B)). No significant effect was detected during extracellular recording of field EPSP amplitudes (E) or slopes of the I/O curve (F). Means ± SEM are shown.
Figure 2.4 Zinc-free Apidra® reduces the AHP ex vivo.

The mAHP amplitude is shown in (A) and the sAHP amplitude is shown in (B). Means ± SEM are presented in response to 1nM Apidra®. These data are taken from young and aged F344 rats. Asterisks indicate significant age-dependent increases in the mAHP and sAHP, and pound signs illustrate significant insulin-mediated reductions in the AHP. Again, the impact of insulin seems more robust on the sAHP compared to the mAHP. These recordings were obtained with 4.5 mM glucose in the ACSF.
Figure 2.5 Intranasal insulin delivery and experimental timeline.

(A) This photograph shows a supine aged F344 rat during intranasal insulin delivery. The animal is held in place temporarily in a decapicone® with the head gently restrained. Note that the pipette tip is not inserted into the naris but presented at the entrance of the right naris. (B) Timeline used for behavioral (Morris water maze – MWM) and electrophysiological characterization presented in figures 1 and 2 using two cohorts of F344 rats. T1, T2 and T3 are initial training days on the MWM and are followed with a 24 h memory recall task (Probe). Each red triangle represents a consecutive day for intranasal insulin delivery in 2 different cohorts of animals. All animals were treated/ handled similarly across cohorts, and received 11 saline or insulin doses. Animals in cohort 2 were also used 2-5 weeks after behavioral characterization, to test the
electrophysiological impact of intranasal Humalog® \textit{in vivo}. Electrophysiology data presented in figures 3 and 4 are derived from Spraque-Dawley and F344 animals, respectively.

\textbf{Table 3. Blood glucose analysis.}

<table>
<thead>
<tr>
<th></th>
<th>Saline (n=5)</th>
<th>10 IU Levemir® (n=5)</th>
<th>20 IU Levemir® (n=5)</th>
<th>40 IU Levemir® (n=5)</th>
<th>40 IU Humalog® (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Dose</td>
<td>73.2 ± 2.9</td>
<td>72.6 ± 6.2</td>
<td>70.8 ± 7.2</td>
<td>75.2 ± 3.8</td>
<td>65.3 ± 2.0</td>
</tr>
<tr>
<td>Post-Dose</td>
<td>82.0 ± 3.7</td>
<td>82.2 ± 4.0</td>
<td>74.2 ± 6.5</td>
<td>78.4 ± 5.3</td>
<td>82.3 ± 6.0</td>
</tr>
</tbody>
</table>

Numbers represent mean ± SEM peripheral glucose levels (mg/dL) taken before (Pre-Dose) and 30-90 min after intranasal insulin Levemir® or Humalog® delivery (Post-Dose). The number in parentheses represents the number of aged animal tested in each group. Intranasal insulin (Levemir® or Humalog®) did not reduce peripheral blood glucose levels.

\textbf{Field potentials:} Rats were anesthetized with Isoflurane® prior to decapitation. Brains were quickly removed and placed in oxygenated artificial cerebro-spinal fluid (ACSF) of the following composition (in mM): 114 NaCl, 3 KCl, 10 Glucose, 1.25 KH$_2$PO$_4$, 26 NaHCO$_3$, 8 MgCl$_2$, 0.1 CaCl$_2$. Hippocampi were sectioned (coronal) with a Vibratome® 3000 (Bannockburn, IL) set at 400 µm. Slices were transferred to an interface-type recording chamber (static) containing oxygenated recording ACSF of the following composition (in mM): 114 NaCl, 3 KCl, 10
Glucose, 1.25 KH$_2$PO$_4$, 26 NaHCO$_3$, 2.5 CaCl$_2$ and 1.3 MgCl$_2$. The recording chamber was kept at 32°C and warm moist 95 % O$_2$/ 5 % CO$_2$ was continually delivered to the chamber. Bipolar synaptic stimulation was delivered through a SD9K stimulator (Astro Med Inc., Grass Instr., Warwick, RI). Input/output curves (I/O) were recorded using a series of increasing voltage steps. The relationship between stimulus intensity and EPSP amplitudes (prior to dendritic spike contamination) was fit (linear, $r^2>$0.9) to yield a measure on the slope of the I/O. Data are also reported as the absolute amplitudes of the EPSPs measured as the maximum deflection from baseline. Insulin (1, 100, 500 nM Humalog®) and/or zinc treatment (130 nM – see AHP-intracellular recordings) under these conditions was initiated 1 hour after placing the slices in the chamber (compounds remained in the chamber for the duration of the experiment.

**AHP-Intracellular recordings**: Hippocampal slices (350 µm) were transferred from the static chamber and placed in a perfusion chamber (RC22C, Warner Instruments, Co., Hamden, CT) and maintained in a continuous flow of oxygenated ACSF pre-heated at 32°C using a TC2Bip/HPRE2 in-line heating system (Cell Micro Controls, Northfolk, VA). The ACSF contained 2 mM CaCl$_2$ and 2 mM MgCl$_2$ and in some experiments (Figure 4 only), the traditional 10 mM glucose concentration was replaced with 4.5 mM glucose. This was done to test whether higher glucose concentrations altered insulin sensitivity. We chose 4.5 mM to help maintain tissue health and physiology for extended hours [388], to approximate values in anesthetized preparations [389], and to consider
fluctuations in brain glucose levels across a day [390]. As previously described, cells were impaled with sharp microelectrodes filled with 2M KMeSO$_4$ and 10mM HEPES, pH 7.4 (tip resistance 90-120 MΩ) [377]. Recordings of membrane input resistance (IR) were obtained in response to 800 ms, 200 pA hyperpolarizing current injections using an Axoclamp 2B amplifier (Molecular Devices, MDS, Toronto, Canada) while holding the cell at -70 mV. To generate an afterhyperpolarization (AHP) cells were held at -65 mV (baseline) and depolarized with a 100 ms current injection to generate four Na$^+$ action potentials. The medium AHP (mAHP) was measured as the peak hyperpolarization immediately after the offset of the depolarizing current injection, the slow AHP (sAHP) was measured 800 ms after the end of the current injection. The AHP duration was measured from the end of the depolarizing step until return to baseline.

Several concentrations and formulations of insulin were tested ex vivo by addition to the solution bathing the hippocampal slices. Humalog® concentrations of 1, 100, and 500 nM were used. A zinc (ZnCl$_2$) concentration of 130 nM was used to mirror levels of free zinc attained in 250 nM Humalog®. Apidra® (insulin glulisine, Sanofi, a zinc-free insulin formulation) was used at 1 nM and results were compared to ACSF. Under these experiments, Apidra® was used to treat slices both in the static chamber and during recording of the AHP. Apidra® was continuously perfused into the flow of the oxygenated recording ACSF using a precision perfusion pump (Orion Research, Model 341B, Boston,
MA) and resulting in a final concentration of 1 nM (diluted in sterile saline once a week from the 600 uM stock). Only neurons with input resistance > 25 MΩ, holding current < 500 pA and action potentials reaching above zero mV were included in the analysis.

**Statistics:** Statistical analyses testing for significance (p<0.05) of main factors and interactions used a 1-way or a 2-way ANOVA as well as a t-test design (some electrophysiology). Fisher’s PLSD was used for post-hoc comparisons. All tests were conducted using StatView statistical package (version 5, Cary, NC) or GraphPad Prism V5 (San Diego, CA). Together the behavioral and electrophysiological data presented here are derived from a total of 106 animals.

2.3 Results

**Blood analyses.** Several studies show that intranasal insulin either has no effect, or a moderate lowering effect (within the euglycemic range) on peripheral blood glucose [31, 367-369, 383, 391, 392]. A 2-way RM ANOVA shows intranasal insulin did not alter peripheral blood glucose levels in a subset of the aged animals (n = 5/group) 30-90 min after intranasal insulin Levemir® delivery (supplemental Table 1). In another group of aged animals, 40 IU Humalog® also was unable to alter blood glucose levels. This result is consistent with transport of insulin to the perivascular space around blood vessels, indicating the hormone may not have crossed into the blood stream [34, 386].
**Behavioral analyses.** All groups learned to find the platform in the MWM as demonstrated by a significant decrease in path length to goal across the first 3 days of training ($F_{(2,116)}=27.2; \ p<0.0001$). During this learning phase, path length (Fig. 1A) however, showed no significant differences across groups with age or intranasal insulin dose (2-way RM ANOVA). As shown previously [387, 393], analysis of swim speed revealed a significant group effect ($F_{(5,58)}=12.5; \ p<0.0001$) with post-hoc tests indicating aged animals swam at slower speeds compared to young animals, but no difference between the insulin treatment groups was noted (Fig. 1D). Latency to target, as predicted from slower speeds in aged animals, was significant across 3 days of training ($F_{(2,116)}=28.9; \ p<0.0001$), with increased latency seen in all aged animals compared to young saline animals (data not shown). Because of this decrease in swim speed we chose to assess search errors and quantify memory performance on the probe day (no platform) using a proximity measure analysis. This approach is less dependent on swim speed than latency and is tabulated using a cumulative search error derived from the subject’s distance to the platform summed over the time to reach the platform and is then subtracted from an ideal path (straight line); smaller values are indicative of better performance [394].

Compared to the learning component of the task, the 24 h memory recall component measured during the probe trial (1-way ANOVA) revealed a significant group effect on path length to goal ($F_{(5,58)}=2.9; \ p<0.05$) and on
cumulative search error ($F_{(5,58)}=3.3; p<0.05$). Post-hoc analyses identified a significant increase on path length and cumulative search error in the aged saline (p<0.05) and aged medium-dose Levemir® (p<0.005) groups compared to the young saline group (Figs. 1B and 1C). A trend (p=0.059) for improved performance was found for the high-dose Levemir® animals on path length measures only. Notably, no significant differences were found between young saline treated and aged animals treated with either low-dose Humalog® or low-dose Levemir® on path length or cumulative proximal distance (Figs. 1B and 1C), indicating that the lower dose intranasal insulin significantly reduced age-dependent memory loss. Additional post-hoc analyses on memory performance in aged animals, comparing combined low dose insulins (Humalog® and Levemir®) with combined medium- and high-dose Levemir®, revealed significant improvement in the low-dose insulin group on path length ($F_{(1,33)}=5.25; p<0.05$) and cumulative search error ($F_{(1,33)}=5.91; p<0.05$). Thus, each of the different insulin formulations (Humalog® and Levemir® at low-doses) improved performance despite being administered to two separate cohorts of aged animals, indicating reproducibility of the insulin effect, and it appears lower, rather than higher doses of insulin are able to redress cognitive decline with aging.

