LINKING INSULIN SIGNALING TO AGE-RELATED COGNITIVE DECLINE:

INSULIN-DEPENDENT REGULATION OF INTRINSIC

EXCITABILITY IN AGING HIPPOCAMPUS

by

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Dedicated to my family and Sophie

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Neha Ramesh Tandon, PhD
The University of Texas at Dallas, 2020

Supervising Professor: Dr. Lucien T. Thompson

The elderly population (age 65 and over) is estimated to double in number by the year 2050. This will increase the socioeconomic burden associated with cognitive decline that accompanies normal aging and pathological aging caused by Alzheimer's disease (AD) and other dementias. While many pharmacological treatments have shown promise in ameliorating age-related cognitive decline, they have severe side effects. Increase in the amplitude of post-burst afterhyperpolarization (AHP) in aging hippocampal pyramidal neurons decreases its intrinsic excitability and leads to impairment in learning and memory formation of hippocampus-dependent tasks. Insulin decreases the amplitude and duration of Ca^{2+}-dependent AHP in aging hippocampus and reveres cognitive deficits. However, the molecular mechanism underlying the insulin-dependent decrease in AHP is as yet unknown.

Abundance of insulin receptors in the hippocampus and insulin-sensitivity of pyramidal neurons, makes insulin a potential therapeutic agent to enhance hippocampus-dependent learning and

memory in aging. While intracerebroventricular (ICV) or intravenous (IV) application of insulin has shown promising results, these methods are not suitable for clinical trials. Intranasal administration has become a preferred method for delivery of small neuropeptides such as insulin directly to the brain. Dysregulation of insulin signaling in the brain has long been implicated with mild cognitive impairment (MCI), AD and aging brain. Intranasal insulin improved verbal memory in a clinical study with MCI and AD patients.

In this dissertation, we utilized chronic (21 days) low dose intranasal insulin application to assess the effects of insulin on hippocampus-dependent spatial and working memory consolidation in aging animals on Morris water maze task. Intranasal insulin significantly enhanced learning and spatial memory in aging rats with cognitive deficits. Next, we sought to study the effect of insulin on proteins involved in Ca^{2+}-dependent intrinsic excitability: (i) the SK2 channel underlying the medium AHP (mAHP), (ii) Ca^{2+} sensor, calmodulin that gates mAHP, and (iii) Ca^{2+} sensor, hippocalcin that gates slow AHP (sAHP). Immunoblots using young and aging hippocampus showed (i) downregulation of insulin signaling (ii) over-expression of SK2 (iii) downregulation of calmodulin and (iv) no change in hippocalcin with aging. *Ex-vivo* insulin stimulation of hippocampal slices was able to (i) restore the insulin signaling in aging, (ii) reverse the age-related overexpression of SK2 and (iii) downregulate the expression of hippocalcin in both young and aging tissue. Our results suggest that manipulation of insulin-signaling in aging can increase hippocampal intrinsic excitability and performance in hippocampus-dependent tasks; and supports the use of intranasal insulin therapy in clinical studies with cognitively impaired aging patients

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LIST OF ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AHP	Afterhyperpolarization
CaM	Calmodulin
CNS	Central nervous system
fAHP	Fast afterhyperpolarization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HpC	Hippocalcin
INI	Intranasal insulin
Ins	Insulin
IR	Insulin receptor
IRS	Insulin receptor substrate
mAHP	Medium afterhyperpolarization
MCI	Mild cognitive impairment
MWM	Morris water maze
RIPA	Radioimmunoprecipitation assay
sAHP	Slow afterhyperpolarization
Sal	Saline
SK	Small conductance calcium-dependent potassium channel
IK	Intermediate-conductance, calcium-dependent potassium channel

CHAPTER 1

INTRODUCTION

1.1. Author Contributions

This chapter was written by Neha R. Tandon (N.T.) and edited by Dr. Lucien T. Thompson (L.T.).

1.2. Aging and cognition

Aging comes with critical changes in the brain activity and function that impacts cognitive function. Cognitive decline with "normal aging" has been attributed to changes in neuroanatomy, neurophysiology, neurogenesis, metabolism and biochemistry, and is commonly referred to as age-related cognitive decline. Various cognitive tests are designed to assess an differentiate cognitive decline in humans due to normal aging and pathological aging such as Alzheimer's disease (AD), mild cognitive impairment (MCI) and other neurogenerative disorders. Studies with healthy older adults demonstrate slower processing speed, decline in executive functioning and mental flexibility due to aging. Age-related learning and memory deficits are more prominent in declarative memory (Harada, Natelson Love, and Triebel 2013). This decline in cognitive function and deficits in memory consolidation make everyday life challenging for older adults. Extensive research is being carried out to understand the underlying causes of age-related cognitive decline and to find therapeutic strategies to ameliorate these cognitive deficits.

Cognitive decline with normal aging has been well characterized in humans as well in apes and rodent models (Bachevalier et al. 1991). Neuroanatomical changes have been mapped in humans

in longitudinal studies. Gradual loss of gray matter volume is reported with normal aging; while white matter volume shows sharp decrease in older adults more than 60 years of age. Hippocampus and cerebellum show more prominent shrinkage with aging. Other regions known to shrink with age are entorhinal cortices, inferior temporal cortex and prefrontal white matter (Raz et al. 2005; Driscoll et al. 2009). Hippocampus plays a critical role in learning and memory consolidation. Hippocampal pyramidal neurons degenerate with aging which also correlates with memory deficits in aging (Landfield et al. 1977). The importance of hippocampus in learning and memory consolidation was shown by Morris et al. in rats with hippocampal lesions. Hippocampectomized rats show poor learning on water maze task, a hippocampus dependent spatial memory task (Morris et al. 1982). Thompson et al. demonstrated age-dependent cognitive decline in learning in rabbits. Aging rabbits took a greater number of trials to learn eye blink conditioning (EBC) task. There is a gradual and continuous decline in cognitive performance with aging. Learning and memory deficits also vary within the same aging group (Thompson, Mover, and Disterhoft 1996).