**Electrophysiology analyses.** Based on improved memory recall observed in aged animals treated with low-dose insulins, we next characterized the effects of
in vivo intranasal insulin on hippocampal electrophysiology in a subset of the Humalog® treated animals that were behaviorally characterized. Supplemental Table 2 shows that passive membrane properties were not different between groups and suggests cells with comparable baseline characteristics were analyzed. Intracellular recording of CA1 pyramidal neurons were obtained from animals treated with intranasal saline or Humalog® (see Methods). Approximately 3-7 hours following intranasal insulin delivery the Ca2+-dependent AHP was not significantly different between groups (Figs. 2B and 2C; t-test). This was true for measures of AHP amplitude (medium and slow AHP) and duration (not shown). Extracellular synaptic potentials also showed no significant difference across groups (Figs. 2D and 2E; t-test). EPSP amplitudes measured at 33% of the I/O were slightly larger in the Humalog® treated animals and I/O slopes based on EPSP amplitude measures were somewhat reduced (Figs. 2D and 2E, respectively, p<0.1). These results are surprising given our prior report that insulin application to hippocampal slices reduces the AHP [380]. In the prior study, however, hippocampal slices were perfused for 15 minutes with insulin and then studied electrophysiologically. The results obtained in the present study raise the possibility that the delay between intranasal insulin exposure and electrophysiological recording (3-7 h) was too long and that the effect of insulin on the AHP may have dissipated. Thus, to confirm that insulin must be present in order to alter hippocampal physiology, we tested a range of ex-vivo insulin
concentrations and formulations on hippocampal slices from a separate group of animals.

We used young animals in the next series of experiments, and examined three different doses of Humalog®, comparing Humalog®’s actions to zinc and ACSF. In most insulin formulations, zinc is used to promote hexamer formation and prolong stability and duration of action \textit{in vivo}. However, zinc is also known to modify ion flux across neuronal membranes, reducing activity at NMDA and GABA\textsubscript{A} receptors, ion channels associated with memory-related processes [reviewed in 395]. Extracellular recordings in the presence of zinc or Humalog® showed that neither EPSP amplitudes, nor \textit{I/O} slopes were significantly altered when compared to ACSF controls (Figs. 3E and 3F; 1-way ANOVA). Similarly to data shown in figure 3, these results indicate little if any, effects of insulin on extracellular postsynaptic potentials.

Intracellular recordings obtained from the same group of animals showed that the Ca\textsubscript{2+}-dependent AHP was sensitive to treatment with zinc or insulin. As previously reported, measures of postsynaptic excitability demonstrated the sensitivity of the AHP to both zinc [396] and insulin [380]. A main effect of treatment on the slow AHP (F\textsubscript{(4,18)}=3.34; p<0.05) and its duration (F\textsubscript{(4,18)}= 4.49; p<0.02) was seen across 5 groups of recorded cells (Figs. 3C and 3D). Albeit being somewhat reduced, the mAHP was not significantly altered by insulin (Fig.
These results suggest a component of the insulin effect on the AHP could well be mediated by zinc found in this insulin formulation. To test the direct impact of ex vivo insulin on the AHP, we next investigated a zinc-free formulation of insulin (Apidra®).

In this next series of experiments we obtained hippocampal slices from young (n=8) and aged (n=9) F344 rats and focused exclusively on intracellular potentials and the response to ex vivo Apidra® exposure (1 nM). Further, a recent study identified that 0.1-100 nM exogenous insulin concentrations could reliably elicit dose-dependent activation of the insulin signaling pathway in human brain tissues [20], and approximated physiological levels near 1 nM. For these reasons, we used 1 nM Apidra®. As previously reported [377-379, 397] and shown here (Fig. 4), mAHP and sAHP amplitudes recorded in CA1 pyramidal neurons were increased (F_{1,24}=17.6, p<0.0005; F_{1,24}=4.7, p<0.05, respectively) in aged, compared to young animals (2-way ANOVA). Treatment with the zinc-free Apidra® insulin significantly reduced the AHP in slices from both young and aged rats as indicated by a main effect of treatment on the mAHP (F_{1,24}=32, P<0.0001) and the sAHP (F_{1,24}=29.1, p<0.0001). However, the Apidra®-mediated AHP reduction was larger in aged animals compared to young animals (Figs. 4A and 4B) as evidenced by a significant interaction term in the 2-way ANOVA (F_{1,24}=10.9, p<0.005 for the mAHP). These results are consistent with our prior work using Humalog® showing that the hippocampal AHP is sensitive to...
insulin [380]. In the experiments presented here however, it is clear that insulin alone has a direct effect on the AHP, and that this effect persists even when lower, more physiological glucose levels are used in the ACSF (experiments in Figure 4 were conducted with 4.5 rather than the typical 10 mM glucose concentration - see Methods). Finally, it is interesting to note that Apidra® did not alter holding current when compared to control cells (sup. Table 2), indicating insulin is neither activating nor inhibiting a tonic current near resting potential.

2.4 Discussion

Studies in humans have shown that intranasal insulin has promising cognition enhancing effects, alleviating, and perhaps even slowing the progression of age-related neurodegenerative disorders. However, the mechanisms underlying insulin’s effects in the brain are unclear. In order to identify potential mechanisms, we treated aged rodents with intranasal insulin, attempting to model aspects of the clinical trials reporting on the beneficial impact of intranasal insulin. Here, we describe physiological actions of insulin in the brain, highlighting a potential new mechanism of action of insulin on hippocampal function in both young and aged rats. This first analysis of intranasal insulin in aged animals demonstrates that intranasal insulin improved memory recall in aged F344 rats. We show that the effect on memory was observed in two separate cohorts of animals, using two different insulin formulations (Humalog® and Levemir®)
delivered at relatively low dose (0.0715 IU/day/rat). Thus, these studies also demonstrate reproducibility of the effect of insulin on behavior. We also show that the Ca2+-dependent afterhyperpolarization (AHP), and in particular the slow AHP, a Ca2+-related biomarker of brain aging which increases with age and cognitive decline, is reduced by acute insulin exposure in aged animals and could well represent a novel target of brain insulin underlying the improvement seen in memory recall.

Long-acting insulin (Levemir®) was as effective as short-acting insulin (Humalog®) on memory recall in aged animals, inducing levels of performance indistinguishable from those seen in young animals. Levemir®’s long duration of action is dependent on the presence of albumin in the periphery and a fatty acid modification of the insulin structure, increasing its’ affinity for albumin. Humalog®’s shorter duration and faster onset are due to amino acid modifications in the β-chain of the insulin molecule. Because both versions of insulin were able to reverse cognitive decline to a similar degree in aged animals, our results suggest both insulins were able to gain access to the brain following intranasal administration despite structural differences. While results from clinical studies in memory-impaired older adults have reported improved word recall within 15 min post intranasal delivery [398], a prior clinical study on younger adults demonstrated the superiority of a faster acting insulin (Novolog®) compared to regular insulin (Actrapid®) on delayed word recall after 7 weeks of intranasal 160 IU/day Novolog® [392]. Using shorter delays (i.e., 10 min) a
recent study reveals 40 IU intranasal insulin delivery to young adult males improves odor-cued spatial memory [399]. Clearly, the memory enhancing effects of insulin are depend on treatment regimen (acute vs. subchronic), but also on the age and gender of the subject [400], as well as on the insulin formulation used [382, 401]. As mentioned above, a single, acute intranasal dose of insulin is capable of enhancing memory in humans and our electrophysiological studies showing an acute action of insulin on the AHP indicate that insulin can have rapid effects on neuronal processes critical to memory (see below). However, in the current study, it is unclear whether the effects on cognition observed here were due to an acute action of insulin (i.e., the dose received 1-3 h prior to memory testing), or the result of the cumulative daily treatments received over the course of 8-11 days.

The decrease in the AHP by insulin was observed in field CA1 of the hippocampus, a synaptic zone which plays a key role in memory processing. Decreasing the AHP in this structure would be expected to increase neuronal excitability and facilitate throughput during physiological activation. Consistent with this, exogenous insulin increases excitatory neurotransmitter receptor trafficking including NMDA and AMPA receptors [88, 358, 359, 402], thus promoting network activation. Furthermore, pure insulin has been shown to activate an inward current and depolarize hypothalamic neurons via activation of transient receptor potential channels, providing supporting evidence that insulin raises excitability [403]. However, recent results provide evidence that insulin or
other therapies that raise insulin (exendin-4 and glucagon-like peptide-1), increase functional GABA_A conductances in CA1 [352] as well as in CA3 pyramidal neurons [404]. This observation is in line with prior reports of enhanced GABA_A function in response to insulin, a resulting decrease in synaptic activity [350, 351] and a long-lasting enhancement of inhibitory post-synaptic currents (IPSCs) [405]. The net result, therefore, of enhancing both steady-state hyperpolarizing (GABA-mediated) and short term depolarizing forces (e.g., reduced AHP or direct depolarization) could well increase synaptic signal-to-noise, thereby improving throughput and neuronal communication. Clearly, as seen in hypothalamic arcuate nucleus neurons, it is important to note that insulin’s modulatory influence on membrane potential/ excitability, is insulin formulation dependent, as well as cell-type specific [403].

To extend slice health and allow for extended neuronal recordings, high glucose concentrations (10 mM) are commonly used in the ex-vivo hippocampal slice. However, this superphysiological glucose concentration may alter insulin sensitivity in the brain. For this reason, we used lower glucose levels in some experiments while maintaining tissue health (see Table 2), and more closely approximating physiological glucose levels. Importantly, significant AHP reduction was seen irrespective of recording conditions (10 or 4.5 mM glucose). Thus, the results presented here appear compatible with potential effects of insulin on hippocampal neurons in vivo.
The placebo group in almost all clinical trials studying the impact of intranasal insulin on memory function is generally exposed to either saline or HOE-31, a dilution buffer that does not contain zinc. Given that all insulin formulations except Apidra® contain zinc, our results showing that Apidra® is capable of reducing the AHP in hippocampal neurons ex vivo could have clinical significance. These data are consistent with insulin per se being able to alter hippocampal physiology, and suggest that insulin and not zinc may be responsible for the CNS effects of intranasal insulin on memory. Clearly further clinical trials with Apidra® appear warranted.