1.3. Post-burst afterhyperpolarization (AHP)

Following a train of action potentials, the membrane potential of a neuron falls below the resting state (hyperpolarization) and slowly comes back to resting membrane potential, this could last up to several seconds. This phenomenon is called post-burst afterhyperpolarization (AHP). The purpose of the AHP is to limit the firing frequency of the neurons. The AHP is divided into three phases- fast AHP (fAHP), medium AHP (mAHP) and slow AHP (sAHP) (Fig. 1.1.B), each mediated by different potassium channels. Fast AHP immediately follows the action potential

and lasts for 2-5 milliseconds (ms). Current underlying fAHP is mediated by voltage and calcium-dependent potassium BK channels. Following the fAHP, there is a longer slowly decaying current that last for several hundred milliseconds to several seconds. Studies showed that this longer AHP is mediated by potassium currents from different channels and was further distinguished as mAHP and sAHP. Medium AHP lasts for 100-200 ms and is mediated by calcium-dependent potassium channels called SK. These channels are voltage-insensitive and effectively blocked by bee venom apamin. Slow AHP can last for 1-2 s and are mediated by voltage-insensitive, apamin-insensitive, calcium-dependent potassium channels. The identity of channel mediating sAHP is still unknown (Faber and Sah 2003). In this chapter, we will go in greater detail about mAHP and sAHP.

1.3.1. SK2 and Calmodulin

There are four known isoforms of apamin sensitive calcium-dependent potassium SK channels: SK1, SK2, SK3 and IK1. Sk1-3 are apamin-sensitive while IK1 is not. SK1-3 channels are localized throughout the central nervous system, while IK1 is predominantly found in periphery. SK1 and SK2 are highly expressed in neocortex and pyramidal cells of CA1 and CA3 regions of the hippocampus (Stocker and Pedarzani 2000; Sailer et al. 2004). Blockade of SK channels using apamin has shown enhancement in learning and memory acquisition in hippocampus-dependent cognitive tasks (Faber and Sah 2003). Apamin reduces the amplitude and duration of mAHP, increasing the neuronal excitability.

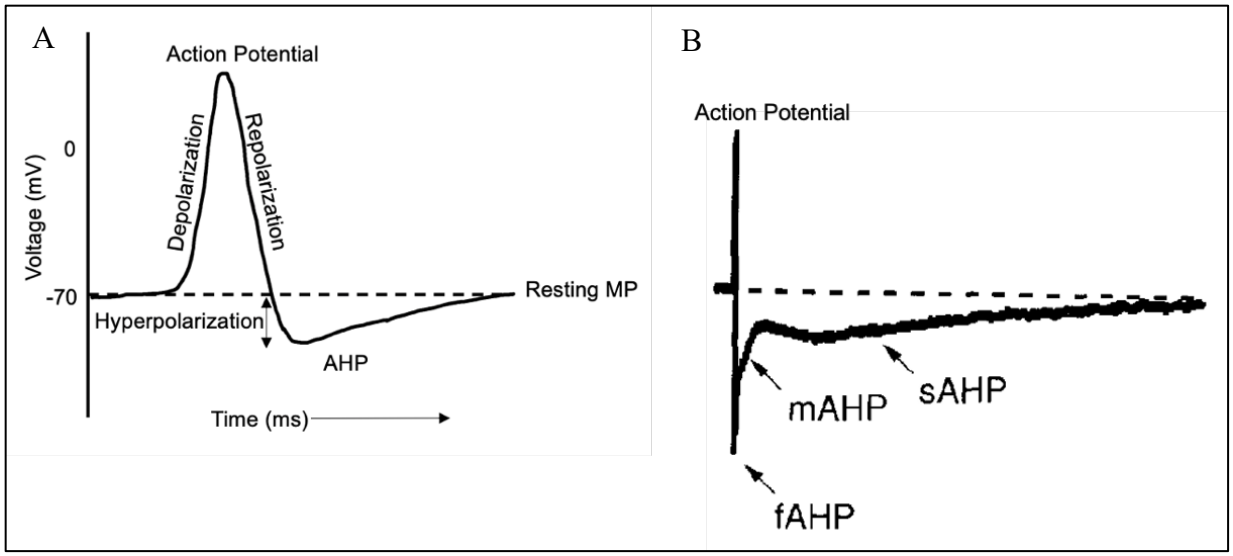

Fig 1.1. Post-burst afterhyperpolarization: Following repolarization phase of action potential, the membrane potential of certain type of neurons falls below the resting membrane potential. This is known as hyperpolarization [A]. After a burst of action potentials, this hyperpolarization phase is elongated generating AHP that limits the frequency of firing subsequent action potentials. The fAHP immediately follows the action potential, lasts for 2-5 ms and is mediated by voltage-dependent potassium channel BK. The mAHP is mediated by calcium-dependent potassium channel SK and lasts for 100-200 ms. The sAHP lasts the longest for 1-2 s, however the identity of the calcium-dependent potassium channels underlying sAHP is unknown. AHP trace representing fAHP, mAHP and sAHP is shown on the right [B], borrowed from (Sah 1996).