Because the AHP is dependent on a Ca2+-activated K⁺ conductance, the insulin-mediated reductions in the AHP may be due to inhibition of Ca2+ influx [406] or inhibition of L-type Ca2+ channel currents as seen in pinealocytes [407]. Taken together, these results suggest insulin acts through the insulin receptor to reduce Ca2+-mediated functions possibly through PI3 kinase- or tyrosine kinase-mediated phosphorylation of Ca2+ channel proteins. Indeed, insulin can enhance mitochondrial function and therefore, Ca2+ homeostasis through PI3 kinase mechanisms in the periphery [193]. Insulin also increases sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) function in heart cells via an Akt (protein kinase B) pathway, and in neurons, insulin has been shown to reduce KV1.3 potassium channel function [408]. Notably, all of these potential mechanisms are consistent with reductions in available Ca2+, and hence, reductions in the amplitude of Ca2+-dependent hyperpolarizing potentials.
Our data cannot address whether the beneficial actions of intranasal insulin on memory recall are mediated, in parts or in whole, by the effects of insulin on the AHP. While suggestive that this may be the case, we do not provide evidence to support this conclusion. Further, because young animals were not tested for the impact of intranasal insulin, future studies will be needed to test the hypothesis that the observed effect is age-selective, or generally nootropic. Finally, although a similar insulin dose yielded quantitatively similar enhancement on memory recall in two cohorts of aged animals, it is not clear that higher Humalog® doses might also be ineffective, similarly to higher Levemir® doses.

The present results shed new light on a previously unrecognized insulin mechanism in the brain. Reductions in the AHP could enhance neuronal communication and might represent a pathway through which low dose Humalog® or Levemir® improve memory recall in aged animals. We show that insulin targeted the AHP, a Ca2+-mediated conductance, and reduced AHP amplitude and duration, elevations of which are characteristic of brain aging. We propose that facilitating insulin signaling restores Ca2+ homeostasis in aged animals, resulting in optimal levels of membrane excitability and synaptic plasticity, in part by limiting the amplitude and duration of the AHP in CA1 neurons (Figs. 4 and 5). During aging, reductions in brain insulin levels and/or insulin receptor function (see Introduction) may help promote neuronal Ca2+ dysregulation, resulting in impaired membrane excitability and reduced synaptic plasticity (e.g., larger AHP). Clearly, given the reductions in insulin receptor RNA
and protein in the aged rodent [27, 111], greater occupation of the leftover insulin receptors with insulin could well offset reductions in signaling through these receptors, or may even slow the age-related reduction in insulin receptor density. Collectively, these results indicate robust associations between brain insulin, Ca2+ homeostasis, and aging-related cognitive decline, and suggest the value of further developing “insulin-raising” strategies for treating cognitive decline in age and disease, perhaps curbing the development of AD.
Chapter 3 Link between studies

3.1 Intranasal insulin and cognition

In the late 1990s, several clinical studies suggested that diabetes is a risk factor for cognitive decline and AD [142, 409, 410]. Also, through population-based studies, it has become clear that dysregulation in insulin signaling increases the risk for cognitive decline with aging and AD [139, 411-417]. In addition, imaging studies have shown that diabetes patients have smaller hippocampi and that brain functional connectivity also changed compared to healthy individuals [418-422]. Furthermore, studies have found that insulin receptors are widely expressed in different regions of the brain and play a vital role in regulating whole-body glucose metabolism, energy balance, and neurotransmission in the brain [423]. In addition, aging can alter insulin and insulin receptor function, reduce pancreatic beta cells function, and reduce insulin secretion by pancreatic beta cells [424, 425]. These studies ignited interest in the effects of insulin in the brain in the context of cognition and led the way for later research confirming that insulin is an important cognitive enhancer.

Interest in the study of intranasal insulin has also stemmed from a number of clinical studies demonstrating that intravenous insulin infusion during clamped glucose conditions improved memory [15, 29, 137, 149]. Clearly, due to the danger of hypoglycemia, insulin infusion peripherally is not a feasible approach for treating AD. On the other hand, it has been shown that intranasal insulin
delivery targets the brain, bypassing the blood brain barrier, and without the risk of hypoglycemia [349, 426]. There are several ongoing intranasal insulin clinical trials, including study of nasal insulin to fight forgetfulness (SNIFF), intranasal insulin in children and young adults at risk of type 1 diabetes, intranasal insulin for the HAND (HIV-associated neurocognitive disorder), and intranasal insulin for stroke patients. My research is focus on the neuronal effects of intranasal insulin on the context of cognitive decline with aging.

Intranasal administration of insulin appears to be one of the best methods to treat cognitive decline due to its minimal effect on systemic glucose levels and its acute improvements on hippocampal-dependent memory. Intranasal insulin enters the brain mainly through olfactory and trigeminal pathways [427-429]. Insulin binds to insulin receptors in various brain regions, including the hippocampus [430]. The hippocampus is well-known as a principle brain region for learning and memory. Normal insulin signaling of neurons within the hippocampus area is typically associated with healthy brain aging. Furthermore, deficiency in brain insulin signaling accelerates pathological brain aging and cognitive decline. The goal of intranasal insulin study in our lab is to achieve a greater understanding of the effects of insulin and insulin signaling pathways that might slow or reverse age-related memory decline and pathologies.

We found that a repeated low dose of intranasal insulin had a positive effect on memory recall in aged F344 rats. However, medium and high doses of insulin did not have a beneficial effect on memory in aged F344 rats. This varying
outcome is in line with clinical intranasal insulin studies. Clinical studies show that intranasal insulin therapy improves memory maintainability in healthy and AD patients [32, 431]. Although intranasal insulin enhances memory in AD patients, varying outcomes arise from its clinical setting usage [431]. Men and women with AD respond differently to intranasal insulin dosages [36]. In addition, some clinical studies have reported that patients carrying at least one copy of the Apolipoprotein E4 (the strongest genetic risk factor for late onset Alzheimer’s disease) did not benefit from intranasal insulin treatment [37]. Hence, these clinical trials revealed that not everybody responds equally to intranasal insulin therapy. Based on both clinical and animal studies, different formulations and dosages of insulin, as well as gender and ApoE status in AD patients increase the outcome variability in intranasal insulin treatment. A clear understanding of the mechanisms underlying insulin actions in the brain will help address these varying outcomes and increase the efficacy and safety of using intranasal insulin to treat cognitive impairment.

3.2 Insulin and Ca2+-dependent after hyperpolarization

It is also well documented that the Ca2+-dependent AHP (Ca2+ related biomarker of brain aging) increases in aged and memory-impaired animals. The greater AHP is correlated with reduced neuronal excitability and learning ability [30, 184, 377, 379, 380, 432-437]. It is widely accepted that reducing the AHP is one viable pathway to enhance learning and memory in aging animals. Data
from our lab suggests that insulin reduces the AHP in CA1 neurons from young and aged rat hippocampal slices [30, 380]. These findings are in line with previous behavioral studies, suggesting that insulin therapy improves memory by reducing the Ca2+ dependent AHP [30]. However, the molecular mechanism(s) that result in such an interaction has not been studied in depth. Ca2+ and potassium are the main ions that influence the amplitude and duration of AHP. Here, I focused on the relationship between insulin and intracellular Ca2+ homeostasis.

Another factor besides insulin that is related to cognitive decline in aging and AD is elevated intracellular Ca2+ concentration [187-191]. The two main sources of intracellular Ca2+ are Ca2+ influx via voltage-gated Ca2+ channels (VGCCs) on the plasma membrane and Ca2+ efflux via ryanodine receptors (RyRs) mediated Ca2+-induced Ca2+-release (CICR) on the endoplasmic reticulum.

3.3 Voltage-gated Ca2+ channels dysfunction with aging
Voltage-gated Ca2+ channels (VGCCs) are responsible for Ca2+ influx into the cytosol in response to membrane depolarization. VGCCs play a critical role in neuronal excitability, neurotransmission and Ca2+ signaling, which is important for learning and memory in healthy and disease states. All types of VGCCs are expressed in the hippocampus and involved in neuronal firing, action potential, and synaptic plasticity [438]. Furthermore, the density of plasma membrane VGCCs increases with aging and results in elevated intracellular Ca2+ levels,
which is toxic to neurons and leads to cell death [226, 439-441]. Earlier studies showed that elevated Ca2+ influx via VGCCs contribute to bigger AHP and cognitive decline in aged animals [184, 224, 437, 442, 444-446]. In addition, voltage-gated Ca2+ channel blockers have been shown to improve cognition in aged animals, as well as in dementia and AD patients [447-450]. Moreover, knockout of voltage-gated Ca2+ channels have a great impact on memory in aged animals [451, 452]. In hippocampal neurons, Ca2+ entry though VGCCs during membrane potential triggers Ca2+-induced Ca2+-release from endoplasmic reticulum via the ryanodine receptors, causing amplification of intracellular Ca2+ levels [220, 377, 435, 453].

### 3.4 Ryanodine receptors dysregulation with aging

Ryanodine receptors (RyRs), an intracellular Ca2+ release channel, is responsible for Ca2+ release from endoplasmic reticulum (ER) and triggers amplification of intracellular Ca2+ levels through RyR-mediate Ca2+-induced Ca2+-release (CICR). RyRs plays a vital role in maintaining intracellular Ca2+ homeostasis. Thus, RyR-mediated Ca2+ release is critical to cellular physiological events ranging from muscle contraction to learning and memory. All RyRs isoforms (RyR1-3) are expressed in the various regions of the brain, including the hippocampus, cerebellum, olfactory region and cerebral cortex, which are located within the presynaptic terminals of neurons. The RyRs channels have been found to show changes in functional efficacy during
aging/AD [136, 377, 435, 453-458] and influence the amplitude and duration of Ca2+ dependent afterhyperpolarization (AHP) [192, 377]. Additionally, increases in RyRs-mediated intracellular Ca2+ release through CICR mechanisms during aging results in loss of intracellular Ca2+ homeostasis, thus greatly influencing cognitive function [192, 377, 459].

Taken together, age-related increases in RyRs-mediated intracellular Ca2+ release mechanisms are also responsible for larger AHP seen in memory impaired aged animals. Ultimately, lowering Ca2+ efflux via RyRs is important for reducing the AHP.