SK channels are calcium-dependent channels but do not directly interact with Ca^{2+} ions. A calcium sensor protein calmodulin (CaM) constitutively binds to C-terminal cytosolic domain, CaMBD (Calmodulin Binding Domain), of SK channel (Fig. 1.2.A). When Ca^{2+} binds to calmodulin, there is a conformational change in SK channel that leads to opening of channel pore (Keen et al. 1999; Xia et al. 1998; Schumacher et al. 2001) (Fig. 1.2.B). Activity of SK2 channels is also regulated by interactions with certain kinases and phosphatases. SK2 binds with CK2 (Casein kinase 2) and PP2A (Protein phosphatase 2) at its cytosolic domains (Fig. 1.2.A). Calmodulin is phosphorylated by CK2, and not SK2, reducing its sensitivity to calcium and

consequently deactivating the channel. On the other hand, calmodulin is dephosphorylated by PP2A increasing its sensitivity to Ca^{2+} (Bildl et al. 2004; Allen et al. 2007). Another kinase PKA (Protein kinase A) has shown to reduce the amplitude of AHP in CA1 neurons of hippocampus in naïve rats. PKA mediated phosphorylation of SK2 channel marks the channel for endocytosis, reducing the number of transmembrane SK2 channels (Pedarzani and Storm 1993; Oh et al. 2009). The role of SK2 channel in hippocampus-dependent memory acquisition and synaptic plasticity is evident. The expression of SK2 increases with aging (Ballesteros-Merino et al. 2012) and increasing SK2 activity impairs learning in rats on a hippocampal-dependent trace eye blink conditioning (EBC) task (McKay et al. 2012).

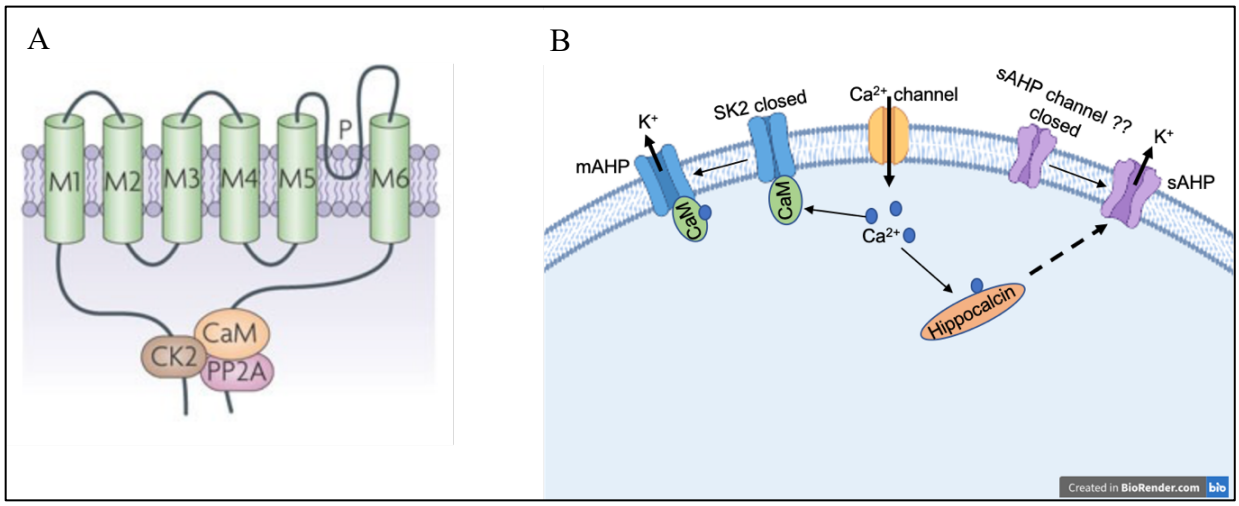

Fig 1.2. Channels mediating mAHP and sAHP and their calcium sensors: SK channels are the calcium-dependent potassium channels mediating mAHP, however SK channel itself does not bind to Ca^{2+}. Calcium sensor protein calmodulin (CaM) is constitutively bound to the calmodulin binding domain (CaMBD) of the SK channel [A]. Binding of Ca^{2+} to calmodulin causes a conformational change in SK channels and activates it [B]. The other protein bound to SK channel -kinase CK2 and phosphatase PP2A- regulate the activity of CaM [A]. While the channel underlying sAHP is still unknown, hippocalcin is identified as the calcium sensor that gates the sAHP [B]. Image [A] borrowed from (Luján, Maylie, and Adelman 2009).

1.3.2. Hippocalcin

Slow AHP is also mediated by calcium-dependent potassium channels, which are apamin sensitive. However, the search for the identity of the channel that primarily mediated the sAHP has baffled scientists for a long time. Just like calmodulin acts as calcium sensor for SK channels, Tzingounis and colleagues identified hippocalcin as calcium sensor for sAHP channel. Hippocalcin is a neuronal diffusible Ca^{2+}-binding protein and is not constitutively bound to any channel (Fig. 1.2.B). Knockout of hippocalcin reduces the amplitude of sIf_{AHP}, which is the current underlying sAHP in hippocampal pyramidal cells, making hippocalcin a one of critical intermediate between Ca^{2+} influx and activation of the unidentified potassium channel responsible for sAHP (Tzingounis et al. 2007).

1.4. Age-dependent increase in AHP

Due to the critical function of hippocampus in learning and memory and the anatomical and physiological changes in hippocampus with age, researchers have extensively focused on studying synaptic plasticity and excitability of hippocampal neurons. Age-related changes in synaptic plasticity and excitability of hippocampus has been studied in different paradigms such as long-term potentiation (LTP), long-term depression (LTD), excitatory post-synaptic potentials (EPSPs), spike frequency accommodation and post-burst afterhyperpolarization (AHP). Hippocampus is a C-shaped structure that is embedded in temporal lobe and is a part of the limbic system. Hippocampus is made of hippocampus proper and dentate gyrus (DG). Neurons in hippocampus proper can be further distinguished into CA1, CA2, CA3 and CA4 regions. CA1 pyramidal cells receive processed information from DG and CA3 in a feed-forward tri-synaptic

circuit and also receives projections from entorhinal cortex. Aging CA1 hippocampal neurons show an increase in post burst AHP amplitude and duration (Landfield and Pitler 1984). Increase in duration of AHP with age reduces neurons excitability and hinders the firing of next train of action potentials needed to process information.

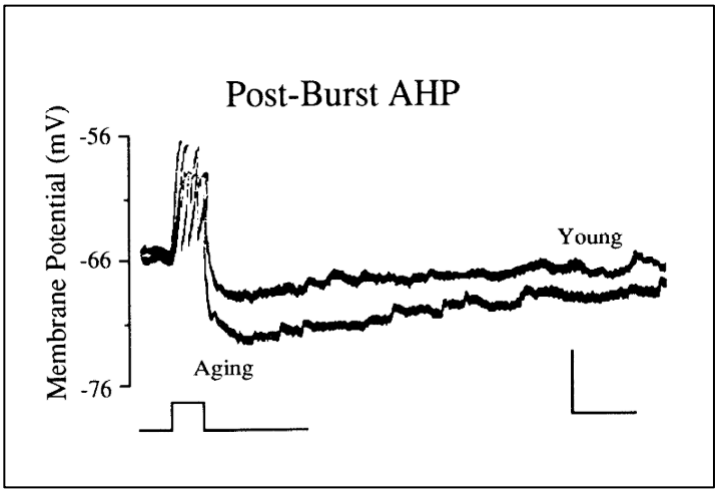

Fig 1.3. AHP amplitude increases with aging: The AHP trace represents the post-burst AHP recorded from young versus aging hippocampus (Moyer et al. 1992). Aging hippocampal pyramidal neurons show increase in amplitude and duration of AHP.

Learning a new task increases neuronal excitability in aging hippocampus. After trace eye blink conditioning (EBC) task, aging rabbits that successfully learned the task showed a decrease in AHP amplitude (Moyer Jr. et al. 2000). Learning and new memory formation increases neuronal excitability in aging and can subsequently reduce cognitive deficits. Calcium-dependent potassium sI_{AHP} current increases with age. Age-dependent increase in AHP amplitude and duration is only observed in sAHP component. Nimodipine, L-type calcium channel blocker, reduces AHP amplitude in aging CA1 neurons. Nimodipine, provided intravenously, improved

learning in aging rabbits on the trace EBC task, which shows the correlation between AHP and learning ability (Moyer et al. 1992; Disterhoft et al. 1996; Power et al. 2002). This was further demonstrated by Tombaugh et al, in F344 aging rats, that increase in sAHP amplitude with age covaries with learning ability aged rats on water maze task. They also used a cAMP analog to inhibit sAHP to measure mAHP and showed that mAHP amplitude does not increase with age. (Tombaugh, Rowe, and Rose 2005).

1.5. Insulin signaling and aging brain

1.5.1. Insulin and insulin receptor in the brain

For a long time, scientists debated the presence of insulin and insulin receptors (IR) and its role in brain. It's only been four decades since the presence of insulin and IR in the brain was first reported. Interestingly, insulin concentration in brain is on average 25 times higher in the brain regions as compared to plasma insulin levels. Hypothalamus, olfactory bulb and cerebellum have abundance of insulin, almost 100 times higher than plasma insulin concentration, while brain stem, cortex and remaining brain tissue has 10-20 times higher insulin concentration than plasma insulin. Also abundant IRs are found in hypothalamus, cerebral cortex and olfactory bulb regions of the brain (Havrankova et al. 1978). This was further confirmed by Marks et al. in 1990 by in-situ hybridization for IR mRNA in rat brain sections. Insulin receptors are localized and abundant across various brain regions such as the hippocampus, cerebellum, olfactory bulb, piriform cortex and hypothalamus. The presence of insulin was first reported in human brain by Sara et al. in 1982 (Sara et al. 1982).