However, a central question of whether insulin reduces VGCCs currents remains unanswered. Furthermore, it is not clear whether insulin affects RyRs function. To address these questions, the activity of VGCCs was recorded from neurons that were treated with acute (10 minute) insulin vs. control solutions. In parallel studies, Ca2+ imaging was used in cultured hippocampal neurons to evaluate the RyRs-dependent intracellular Ca2+ level changes in insulin treated neurons. Our approach helped us to delineate one of the many neuronal molecular mechanisms underlying insulin’s effect on memory through the reduction of AHP via regulating intracellular Ca2+.

The following manuscript has been submitted to Neuroscience. In this manuscript, I continue to study the underlying mechanisms responsible
for the memory enhancing effects of intranasal insulin in aged animals. It is clear that Ca2+-dependent AHP is one of insulin’s neuronal targets from the previous study presented in this dissertation (Chapter 2). Those data led me to focus further on the relationships between insulin and hippocampal intracellular Ca2+, which influence the amplitude and duration of AHP. Voltage-gated Ca2+ channels and ryanodine receptors are major factors that contribute to dysregulation of intracellular Ca2+ homeostasis present in aged animals. Hence, I tested acute insulin’s effect on single live hippocampal neurons in culture using whole-cell patch clamping. In addition, insulin effects on resting and depolarization mediated intracellular Ca2+ transients were examined in the single live neurons using Ca2+ imaging techniques.
Chapter 4 Novel Ca\(^{2+}\)-related targets of insulin in hippocampal neurons

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Synopsis

Both insulin signaling disruption and Ca\(^{2+}\) dysregulation are closely related to memory loss during aging and increase the vulnerability to Alzheimer’s disease (AD). In hippocampal neurons, aging-related changes in Ca\(^{2+}\) regulatory pathways have been shown to lead to higher intracellular Ca\(^{2+}\) levels and an increase in the Ca\(^{2+}\)-dependent afterhyperpolarization (AHP), which is associated with cognitive decline. Recent studies suggest that insulin reduces the Ca\(^{2+}\)-dependent AHP. Given the sensitivity of neurons to insulin and evidence that brain insulin levels are reduced with age, insulin-mediated alterations in Ca\(^{2+}\) homeostasis may underlie the beneficial actions of insulin in the brain. Indeed, increasing insulin signaling in the brain via intranasal delivery has yielded promising results such as improving memory in both clinical and animal studies. However, while several mechanisms have been proposed, few have focused on regulation on intracellular Ca\(^{2+}\). In the present study, we further examined the effects of acute insulin on Ca\(^{2+}\) pathways in primary hippocampal neurons in culture. Using the whole-cell patch-clamp technique, we found that acute insulin delivery reduced voltage-gated Ca\(^{2+}\) currents. Fura-2 imaging was
used to also address acute insulin effects on spontaneous and depolarization-mediated Ca2+ transients. Results indicated that insulin reduced Ca2+ transients, which appears to have involved a reduction in ryanodine receptor function. Together, these results indicate insulin regulates pathways to control intracellular Ca2+ which may be responsible for reducing the AHP and improving memory. This may be one mechanism contributing to improved memory recall in response to intranasal insulin therapy in the clinic.

**Keywords:** diabetes, intranasal, excitability, imaging, electrophysiology, aging
4.1 Introduction

Aging is a major risk factor for Alzheimer’s disease (AD), and both brain aging and AD are characterized by a progressive decline in cognitive and memory function. A promising treatment for cognitive impairment seen in AD is to maintain or enhance insulin signaling in the brain. Insulin is a cognitive and neural modulator that is synthesized by pancreatic β-cells and enters the brain through the blood brain barrier [460-463]. In the periphery and in the brain, insulin has been shown to bind to insulin receptors and to regulate glucose uptake by inducing translocation of glucose transporters to the plasma membrane [464]. In aging or AD, declining insulin levels, insulin receptor numbers, and/or glucose transporters can lead to dysregulation in glucose uptake. This is evidenced by nearly two decades of research showing strong associations between diabetes, cognitive decline, and AD [16, 141-144]. At the cellular level, brain insulin deficiency and reduction in insulin signaling, perhaps mediated by insulin resistance, could represent one of the altered pathways linked to altered memory function or synaptic communication during aging and AD [15-20, 30]. Underlying this relationship is evidence that insulin receptor numbers and their functions are decreased in aging and AD animal models [111, 346, 465, 466]. As such, interruption of insulin production or in insulin receptor activity may cause cognitive decline [323]. One approach designed to combat this reduction in insulin signaling that has received much interest in the clinic is the use of intranasal insulin delivery to selectively increase ligand concentration in the brain [349].
Intranasal insulin therapy has been shown to improve memory function in AD patients [32, 431]. The intranasal route of insulin administration raises insulin acutely in the central nervous system without much risk of peripheral hypoglycemia [30, 367, 426, 467, 468]. Importantly, it significantly enhances memory in both healthy individuals and AD patients [31, 33-35, 37]. In aging and AD animal models, the positive impact of intranasal insulin in combating cognitive decline has also been reported [30, 381, 467, 469-473]. The mechanisms of action in the brain, and specifically on neurons of the hippocampus where insulin plays a recognizable role in learning and memory [28, 111], remain however, largely unknown.

Because previous work on mechanisms of cognitive aging has focused on Ca2+ dysregulation in hippocampal neurons providing evidence of enhanced Ca2+ levels [377, 439, 445, 474-476] and voltage-gated Ca2+ channels (VGCC) activity [224, 432, 445, 477], we focused on studying two well-characterized Ca2+ sources in hippocampal neurons in culture. The rationale for this approach was based on recent evidence from our lab that insulin acutely reduces the Ca2+-dependent afterhyperpolarization (AHP) in neurons recorded from hippocampal slices [30, 380]. The AHP is a hyperpolarization potential that is enhanced in aging, limits neuronal firing, and is associated with cognitive decline [379, 380, 433, 478]. The larger AHP seen in aging is mediated in part by an increase in the density of L-type voltage gated Ca2+ channels (L-VGCC) [224] and by Ca2+-induced Ca2+-release (CICR) through activation of RyRs [377, 479, 480]. Together, these two sources of Ca2+ contribute to elevated
intracellular Ca2+ levels, larger AHPs and altered synaptic communication. Thus, here, we tested the hypothesis that acute insulin (glulisine, zinc-free and fast acting) could reduce Ca2+ levels, or stabilize Ca2+ homeostasis by altering VGCC function and/or ryanodine receptor (RyR) function. To address these questions, we used patch clamp recording of VGCCs with rapid drug delivery, as well as Fura-2 imaging during depolarization in the presence or absence of RyR blocker. Our results indicate that insulin is able to reduce Ca2+ levels during periods of neuronal depolarization and that this is mediated, at least in part, by reductions in VGCC and RyR function. These two aging-sensitive neuronal Ca2+ targets are therefore also sensitive to the actions of insulin and could represent novel therapeutic targets for cognitive decline in aging and/or AD. Re-establishing Ca2+ homeostasis represents a mechanism by which insulin and by extension, intranasal insulin, may offset learning and memory dysregulation in vivo.

4.2 Experimental procedures

Cell culture: Hippocampal mixed (neuron/glia) cultures were prepared as described previously [439, 481, 482] and established from (E18) Sprague-Dawley rats. E18 pups and hippocampi were dissected under a microscope in ice-cold Hank’s balanced salt solution (Thermo Fisher Scientific Inc., MA, USA) supplemented with 4.2 mM NaHCO3, 10 mg/L gentamicin and 12 mM HEPES (pH 7.3). Hippocampi were transferred to a 37°C 0.25% Trypsin EDTA solution.
(Thermo Fisher) and left at room temperature for 11 minutes. Trypsin was removed and the hippocampi were washed three times with Minimum Essential Medium (MEM). Hippocampi were then titrated, and diluted with MEM to the desired final concentration (5-7 x 10^5 neurons/ml) before being plated onto 35 mm poly-L-lysine coated dishes. Cultured neurons were incubated (36°C, 5% CO₂, 95% O₂) for 24 h before the first medium exchange. At this time, half of the medium was replaced with 90% SMEM supplemented with 10% Horse serum. After three days in vitro (DIV), half of the medium was replaced with SMEM, horse serum, 5-Fluoro-2-Dioxynuridine and uridine to stop glial cell growth. At DIV 10, a sodium bicarbonate solution (200 uL) was added to help maintain pH and limit evaporation.

For whole-cell recording experiments, plastic culture dishes were used (Corning Inc., Corning, NY, USA) and for Ca²⁺ imaging experiments, glass bottom culture dishes (Mattek Crop., Ashland MA, USA) were used. All data presented were collected between DIV 13 and 17, and experiments were conducted following a 24 h exposure to lower glucose-containing MEM (5.5 mM; MEM with no added glucose). This was done to maintain normal glucose oxidation rates and insulin sensitivity [482]. All data presented were obtained at room temperature.

**VGCC recording solution:** For whole-cell, external recording solution of VGCC currents, was as follows (in mM): 111 NaCl, 5 BaCl₂.H₂O, 5 CsCl, 2 MgCl₂, 10 glucose, 10 HEPES, 20 TEA.Cl.H₂O, pH 7.35 with NaOH, and 500 nM tetrodotoxin (TTX) was added before recording to inhibit Na⁺ channels. The internal pipette solution (in mM): 145 CH₄O₃S-methanesulfonic acid, 10 HEPES,
3 MgCl₂, 11 EGTA, 1 CaCl₂, 13 TEA.Cl.H₂O, 14 phosphocreatine Tris-salt, 4 Tris-ATP, 0.3 Tris-GTP, pH 7.3 with CsOH. All solutions were sterile filtered.

**Drugs and solution application:** External solutions were delivered using a rapid solution exchange system (SF77A - Warner Instruments Corp. Hamden, CT, USA) positioned approximately 400 um above the cell being recorded. Flow rate was set to 0.3 mL/min and a control solution supplemented with TTX was used to establish baseline recording (~10 min) during which passive cell membrane properties were obtained (e.g., membrane resistance, capacitance, access resistance), and currents were allowed to run up. Rapidly switching the position of the tubing delivering the solutions above the recorded or imaged cell allowed us to tightly control the environment and deliver either insulin, KCl (50 mM), ryanodine (20 uM), a high-affinity small peptide interacting with insulin (affibody, ab31906, Abcam, Cambridge, MA, USA; 100 ng/mL, 500 ng/mL, or 1 ug/mL), or an insulin receptor antibody (S961, Phoenix Pharmaceuticals Inc., Burlingame, CA., USA; 500 ng/mL). Insulin glulisine (10 nM, a fast acting, zinc-free insulin) was prepared weekly in external recording solution from a 6 uM stock (Apidra® 600 uM, Sanofi-Avantis US. LLC, diluted in sterile saline). This concentration was chosen based on our previous results from our lab [30, 380].

**Whole-cell recording and analysis:** To minimize capacitance artifacts, whole-cell patch-clamp electrodes were coated with polystyrene Q-dope before recording (see Table 1 for membrane and electrode properties). The culture dish was rinsed with external solution twice and then supplemented with TTX. To allow for currents to stabilize, all data were recorded 5-10 min after the whole-cell
configuration was achieved. Current/voltage (I-V) relationships (-60 to + 30 mV) were used to identify the voltage step eliciting the maximal current amplitude. This voltage was used for each cell in the study in order to compare data at the peak of the I-V relationship (150 ms depolarization). Cells were either held at -70 mV (150 ms) or -40 mV (350 ms) and currents were elicited at the maximal peak response. All currents were leak subtracted using 5-8 scaled hyperpolarizing sub-pulses. Because insulin may alter cell size, we report on measures of current densities (pA/pF), derived from dividing peak current amplitude by membrane capacitance (measured in pClamp) for each cell. All recordings were conducted on the stage of an epifluorescence microscope (E600FN - Nikon Inc., Melville, NY, USA) placed on an anti-vibration table. An amplifier (Axopatch 1D - Molecular Devices, Sunnyvale, CA, USA) in combination with an A/D board (Digidata 1200 - Molecular Devices) and acquisition software (pClamp 7 - Molecular Devices) were used for electrophysiology acquisition. Data were digitized at 5-10 KHz and low-pass filtered at 2-5 KHz and were quantified in Clampfit 7 (Molecular Devices).

**Ca2+ imaging and analysis:** Cultures were incubated in Ca2+ imaging solution (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 CaCl₂, 1MgCl₂, 0.01 glycine, pH 7.3 with NaOH for 30 min in the dark. This solution contained 2 uM Fura-2 AM (F1221 – Invitrogen) and was made monthly with fresh DMSO (0.085%) and Pluronic® F-127 (0.015%, weight/ volume). Each culture dish was then rinsed three times with Ca2+ imaging solution containing no indicator and placed in the dark for 20 min (de-esterification period). The dish was then placed
on the stage of the microscope. Intracellular Ca2+ transients were visualized by exciting Fura-2 at 340 +/-20 nm and 380 +/- 20 nm using a high-speed filter changer (Lambda DG4, Sutter instruments, Novato, CA, USA) to obtain a ratiometric value independent of indicator concentration. Emitted light was passed through a dichroic filter (400 nm high pass) and an emitter filter (520 +/-30 nm; Chroma Technology) and was digitized onto the sensor of an EMCCD camera (iXon-Andor Technology, Belfast, Ireland). Ca2+ transients were measured and analyzed using Imaging Workbench 5.0 (INDEC BioSystems Santa Clara, CA) as previously published [481, 482]. The greater ratio (340/380 nm) values reported reflect higher Ca2+ levels. The gray value for both excitation wavelengths of each cell measured (region-of-interest - ROI drawn around soma) was background subtracted from an area devoid of cellular components. When imaging Ca2+ during periods of depolarization (50 mM KCl), the Ca2+ imaging solution was modified with a reduction in NaCl to 97 mM in order to control for osmolarity changes.

**Statistics:** Data were analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Paired t-tests and repeated measures ANOVA were used to test for group differences with significance set at p< 0.05. For electrophysiology experiments, data from each recorded cell (perfusion conditions precluded recording 2 cells per dish) was considered a single data point (*i.e.*, n=1). For Ca2+ imaging data, 5-10 cells in the field of view were averaged and considered a single data point (*i.e.*, n=1).
4.3 Results

4.3.1 Effect of insulin on whole-cell Ca2+ currents.

Ca2+ currents were recorded before and after a 10 mins perfusion of either external solution (time control; n=19), or external solution supplemented with 10 nM insulin (n=17) using the SF77A fast perfusion system. Cells were held at -70 mV and were stepped to the peak voltage determined from the I-V relationship. Peak and late currents (obtained immediately prior to termination of the voltage step) were quantified and are presented normalized to cell size (pA/pF). A significant decrease in peak (-20%; p<0.0001) and late (-31%, p<0.002) Ca2+ currents in response to acute insulin application was seen (Figure 1A-C). Based on a subset of the cells recorded (n=7 per group), there was no change in the I-V relationship (Figure 1D).

To determine if the actions of insulin were selective for L-type Ca2+ channels, cells were held at -40 mV and stepped (350 ms) to peak voltage. Whole-cell recording analysis indicated that similarly to reductions seen from a holding potential of -70 mV, L-type-enriched Ca2+ currents were significantly reduced (Figure 1E and F) both at the peak (-22%; p<0.0004) and during the late current phase (-33%; p<0.005). The data suggest that insulin did not alter the inactivation rate of currents (Figure 1A), and did not show a greater inhibitory effect on currents elicited from -40 mV. This indicates insulin does not selectively reduce L-VGCCs but instead, may reduce all VGCCs.
Figure 4.1 Ca2+ channel currents are reduced in response to acute insulin application in mixed hippocampal cultured neurons.
A. Example of VGCC currents recorded during recording solution perfusion (black) and after 10 min insulin (grey) during maximal step depolarization from -70 mV. B.-C. Quantification across groups of cells following 10 min of insulin perfusion (Insulin) versus recording solution perfusion (Time Control) shows peak and late Ca2+ currents were significantly reduced (p<0.05). D. In a subgroup of cells, current-voltage relationships were not significantly altered. E. Example of the effects of insulin on L-VGCCs recorded from a holding potential of -40 mV during recording solution perfusion (black) and after 10 min insulin perfusion (gray). F.-G. Compared to time control, recordings from -40 mV, max peak (F) and late Ca2+ current (G) were significantly reduced by insulin (p<0.05). The effect of insulin on VGCC does not appear to be L-VGCC selective. All data represent mean +/- SEM and are normalized to cell size (density, pA/pF), asterisks indicate significance at the p<0.05 level.

Table 4. Patch clamping parameters.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cm (pF)</th>
<th>Rm (MΩ)</th>
<th>Ra (MΩ)</th>
<th>HC (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bath (n=17)</td>
<td>50.8 ± 3.89</td>
<td>432.9 ± 32.0</td>
<td>10.6 ± 0.85</td>
<td>-102 ± 8.6</td>
</tr>
<tr>
<td>10 nM Insulin (n=19)</td>
<td>56.2 ± 3.5</td>
<td>415 ± 34.8</td>
<td>10.7 ± 0.77</td>
<td>-100 ± 8.7</td>
</tr>
</tbody>
</table>

All data presented as means± SEM on measurements of patched neurons membrane capacitance (Cm), membrane resistance (Rm), access resistance (Ra), and holding current (HC) (control n= 17, insulin n=19). No significant
difference was found between control and insulin treated neurons in any parameters measurements (p>0.05). Comparisons made using unpaired student t-test.

4.3.2 Effect of insulin on spontaneous Ca2+ transients.
We then used ratiometric Ca2+ imaging to test whether insulin could alter spontaneous activity in cultured neurons. We used the similar experimental time frame as presented in Figure 1 and monitored Ca2+ oscillations during 10 min of Ca2+ imaging solution perfusion (time control; n=11) and during the next 10 minutes when the solution was supplemented with 10 nM insulin (n=14). We show here that resting Ca2+ levels were not altered by acute insulin exposure (Figure 2D; p>0.05). Similarly, insulin did not change spontaneous network activity (no TTX) derived from measures of spontaneous Ca2+ events (Figure 2A – right). Those include measures of area-under-the curve (AUC; Figure 2 B; p>0.05), peak amplitude (Figure 2C; p>0.05), and the number of spontaneous events detected (Figure 2E; p>0.05) in control conditions and in insulin-treated experiments. These spontaneous Ca2+ transients were blocked by TTX (data not shown).
Figure 4.2 Spontaneous Ca2+ transients are not altered in response to acute
insulin exposure.

A. Example of neurons and ROI selection (green circles and numbered arrows-left). Right shows the three ROIs measuring somatic Ca2+ across time. This approach was used to quantify changes during 10 min of imaging solution perfusion (Time Control) as well as during 10 min of insulin perfusion (Insulin).

B.-E. Insulin perfusion did not change spontaneous Ca2+ activity based on measures of AUC, peak transient amplitude, or the number of events detected. Resting Ca2+ levels also were not altered by insulin. All data represent mean +/- SEM.

4.3.3 Effect of insulin on KCl-mediated Ca2+ transients.

We used potassium-mediated depolarization (30 s) in Ca2+ imaging experimental conditions to investigate the impact of insulin on overall neuronal Ca2+ homeostasis. This approach causes membrane depolarization with subsequent activation of VGCCs and Ca2+ influx through the plasma membrane, which in turn, activates Ca2+-induced Ca2+-release (CICR) from the endoplasmic reticulum through activation of RyRs [482-484]. Potassium-induced Ca2+ transients were reduced by 10 min of insulin perfusion (n=23) compared to 10 min of Ca2+ imaging perfusion (time control; n=14; Figure 3A and B). Consistent with the results on spontaneous Ca2+ fluctuations, insulin did not reduce resting Ca2+ levels measured immediately prior to the depolarization (Figure 3D). While a trend in reduction of the peak Ca2+ transient amplitude was
noted in response to insulin (Figure 3C; p=0.08), measures of the AUC of the depolarization envelope unmasked a significant insulin-mediate reduction (Figure 3B; p<0.0001). Notably, the reduction in the potassium-mediated Ca2+ response seemed to selectively reduce the delayed Ca2+ component or “hump”, present after the peak response (Figure 3A, bottom right). This brought to our attention a potential link to CICR, given that this response by nature, is often delayed compared to an initiating Ca2+ spike.
Figure 4. Effects of insulin on KCl-induced depolarization.
A. Pseudocolored images of cells in culture during Fura-2 imaged at rest (left) and during peak depolarization (right). Red colors indicate greater levels of Ca2+ (higher ratios). Two sequential KCl depolarizations were separated by 10 minutes of either imaging perfusion or insulin. Representative Ca2+ transients are shown and highlight measures of resting Ca2+, peak Ca2+ and AUC. Note the selective effect of insulin on reducing the late phase of the Ca2+ transient, essentially eliminating the “hump”. B. Ratiometric quantification during time control experiments or after insulin, shows the AUC resulting from KCl-induced depolarization was significantly decreased by insulin. C.-D. Peak ratio or resting ratio measures were not altered by insulin. A trend for peak ratio levels in response to insulin was noted. All data represent mean +/- SEM. Asterisks indicate significance at the p<0.05 level.
4.3.4 Insulin appears to reduce CICR function.