1.5.2. Insulin signaling pathway

Insulin and insulin signaling is mainly known for maintaining blood glucose level. But apart from glucose homeostasis, insulin signaling affects many physiological processes such as lipid and protein metabolism, gene expression and protein synthesis, cell division and growth and cognition. Peripheral insulin is a peptide hormone synthesized by beta cells of the pancreas and can readily cross the blood brain barrier (BBB). Insulin resistance and insulin signaling inhibition in the brain affect synaptic plasticity, neurogenesis and cognition. Insulin receptors are tyrosine receptor kinases with two alpha and two beta subunits. Insulin binds to extracellular alpha subunit of dimeric IR causing autophosphorylation of tyrosine residues on transmembrane beta subunits and activates it. Phosphorylation of IR initiates different signaling cascades (Fig. 1.4). The immediate substrate of IR kinase activity is insulin receptor substrate (IRS). Out of the two IRS isoforms- IRS1 and IRS2, IRS1 is recruited by IR-IR dimer receptor and induces its tyrosine phosphorylation. While serine phosphorylation of IRS inhibits its recruitment to the receptor, inhibiting the insulin signaling (Gual, Le Marchand-Brustel, and Tanti 2004; Copps and White 2012). Tyrosine phosphorylation of IRS1 induces recruitment and activation of IRS1-PI3K-Akt/PKB cascade. Activation of this pathway induces translocation to glucose transporter (GLUT4) from the cytosol to the plasma membrane. Along with GLUT 4, GLUT 1 and GLUT 3 are abundant in various brain regions and participate in glucose homeostasis, feeding behavior and cognition (Maher et al. 1991; Uemura and Greenlee 2006; Pearson-Leary and McNay 2016). Activated Akt phosphorylates and inhibits GSK3beta that controls many neuronal functions such as neurogenesis, synapse formation and synaptic plasticity (Cole 2012). Mitogen-activated protein kinase (MAPK) is another signaling cascade activated by insulin signaling, which

regulates gene expression and cell proliferation. Insulin resistance in the brain is one of the major implications on diabetes-related cognitive decline, age-related cognitive decline, and in other neuropathological disorders such as Alzheimer's disease (AD), Parkinson's disease and epilepsy.

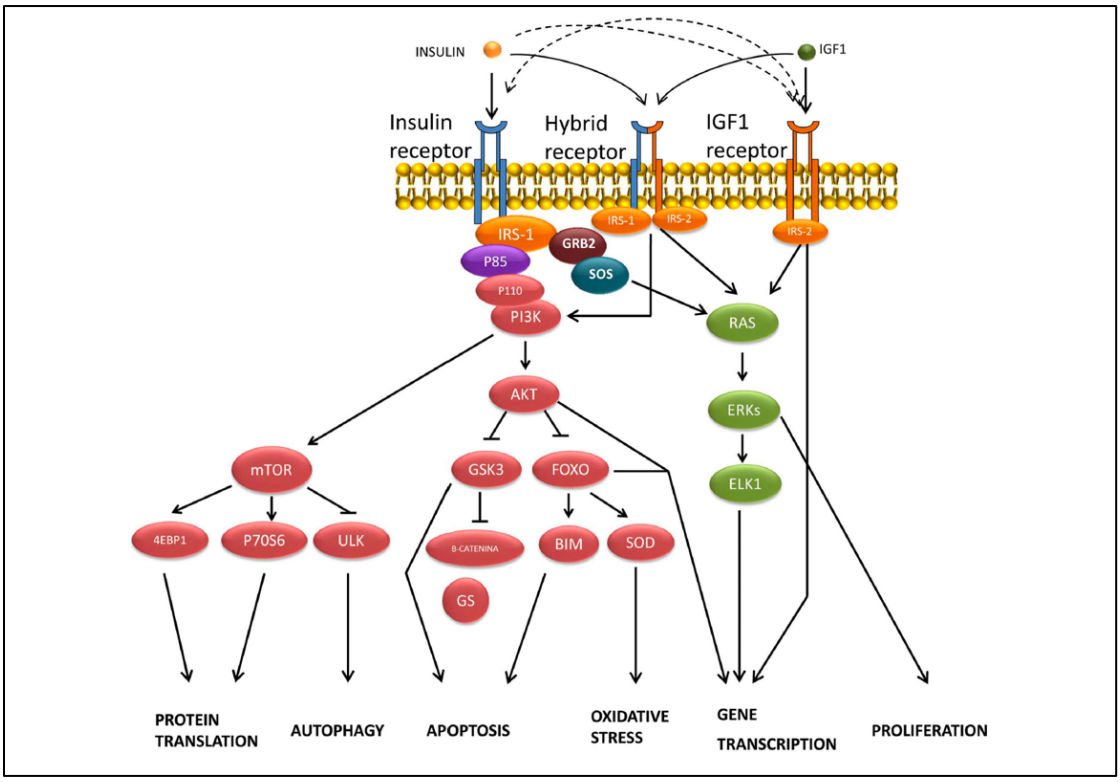

Fig 1.4. Signaling cascades and biological processes regulated by activation of insulin signaling.

1.5.3. Insulin signaling inhibition in aging brain

However, insulin and IR density in the brain decreases with aging and other neuropathological disorders. Insulin-IR binding affinity also decreases with age (Zaia and Piantanelli 1996). Insulin concentration decreases in all cortical regions of aging brain, while IR densities decrease only in frontal and parietal cortex (Frölich et al. 1998). Due to the decrease in insulin and IRs and their

binding affinity with aging, the insulin signaling pathway is downregulated in aging hippocampus (Solas et al. 2013).

1.6. Insulin enhances memory and synaptic plasticity

There is an abundance of IRs in hippocampus and the insulin-IRs binding affinity in the hippocampus is higher than other regions of the brain. Insulin plays important role in hippocampal dependent learning and memory. Insulin has repeatedly shown to modulate hippocampal plasticity and improve cognition in aging, AD and diabetes mellitus related dementia. In ex-vivo electrophysiological recording from pyramidal neurons of hippocampus, insulin leads to inhibition of spontaneous firing (Palovcik et al. 1984; Phillips and Palovcik 1989). This was the first evidence that demonstrated that insulin causes increase in neuronal excitability in hippocampus. Similarly, insulin application during ex-vivo recordings from CA1 pyramidal neurons of young rats reduced the amplitude and duration of mAHP and sAHP (Fig. 1.5) (Underwood and Thompson 2016). Zhao et. al. recently reported insulin-dependent changes in other variables of synaptic plasticity in hippocampus. Insulin application mediated inhibition of fEPSPs, stimulated LTP induction and LTD recovery (Zhao et al. 2019).