To test whether the reduction in the “hump” during potassium-induced depolarization reflected inhibition of the CICR component, we used high concentrations of ryanodine (20 uM) which significantly reduce CICR [377, 474]. These experiments required three 30 s repeated potassium depolarizations each separated by 10 min (time control; Figure 4). To test for the impact of ryanodine and/ or insulin, the first depolarization was triggered after 2 min of imaging solution perfusion, the second depolarization occurred after 10 min of ryanodine perfusion, and the third depolarization was after an additional 10 min of insulin perfusion (Figure 4A). As shown in Figure 4A and B, blocking CICR with ryanodine during potassium mediated depolarization (n=10) significantly reduced the “hump” after the initial high amplitude transient (Figure 4B, F_{(2,29)} = 53.8; p <0.0001). As consistently shown throughout our studies, time control experiments show no significant difference in the variables measured (n=11). Post-hoc analyses revealed no significant difference in the AUC obtained under ryanodine or insulin conditions. Thus, addition of insulin had no further impact on this Ca2+ plateau, indicating that high ryanodine concentration may have occluded the impact of insulin. The lack of an additive effect between insulin and ryanodine actions suggests insulin may reduce CICR by inhibiting ryanodine receptors.
Figure 4.4 Ryanodine receptors are likely targets of insulin actions.

A. Three sequential KCl-mediated depolarizations were used in these experiments and were triggered under imaging solution perfusion (left) and during perfusion with ryanodine, or insulin (right). Representative ratiometric Ca2+ transients highlight the impact of inhibiting CICR on the “hump”. Addition of insulin did not further reduce the “hump”. B. Quantification across the groups of cells imaged shows the significant insulin-mediated reduction in the AUC as in Figure 3. As expected, ryanodine (Rya) also significantly reduced the “hump”, indicating ryanodine and insulin may both be working on inhibition of RyRs. All data represent mean +/- SEM. Asterisks indicate significance at the p<0.05 level.
4.3.5 Insulin actions occur via occupation of the insulin receptor.

To investigate whether 1) components other than insulin in the glulisine formulation, or whether 2) the actions of insulin were mediated via occupation of the insulin receptor, we used an insulin binding peptide (i.e., affibody) and an anti-insulin receptor antibody, respectively. For these experiments, we used the same experimental protocol as presented in Figure 3 with two potassium-mediated depolarization 10 min apart. Either the affibody peptide (n=6-16 per condition) or the antibody (n=9) was used. The results are again compared to time controls with perfusion of imaging solution (n=16). While lower doses of the affibody (100 ng and 500 ng/mL) did not prevent the insulin-mediated reductions in the Ca2+ AUC (Figure 5A, p<0.01 and p<0.05, respectively), a higher dose (1 ug/ml) was able to completely inhibit this effect such that the reduction in AUC was no longer present (p>0.05). Doses used are modelled after in vivo studies [485, 486]. These results suggest that the effects of insulin on hippocampal Ca2+ levels were mediated through insulin receptors (Figure 5B - right).
Experiments were conducted as in Figure 3, with 2 sequential KCl depolarizations. **A.** High-dose (1 ug/mL) anti-insulin affibody neutralized the effect of insulin on KCl-induced Ca2+ transients while lower concentrations (100 ng/mL and 500 ng/mL) did not. **B.** 500 ng/mL insulin receptor antibody also was able to inhibit insulin actions on the KCl-mediated Ca2+ transients. These results indicate the actions reported here on Ca2+ homeostasis are mediated by insulin working on insulin receptors. All data represent mean +/- SEM. Asterisks indicate significance at the p<0.05 level.
4.4 Discussion

The experiments described here were intended to characterize actions of acute insulin exposure on neuronal Ca2+ currents and Ca2+ levels. The techniques used provide the most direct approach for analyses on the impact of insulin on hippocampal neurons without the confounding interactions of blood vessels and other cell types. Cultured neurons are detached from hormonal and perfusion influences, providing for a simplified environment where acute drug exposure is rapid and direct, free from the diffusion hindrance of the brain parenchyma or extra-neuronal interactions. Similar approaches in our lab and others have revealed significant connections between the actions of several hormones and neuronal function [474, 482]. Our results provide evidence that Apidra®, a zinc-free insulin formulation, is able to reduce VGCC currents and Ca2+ levels attained during neuronal depolarization. The mechanism is dependent on insulin receptor activation, and also appears to engage ryanodine receptors as CICR contributions were reduced in response to acute insulin treatment.

Ca2+ currents are a target of acute insulin in neurons

The insulin-mediated reduction in whole-cell currents does not appear to be specific to one subtype VGCC. This is supported by evidence of currents recorded from -70 mV before and approximately 10 minutes after the addition of 10 nM insulin, showing the same degree of inhibition for the peak and late currents (i.e., similar inactivation rates in Figure 1A). Furthermore, the reduction in currents elicited from a holding potential of -40 mV, which favors L-type Ca2+
currents, was not significantly greater in amplitude when compared to the inhibition seen from -70 mV, arguing against a selective effect of insulin on L-type Ca2+ channels. Previous work on regulation of VGCC in different cell types also notes that the effect of insulin may not be selective for a particular type of VGCC (e.g., N- or L-type VGCCs; [487, 488].

This insulin mediated reduction in VGCC was not accompanied by a shift in the I-V relationship (Figure 1D), indicating the mechanism likely impacts flux of the charge carrier through the channels rather than alterations in the voltage sensitivity or inactivation properties of the channels. This result is aligned with prior work in pinealocytes and photoreceptors, showing that tyrosine phosphorylation can reduce L-VGCC function [335, 407]. Evidence also suggests that the pore-forming alpha subunit of some VGCCs can be regulated directly by tyrosine residue phosphorylation [487-489]. Prior work has shown that acute insulin can alter other ion channels including large-conductance Ca2+-activated potassium channels (BK-type). Under these conditions, insulin reduced somatic Ca2+ levels during spontaneous oscillations in cultured hippocampal neurons [406]. Further, given the evidence that reductions in Src binding to the alpha 1 subunit of the L-VGCC significantly reduces channel current amplitude in cardiac myocytes [490] and that insulin can reduce Src activity [491], this signaling pathway likely highlights a potential mechanism by which insulin reduces VGCC current flux.

Still, it should be noted that some cells respond to tyrosine kinase phosphorylation with enhanced VGCC function and potentiated Ca2+ levels [492,
and that given the complexity of second messenger systems working through many kinases and phosphatases [494], the exact impact of insulin on VGCC likely depends on the presence and state of activation of other interacting pathways (i.e., PKA, PKC, AKAP, phosphatases, etc.). Irrespective of the mechanism of inhibition of VGCC by insulin, it is clear that VGCC are a target of insulin in neurons. The effect described here, therefore, is likely to mediate the beneficial impact of the hormone on cognitive function following intranasal delivery in animal models [30, 467, 469, 470] and in early AD subjects [32, 33, 35, 398, 401, 495]. Indeed, according to the Ca2+ hypothesis of aging and dementia [188, 443, 496], reducing Ca2+ levels in postsynaptic neurons is a favorable approach to offset cognitive decline in aging and AD (see below).

**Other potential targets of insulin actions**

Previous work shows insulin can increase cell surface expression of NMDA receptors [89] and can reduce surface expression of AMPA receptors [88, 332]. These studies employed similar insulin exposure time as used in the current study, but concentrations were 50-1000-fold higher. Thus, it is not clear whether transient changes in NMDA and AMPA surface expression would manifest using 10 nM insulin in the absence of TTX. As reported in Figure 2, the nearly physiological insulin concentration used here does not necessarily alter network excitability. This is evidenced by a lack of insulin action on resting Ca2+ levels, peak amplitude of the spontaneous Ca2+ responses, the AUC of the spontaneous events, as well as the number of spontaneous events. Our work,
therefore, may suggest that higher concentrations of insulin are needed to alter network oscillations, overall excitability and spontaneous activity. Although not tested directly here, our results do not provide evidence that insulin can increase surface expression of GABA<sub>A</sub> receptors [92, 405] which should have reduced excitability. Within the time frame tested here (~10 minutes) and at the low concentration used, we did not see evidence of depression in spontaneous Ca<sub>2+</sub> events (Figure 2). Thus, further studies focusing on insulin concentration-responses and time of exposure are needed to clarify these discrepancies. Of course, it will also be important to address the different types of insulin formulations used and to include the adequate zinc controls, given that almost all insulin formulations are zinc-based.

While insulin did not alter spontaneous Ca<sub>2+</sub> activity and Ca<sub>2+</sub> events amplitudes within the network of cells in culture (Figure 2), we did notice a significant alteration in the shape of the potassium-mediated Ca<sub>2+</sub> responses following insulin treatment (Figure 3). The disappearance of the “hump” following the peak Ca<sub>2+</sub> rise revealed the possibility that RyRs might be sensitive to insulin. We tested this hypothesis by pretreating the cultures with high concentrations of ryanodine to inhibit Ca<sub>2+</sub>-induced Ca<sub>2+</sub> release (CICR). As shown here, ryanodine pretreatment precluded further insulin-mediated inhibition of the Ca<sub>2+</sub> response to depolarization (Figure 4). This suggests insulin is capable of reducing CICR likely through tyrosine mediated inhibition of the RyR. This result is in line with prior evidence that neuronal store-operated Ca<sub>2+</sub> channels (SOCCs) responsible for a small but significant secondary Ca<sub>2+</sub> entry
pathway for replenishing the endoplasmic reticulum, are also a target of protein tyrosine phosphorylation [497]. It should be noted, however, that this result is contrary to what is seen in striated muscle cells, where insulin elicits an increase in Ca2+ release through activation of RyR [498]. Future studies are necessary to identify whether the different RyR subtypes can respond differently to insulin, or whether these effects are tissue specific, and dependent on particular signaling pathways.

We suggest that the novel finding of insulin actions on CICR could be highly beneficial to neurons and to Ca2+ dysregulation seen in aging and/or AD, and that this new target potentially offers a secondary level of protection from sustained Ca2+-related actions in neurons. While it is not clear that the reduction in the “hump” (Figure 4) may provide a neuroprotective function to neurons, the insulin-mediated reduction in depolarization observed during this Ca2+ envelope could well keep neurons engaged during periods of activation.

Learning and memory selective actions of insulin in the brain

Past work studying insulin function in the brain identified critical links to learning and memory. Whether exogenous insulin on hippocampal neurons worked through changes in GABA, NMDA, AMPA, or even glucose transporters, it was understood that insulin could have a specific and selective impact on learning and memory and conversely, that learning also could have an impact on insulin receptors [28, 499]. More recent analyses of insulin action provide evidence that large and diverse areas of the CNS are indeed sensitive to insulin. Previous work shows insulin is a dynamic hormone with a rich influence on brain function.
Insulin stimulates the translocation of glucose transporter-4 to plasma membranes of hippocampal neurons within 15-30 min [100]. This rapid process is likely to regulate neuronal metabolic demands and the energy needed for learning and memory processing. In addition, insulin enhances synaptic plasticity by increasing the expression of dendritic postsynaptic density scaffolding protein (PSD-95), a key element of postsynaptic junctions in hippocampal neurons [334]. Together these functions and their sensitivity to insulin undoubtedly contribute to improved encoding of synaptic information for the storage, and perhaps, the retrieval of memories.

Equally concrete examples of the links between insulin and memory come from evidence that insulin can acutely reduce the hippocampal AHP, a Ca2+-dependent hyperpolarizing potential, which maintains hyperpolarization and precludes action potential threshold from being reached [30, 380]. The AHP is larger in aged compared to young animals [184, 379, 433, 453, 476, 500, 501], thus reducing the amplitude or duration of this hyperpolarizing potential may help combat age-related memory dysregulation by allowing cells to participate within a functional network [502]. Indeed, it has been shown that elevating potassium channel activity impairs learning [503], while lowering L-VGCC reduces the AHP [432, 452] and enhances learning [432, 504]. Moreover, increasing cholinergic neurotransmission which enhances cellular excitability also can enhance learning [436, 505-507].

Still, the evidence of robust insulin actions linked to memory processes or processes capable of facilitating memory encoding (e.g., metabolism, blood
flow), does not detract from the traditional role of insulin in homeostasis [508, 509]. Indeed, it is becoming very clear that once insulin in the brain, the hormone is not limited in function to a single or even a few target areas [366, 510], strengthening its position as a potential therapeutic target which requires greater research emphasis.

4.5 Conclusions
We used imaging and electrophysiology techniques to show that zinc-free insulin could have rapid and selective effects on Ca2+ sensitive functions in neurons, and that the effects are dependent on insulin activating the insulin receptor (Figure 5). This clearly engages a series of intracellular signals that through uncharacterized pathways or mechanisms, lead to rapid Ca2+ reductions during neuronal depolarization. Clearly, more research will need to be conducted to fully characterize the mechanism underlying this phenotype and to address the selectivity of the effect using different insulin formulations. Importantly, we remark that reducing Ca2+ dysregulation in brain aging, AD and in neuronal cultures has generally been associated with positive and neuroprotective outcomes, from improving cognitive functions to reducing cellular toxicity. We present evidence here that insulin could well be an endogenous modulator of these functions. Reductions in insulin levels or in insulin signaling in the brain during aging and in AD may highlight one of the mechanisms that could reduce metabolism, thereby reducing cognitive function. However, our results also suggest that replacing insulin or enhancing insulin signaling in the brain of aging, or early AD subjects
may be a valuable approach. We emphasize that the novel impact of insulin on neurons presented here could well underline the beneficial impact of intranasal insulin on cognitive enhancement in the clinic and in animal studies. If insulin is able to reduce Ca2+ levels \textit{in vivo}, the noted reductions in insulin-sensitive Ca2+ responses may offset brain aging processes and ultimately, offset the impact of dementia on brain function.
Chapter 5 Discussion and conclusion

The data collected for this dissertation project is presented in the form of two manuscripts. The purpose was to test the hypothesis that intranasal insulin could offset cognitive decline in aged animals and to characterize the impact of insulin on neuronal calcium status. The first manuscript (see chapter two) has been published in the Journals of Gerontology Series A in 2016 [30] and the second manuscript (see chapter four) has been submitted to Neuroscience (Maimaiti et al., 2017).

5.1 Effects of insulin on memory and Ca2+-dependent AHP

In the first study, to test whether intranasal insulin could reverse aging-related memory decline, we used the Morris water maze, a hippocampal dependent spatial learning and memory task, to evaluate insulin effects on memory in young and aged F344 rats. Aged animals were divided into five groups; each group repeatedly received saline, a low dose of Humalog® and a low, medium, and high dose of Levemir®. Also, the young control group received saline, used as a normal young baseline. The behavioral study results showed that intranasal administration of two different formulations of insulin (Humalog®, a short-acting insulin analogue, or Levemir®, a long-acting insulin analogue) reversed the memory decline in aged F344 rats compared to aged rats in the saline controls. We found that while a low dose of Humalog® and Levemir® enhanced the memory, a medium dose of Levemir® worsened the memory, and a high dose of
Levemir® did not change the memory. Therefore, it is clear that selective doses and formulations of insulin have beneficial effects on the memory in the aged animals. In addition, controversial clinical study results exist regarding insulin dosages and formulations for use in AD patients. In animal and clinical studies, different dosages and formulations of insulin have various outcomes. These varied results led to further investigation of the underlying mechanisms responsible for intranasal insulin's beneficial effects on memory as seen in select animals and human studies, highlighting the importance of enhancing insulin action in the brain.

So far, three main methods (insulin sensitizers, intravenous insulin infusion, and intranasal insulin) have been used for increasing brain insulin levels. Due to the poor permeability of insulin sensitizer (Thiazolidinedione) to the blood brain barrier [511, 512], and the high risk of hypoglycemia from intravenous insulin infusion (see chapter three), however, intranasal insulin appears to be the best method to increase brain insulin levels. Nevertheless, the underlying mechanisms responsible for intranasal insulin's positive effects are still poorly understood. One mechanism responsible for memory decline with aging is hippocampal Ca2+ dysregulation.

It has been more than 30 years since researchers have focused on Ca2+ dysregulation in the aging brain, which is measured by recording Ca2+-dependent afterhyperpolarization (AHP) in the hippocampus neurons. Therefore, we examined the effects of insulin on Ca2+-dependent AHP, one of the well-known mechanisms that increases with aging and contributes to memory decline.
Our results show that treatment of hippocampal slices with insulin reduced Ca2+-dependent AHP in hippocampal neurons to the levels seen in young neurons. It is well documented that Ca2+-dependent AHP (a key biomarker of brain aging) increases in aged, and memory-impaired animals [30, 184, 377, 379, 380, 432-434]. Thus, insulin-mediated reduction in Ca2+-dependent AHP may be responsible for some of the beneficial effects of insulin on cognition.

Most insulin formulations were designed by combining insulin with zinc. In fact, zinc itself plays a vital role in the physiological functions of cells, and is like Ca2+, an essential ion to regulate intracellular signaling. Therefore, it is important to test insulin and zinc effects separately on brain Ca2+ regulatory processes. Thus, when the effects of Apidra® (fast acting zinc-free human insulin) and zinc on the AHP were tested separately, both were found to reduce the AHP. Therefore, we used the zinc-free human insulin Apidra® to study specific insulin actions in hippocampal neurons without the influence of zinc.

The next question we asked was how does insulin reduce the Ca2+-dependent AHP? The greater AHP seen in aged memory-impaired animals positively correlated with elevated intracellular Ca2+ concentration. This alteration results from increased Ca2+ influx via voltage-gated Ca2+ channels and Ca2+-induce Ca2+-release via RyRs (see chapter three). Conversely, it is possible that a reduction in AHP with insulin treatment may mediate through the reduction in intracellular Ca2+ levels via VGCCs and RyRs.
5.2 Whole-cell patch clamping and Ca2+ imaging in hippocampal neurons

In the second manuscript, we aimed to identify the effects of insulin on hippocampal Ca2+ levels in cultured neurons. The significance of this data is showing that insulin reestablishes hippocampal Ca2+ homeostasis. Aging and AD animal models have shown that elevated intracellular Ca2+ levels in the hippocampus contribute to poor spatial memory [180, 453]. Reestablishment of intracellular Ca2+ homeostasis might be one of the possible neuronal mechanisms responsible for the beneficial effects of insulin on cognition. Two main sources of intracellular Ca2+ are Ca2+ influx through voltage-gated Ca2+ channels on the plasma membrane and internal Ca2+ efflux from the endoplasmic reticulum via ryanodine-mediated Ca2+ release in the hippocampus. It is well documented that both voltage-gated Ca2+ channels and ryanodine receptors undergo functional changes with aging and that lead to elevated intracellular Ca2+ levels, which is toxic for neurons (see chapter three). It is widely accepted that reducing the elevated intracellular Ca2+ levels is one of the established approaches to improving memory in the aging and AD population [377, 435, 453]. Therefore, insulin effects on both voltage-gated Ca2+ channels and ryanodine receptors were tested. Insulin affected two main intracellular Ca2+ sources, including Ca2+ influx via voltage-gated Ca2+ channels and RyRs-mediated Ca2+ release. After 10 minutes of insulin perfusion to patched single neurons, whole-cell patch clamp recording results show that both VGCCs and L-type VGCCs currents decreased in hippocampal neurons. We found that acute insulin perfusion to neurons resulted in the same level of reduction in both
VGCCs and L-VGCCs. This data suggests that insulin uniformly affects all type of voltage-gated Ca2+ channels. It is well documented that an elevated intracellular Ca2+ results from increased L-VGCCs, which has been linked to memory decline in aged animals (see chapter three). Therefore, insulin-mediated reduction in Ca2+ influx via this channel may be an underlying mechanism partially responsible for insulin’s memory-enhancing effects seen in animal and clinical studies. In addition, Ca2+ imaging data show that acute insulin perfusion did not change resting Ca2+ transients, and significantly reduced ryanodine-mediated Ca2+ transients. Intracellular Ca2+ concentration is amplified due to enhanced ryanodine-mediated Ca2+-induced Ca2+-release from the endoplasmic reticulum in animal models of aging and AD [377, 435, 513]. Reduction in RyRs-mediated Ca2+ transients may underlie neuronal mechanisms responsible for positive actions of insulin in hippocampal neurons. Furthermore, this result indicates that RyRs inhibitors will improve the memory in AD patients.