Insulin dependent changes in AHP, EPSP and other correlates of synaptic plasticity indicate insulin strong potential role in improvement of hippocampal function and hippocampal dependent learning and memory. Intracerebroventricular (ICV) application of insulin in young rats improved their performance on passive-avoidance task as compared to young rats receiving ICV of saline (Park et al. 2000). ICV is an invasive technique and cannot be performed on

humans to test the efficacy of insulin on cognition in humans. Intravenous (IV) application of insulin will lead to hypoglycemia, hyperinsulinemia and other related metabolic side-effects. Kern et al. performed IV insulin in healthy adult males, under euglycemic controlled conditions to avoid hypoglycemia. Increase in plasma insulin lead to a spike of CSF insulin as insulin crosses the BBB. Subjects receiving insulin showed improved performance and working memory after assessment on a word recall task (Kern et al. 2001).

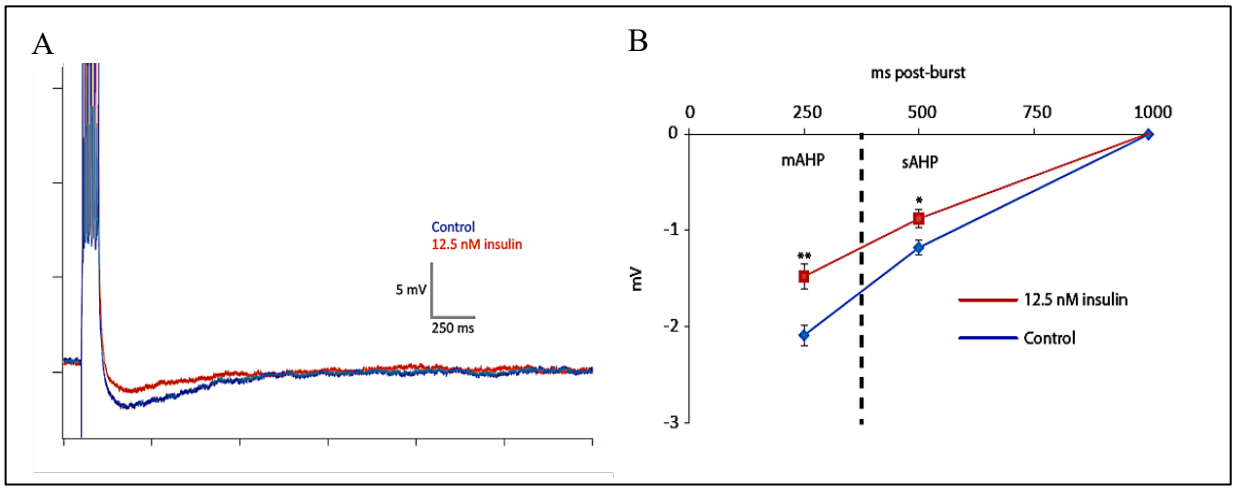

Fig 1.5. Insulin-dependent decrease in AHP: Application of insulin in aCSF bath during *ex-vivo* electrophysiological recordings from young hippocampal pyramidal neurons reduces the amplitude and duration of mAHP and sAHP [B]. Representative AHP trace shown in [B]. Images adapted from (Underwood and Thompson 2016).

However, there has been contradicting data in the literature regarding cognitive improvement effect of insulin in aging. Depending on the type of insulin used, insulin concentration and length of treatment (number of days) the results vary. ICV application of insulin Humulin of 20mIU (International Units)/day for 5 consecutive days, showed no cognitive improvement in aging rats

on hippocampal-dependent spatial memory task of Morris water maze (MWM). Interestingly, ICV insulin application (20mIU for 5 days) upregulated IR expression, phosphorylation of GSK3beta, increased mTOR and showed increase in NeuN staining, suggesting insulin signaling plays a role in neurogenesis (Haas et al. 2016). Another study showed that long term consistent application of insulin via ICV has detrimental effects on synaptic plasticity. ICV application of insulin Velosulin of 20mIU/hour over 4 weeks, showed an increase in sAHP amplitude in young rats. Long term high dose of insulin can lead to insulin desensitization in hippocampus (Kamal et al. 2012).

All these reports show a need of wide research to find the correct insulin dose and application method for insulin delivery to efficiently improve learning and memory. ICV and IV are invasive with serious side-effects and not the practical to be used as therapeutic approach in humans. Intranasal insulin (INI) application, delivers insulin from nasal cavity to the CSF and brain tissue, bypasses the BBB and has minimal side-effects. INI has become a widely accepted approach for delivery of insulin to the brain in human and animal models.

1.7. Intranasal insulin

Born at al. tested the intranasal delivery of small neuropeptides and its accumulation in the CSF in healthy human adults. Insulin peaked in CSF at 30 mins, while there was no change in peripheral plasma insulin or glucose levels (Born et al. 2002). Insulin is transported from nasal cavity to CSF and brain regions via the olfactory bulb via 3 possible pathways: 1. endocytosis by the olfactory epithelial cells, 2. transport via intracellular clefts in olfactory epithelium and 3.

endocytosis by olfactory neurons and axonal transport to olfactory bulb (Illum L 2000). As insulin peaks in CSF within 30 mins, the transport is considered fast and most likely transported by passive absorption via intercellular clefts in olfactory epithelial cell layer.

Clinical study in adults with early onset of AD and signs of MCI , INI application improved their verbal memory (Reger et al. 2006). In another study, INI improved declarative memory and mood in young (18- 34 yrs) healthy adults (Benedict et al. 2004). INI application in AD model 3xTG mice showed reductions in amyloid beta (Aβ) plaques, regulation of insulin signaling and increase in synaptic proteins- synapsin and PSD-95 (Chen et al. 2014). INI application of Humalog insulin (a fast-acting human insulin) improved spatial memory and learning in aging rats on the MWM task. Insulin application in artificial CSF bath during ex-vivo recording reduces amplitude of mAHP and amplitude and duration of sAHP in CA1 pyramidal neurons of aging hippocampus (Maimaiti et al. 2016). Most of the insulin formulations contain zinc and scientist are arguing regarding the role of zinc in learning and memory improvement. In an interesting report, Apidra, a zinc free insulin formulation showed upregulation of insulin signaling in some regions of the brain, however no effect on cognition in aging (Anderson et al. 2017).

1.8. Significance of study

There is enough evidence of the benefits of insulin in improving cognition and intrinsic excitability in aging hippocampus. Intranasal insulin has shown to improve memory in MCI and AD subjects. Intranasal insulin therapy in treating age-related cognitive deficits has not been

tried in humans yet. Acute (4 days) intranasal insulin therapy improved memory recall in aging rats but did not show any effect on learning on Morris water maze task. We propose chronic low dose intranasal insulin treatment would show a more pronounced effect on learning and spatial memory consolidation in aging rats on Morris water maze task. It is also well-established that insulin-mediated decrease in post-burst AHPs in hippocampal neurons, increases neuronal intrinsic excitability. However, a better understanding of the insulin signaling pathway and mechanism that induces the reduction of AHP amplitudes is necessary. We aim to assess the age-related changes in insulin signaling pathway and changes in proteins associated with AHP – SK2 channel, calmodulin and hippocalcin. And further analyze the effect of insulin on the expression of SK2, calmodulin and hippocalcin in aging hippocampus. The molecular mechanism of insulin-dependent increase in intrinsic excitability will open doors to novel therapies involving manipulation of insulin signaling to enhance hippocampal-dependent memory and further support clinical studies using insulin therapy to alleviate cognitive decline in normal aging, AD and other dementias.

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CHAPTER 2

INTRANASAL INSULIN IN AGING RATS CAN RESTORE SPATIAL MEMORY AND ALTER EXPRESSION OF CRITICAL PROTEINS UNDERLYING HIPPOCAMPAL INTRINSIC EXCITABILITY

2.1. Author contributions

N.T. conceived of this study and experimental design, conducted the experiments, performed data analysis and drafted this chapter. L.T. assisted with experimental design and editing the chapter. This chapter will be submitted as manuscript to a peer-reviewed journal for publication.

2.2. Abstract

Intranasal insulin has been shown to improve cognitive deficits in clinical trials with AD and MCI patients, with few reported side effects. Examining underlying mechanisms, Maimaiti *et al*. (2013) showed cognitive improvement in aging rats treated with intranasal insulin. They also showed insulin-dependent decreases in the amplitude and duration of hippocampal post-burst afterhyperpolarizations (AHPs), which are significantly increased in aging hippocampus leading to reduced intrinsic excitability. However, the molecular targets of insulin in hippocampal neurons leading to insulin-dependent decreases in AHPs in aged rats remained to be shown. In the current study, we utilized chronic low dose intranasal insulin (2 IU/d Humalog) application for 21 d. Aging (22-24 mo) male rats treated with intranasal insulin showed significantly improved learning and spatial memory consolidation on the Morris water maze task. Immunoblots performed with aging and with young whole hippocampal lysates showed age-

dependent (i) increased expression of phosphorylated insulin receptor substrate 1 (p-IRS1), (ii) decreased expression of phosphorylated Akt (p-Akt_{Ser473}), (iii) increased expression of the Ca2+ dependent K+ channel SK2 which mediates the mAHP, and (iv) decreased expression of the associated calcium sensor calmodulin (CaM). *Ex-vivo* insulin stimulation of hippocampal slices reversed age-dependent (i) upregulation of p-IRS1, (ii) downregulation of p-Akt, and (iii) upregulation of SK2, while it (iv) downregulated hippocalcin expression modulating the sAHP in both young and aging slices. Manipulation of insulin-signaling can benefit hippocampal-dependent memory in aging, and these effects on intrinsic excitability support further clinical study of the benefits of insulin therapy in aging populations.

2.3. Introduction

It has been only four decades since the presence of insulin receptors (IR) in the rat CNS was first reported (Havrankova et al. 1978). IRs are also found in human brain (Sara et al. 1982), and are expressed in high densities in hippocampus, dentate gyrus, pyriform cortex, cerebellum, olfactory bulb, hypothalamus and choroid plexus (Marks et al. 1990). Considering the abundance of IRs in the hippocampus and the insulin sensitivity of pyramidal neurons of hippocampus (Palovcik et al. 1984), it has been postulated that insulin and insulin-signaling pathways play an important role in the learning and formation of hippocampal-dependent declarative memories and spatial memories. This was demonstrated in rats (Park et al. 2000), where intracerebroventricular application of insulin improved memory formation and performance in a passive-avoidance task.