5.3 Insulin improves memory by regulating intracellular Ca2+ levels

Together, these two studies combine intranasal insulin approaches, hippocampal-dependent memory and learning task, sharp electrophysiology, whole-cell patch clamp, spontaneous and depolarization mediated Ca2+ imaging to highlight potential neuronal mechanisms of insulin-mediated cognitive improvement seen in clinical studies. We show that hippocampal Ca2+-dependent AHP, voltage-gated Ca2+ channel currents, and Ca2+ transients are
increased in aged rats—all major factors influencing memory decline with aging—are significantly reduced by insulin treatment. Furthermore, this reduction in voltage-gated Ca²⁺ channel currents and ryanodine-mediated Ca²⁺ transients were correlated with reversal of Ca²⁺ dysregulation and memory impairment observed in aged rats.

These study results support the concept that brain insulin resistance/deficiency present in aged, diabetic and AD models of rats could be a major contributor to aging-related cognitive decline. Indeed, increasing brain insulin levels using the intranasal route improves memory by reducing AHP via regulating intracellular Ca²⁺ levels. Thus, taken together, these findings suggest that insulin is a key hormone influence over neuronal Ca²⁺ regulation and strengthen the brain insulin-deficiency mechanistic hypothesis of pathological aging.

### 5.4 Aging-related changes in memory and hippocampal Ca²⁺ levels

Insulin treatment reversed both aged-related memory decline and Ca²⁺ dysregulation in hippocampal neurons. It has been decades since our lab and others have focused on the relationship between cognitive decline and increased Ca²⁺ dysregulation in aged animals [30, 224, 433, 435, 514]. Ca²⁺ dysregulation means elevated intracellular Ca²⁺ concentration, that gives rise to a larger AHP in aged animals and is considered one of the neuronal mechanisms underlying memory decline with aging. In addition, researchers identified pharmacological agents such as Ca²⁺ channel blockers (inhibiting the elevation of intracellular Ca²⁺ concentration) that reduce the amplitude and duration of the AHP to
improve cognitive function in aged animals [515]. Furthermore, previous studies from our research group using gene transfer approaches to increase and/or decrease FKBP1b (a small immunophilin that stabilizes RyR-mediated Ca2+ release) expression in the hippocampus showed a similar correlation between memory impairment and Ca2+ dysregulation [435]. In the present study, we found a consistent association between age-related changes in hippocampal-dependent memory, and Ca2+ levels. Most importantly, insulin treatment improved memory in aged rats, reduced the AHP, Ca2+ channel currents, and Ca2+ transients. Insulin in the brain may be a key element for maintaining hippocampal Ca2+ homeostasis and cognitive function during aging.

5.5 Potential relevance to healthy and pathological aging

Biologists suggest that brain function starts to decline from the third decade of life leading to either healthy or pathological aging, suggesting that some changes in natural brain aging give rise to pathological neurodegeneration and AD. These changes include brain insulin deficiency and loss of neuronal Ca2+ homeostasis. Disruption of brain insulin signaling contributes to the AD clinical symptoms (memory impairment) and pathology [137, 149]. In addition, increased intracellular Ca2+ concentration from the dysfunction of VGCCs and RyR receptors markedly influence neuronal survival, communications, and function, which have been considered major risk factors for AD [185, 435, 516, 517]. Conversely, brain-specific enhancing insulin signaling improved cognitive function in selective AD patients, and improved memory in experimental aged
animals. In addition, our study results show that intracellular Ca2+ levels decreased with insulin treatment in hippocampal-cultured neurons. Importantly, brain insulin deficiency during aging together with other factors may accelerate Ca2+ dysregulation and stimulate amplification of intracellular Ca2+ levels, increasing the vulnerability of AD. Thus, studies presented here support that increasing brain insulin levels may be a vital approach for promoting healthy aging and, possibly, reversing cognitive decline present in aging and AD populations.

5.6 Study limitations

In the second study, all the whole-cell patch clamping and Ca2+ imaging data were obtained from the hippocampal cultured neurons in response to acute insulin treatment. Cells in culture are not expose to same multitude of signals environment that is present in functional human body. Thus, those data cannot directly translate into a clinical AD study. However, it provides potential mechanisms for insulin actions on hippocampal neurons. Primary hippocampal cultured neurons have been used as a valid model for studying the neuronal molecular mechanisms underlying the age-related changes in Ca2+ channels and neuronal survival [439], as well as for visualizing protein expression, trafficking, and localization [518]. We used primary hippocampal cultured neurons to study insulin action on hippocampal Ca2+ levels. Intracellular Ca2+ homeostasis is important for proper neuronal function throughout life, and it influences a wide range of neuronal development processes, including
formation of neurotransmitters, ion channels, neurite outgrowth, synaptogenesis, and intrinsic firing patterns [519-522]. Intracellular Ca2+ homeostasis is dependent on the proper function of voltage-gated Ca2+ channels on the plasma membrane and ryanodine receptors on the endoplasmic reticulum. In addition, both these channels have shown functional changes during aging. The voltage-gated Ca2+ channel density increases with aging, and this negatively correlates with neuronal survival in cultured hippocampal neurons [439]. Hippocampal neurons in the primary culture undergo time-dependent development and changes as well [183, 523]. From day in vitro (DIV) 1 to DIV 6, neurons rapidly attach to the dish, grow, and establish networks. From age DIV 10 to DIV 28, unhealthy and dead neurons increase as a function of age in the culture. In addition, a whole-cell Ca2+ currents recording from hippocampal neurons at different ages in the culture show that Ca2+ current density increases as neurons are aged in the culture [439]. Hippocampal cultured neurons help us to study the effects of insulin on hippocampal neurons in a single isolated environment that eliminates other factors. Thus, hippocampal cultured neurons seem to be an appropriate model for studying the link between insulin and hippocampal Ca2+ levels.

As I mentioned earlier, Ca2+-dependent AHP depends on both Ca2+ and K+ ions. In my dissertation, I focus only on the Ca2+; therefore, the K+ component still needs to be studied. Therefore, those data partially explain the mechanism of insulin actions on the AHP and memory.
5.7 Future directions

These studies have shown that insulin reduces the Ca2+-dependent, K+-mediated slow afterhyperpolarization (sAHP), reduces the VGCCs currents, and reduces RyRs-mediated Ca2+ transients. This study provided a clear link between insulin and intracellular Ca2+ concentration (mainly through voltage-gated Ca2+ channels and RyRs mediated Ca2+-induced Ca2+-release) in hippocampal neurons. However, the effects of insulin on potassium channels in hippocampal neurons in the context of cognitive functions have not yet been studied. There are a few studies showing a connection between insulin and potassium channels in the kidney [524] but not in the brain. In fact, “Ca2+-activated K+ channels play an important role in the control of neuronal excitability via the generation of the AHP” (Davies et al., 2006). Furthermore, earlier studies suggest that potassium channels contribute to the generation of sAHP in hippocampal CA1 neurons [525-529]. In addition, potassium channels undergo functional changes with aging and influence neuronal function and sAHP [530, 531]. From these findings arose the idea that insulin may be able to rebalance potassium channel functions, which may further reduce amplitude and duration of the sAHP. Other graduate students in the lab could look at the relationship between insulin and potassium channels in the hippocampal neurons.

Earlier research intensely focused on RyRs’ role in heart and skeletal muscle excitation-contraction. Some studies suggest that the cardiac ryanodine
receptor (RyR2) is a promising drug target for treating arrhythmogenesis [532]. Moreover, Rycals™ is a drug available on the market to inhibit cardiac and skeletal muscle specific RyRs [533]. In addition, dantrolene, an RyRs blocker, is a widely used drug to treat malignant hyperthermia in clinic. This dissertation showed that insulin reduced the RyRs-mediated Ca2+ transients in hippocampal neurons. This data suggests that the inhibition of RyRs channels positively correlates with memory improvement in aged animals. Therefore, RyRs’ role is not limited to the periphery, but also plays a vital role in the brain in the context of memory. Specifically, in the hippocampal, RyRs plays an important role in regulating intracellular Ca2+ levels and maintaining cognitive function [435, 453]. A few researchers are promoting the idea that hippocampus-specific RyRs inhibition is a therapeutic target for AD. Furthermore, this study strongly supports the hypothesis formulated by Dr. Grace Stutzmann that RyRs blockers are an effective anti-AD therapy. Thus, RyRs could be a drug target for inhibiting pathological intracellular Ca2+ leaks in the hippocampal neurons, and, more importantly, for reversing Ca2+ dysregulation and memory impairment with aging and AD.
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- Local bluegrass chapter for the Society for Neuroscience 2012 - Present
- National member of the Society for Neuroscience 2014 – Present
1. Bruce A. Berkowitz\textsuperscript{a,b,1}, Jacob Lenning\textsuperscript{a}, Nikita Khetarpal\textsuperscript{a}, Catherine Tran\textsuperscript{a}, Johnny Y. Wu\textsuperscript{a}, Ali M. Berri\textsuperscript{a}, Kristin Dernay\textsuperscript{a}, E. Mark Haacke\textsuperscript{c}, Fatema Shafie-Khorassani\textsuperscript{d}, Robert H. Podolsky\textsuperscript{d}, John C. Gant\textsuperscript{a}, Shaniya Maimaiti\textsuperscript{a}, Olivier Thibault\textsuperscript{a}, Geoffrey G. Murphy\textsuperscript{f}, Brian M. Bennett\textsuperscript{g}, and Robin Roberts\textsuperscript{a}. In Vivo imaging of prodromal hippocampus CA1 subfield oxidative stress in models of Alzheimer's disease and angelman syndrome. Research communication for FASEB J. 2017