In humans, intraventricular (IV) application of insulin under euglycemic controlled conditions showed an improvement in declarative memory following acquisition of a word recall task (Kern et al. 2001). Long term IV infusion of insulin in humans is impractical and comes with serious side effects on peripheral glucose regulation and metabolism. However, (Born et al. 2002) demonstrated that intranasal (IN) application of small peptides like insulin increases the mean cerebrospinal fluid (CSF) concentration within 30 mins after application, while plasma insulin concentrations remained unchanged. Intranasal insulin application can bypass the blood-brain barrier (BBB) and enter the CSF from the nasal mucosa via intercellular clefts along the first cranial nerve and the olfactory bulb (Illum L 2000). Intranasal insulin application improves memory in patients with mild cognitive impairment and also in healthy adults (Benedict et al. 2004; Hölscher 2014). To probe the underlying mechanism of beneficial effects of insulin, animal models of AD and aging have been utilized (Maimaiti et al. 2016; T. J. Chen et al. 2014). However, the potential molecular targets of insulin in the hippocampus that leads to cognitive benefits has remained unknown.

Calcium-dependent post-burst afterhyperpolarization (AHPs) evoked by a burst of actions potentials is modulated in CA1 pyramidal neurons of hippocampus during learning and memory consolidation. *Ex-vivo* electrophysiological recordings from CA1 neurons of aging hippocampus shows increase in AHP amplitude, which reduces neuronal excitability and underlies the age-related cognitive decline (John F Disterhoft et al. 1996). The AHP is defined by three phases: Fast, medium and slow AHP. The increase in amplitude and duration of AHP in age-related cognitive decline is consistently observed in the slow AHP (sAHP) component (Power et al.

2002; Tombaugh, Rowe, and Rose 2005; M. Matthew Oh et al. 2009). Insulin application during *ex-vivo* recording from CA1 neurons reduces amplitude and duration of sAHP, in both young and aging rats (Pancani et al. 2013; Maimaiti et al. 2016). Underwood (Underwood and Thompson 2016) demonstrated that insulin reduces both mAHP and sAHP amplitude in young rat hippocampal slices.

The channel underlying mAHP is apamin-sensitive SK channel which mediates the calcium activated potassium current. There are four known isoforms of SK channel (SK1-4) out of which SK2 channel is most abundant isoform in hippocampus. SK2 channels are neuronal membrane bound proteins which are translated in the cytosol and translocated to the membrane (Lee et al. 2003). Calmodulin is a transient calcium binding messenger protein constitutively bound to SK2; which when occupied by calcium ions undergoes a conformational change leading to opening of the SK2 channel (Xia et al. 1998; Schumacher et al. 2001). The sAHP is also mediated by calcium activated potassium current, but the channel that underlies this current is yet unknown. It has been recently reported that knockout of neuronal calcium binding protein hippocalcin abolished the sAHP in the hippocampus (K. S. Kim et al. 2012). Hippocalcin is the calcium sensor that aids the channel mediating sAHP current.

It is important to assess the age-related changes in AHP-associated proteins- SK2, calmodulin and hippocalcin and to understand the molecular mechanism of insulin dependent AHP regulation. The potential link between insulin signaling and AHP can lead to a more efficient therapeutic target to ameliorate age-related cognitive decline.

2.4. Methods and Materials

2.4.1. Subjects:

The F1 hybrid of Fisher-344 females and Brown Norway males (Fisher-Brown Norway: FBN), a model system used in multiple laboratories studying aging, were utilized for this study. The locally bred FBN rats were socially housed on a reverse 12 hr light/dark cycle with *ad libitum* access to food and water, and environmental enrichments added to their home cages on a recurrent basis. The rats were divided into two cohorts: young (4-6 mo) and aging (22-24 mo). Daily records of weight were maintained throughout the study, and rats were repeatedly handled across their lifespan. All procedures were conducted in accordance with the current Institutional Animal Care and Use Committee (IACUC) guidelines of The University of Texas at Dallas, as well as all guidelines of the USDA.

2.4.2. Behavior:

A hippocampal-dependent spatial memory behavior task in the Morris water maze was performed to analyze spatial acquisition and reference memory (Vorhees and Williams 2006). Naïve young (n = 10) and aging rats (n = 15) which received no intranasal treatment were handled by the experimenter for 15 min daily for 3 d in the holding room, followed by 2 d of handling and habituation in the testing room. During habituation, rats were placed individually in covered empty cages in the testing room. Water maze testing was then performed in 3 stages: Day 0 (training), Days 1- 4 (spatial acquisition phase), and Day 5 (probe testing to assess spatial reference memory). On day 0, a 12 x 12 cm platform was completely submerged in water

(temperature maintained between 19-22º C) opaqued with white tempera paint but made visible by attaching an upright object (plastic spoon) that served as a visual cue for the platform's location. Each rat was given 2 min exploration time to find and climb onto the platform, 15 s spatial orientation acquisition time once they climbed atop the platform, then removed from the platform for a 1 min inter-trial interval. Each rat went through 4 trials in succession on Day 0, each trial with a unique start position and platform location. This training day data was used to identify and eliminate any rats with vision and motor disability (none were eliminated). On Days 1 to 4, spatial learning acquisition and memory was recorded by keeping the platform hidden in one quadrant (i.e. zone 4, Fig. 1F) and the rats were given 2 min to search for the platform from randomized different starting positions (S, SE, N, NW). Each rat underwent 4 trials in succession, with 15 s acquisition times and 1 min inter-trial intervals. During the probe testing on Day 5, rats were allowed to explore the water maze for 30 s, in the absence of the platform, to assess the reference memory. All spatial behaviors in the water maze were video-recorded, and performance assessed with ANY-maze behavioral tracking software.

2.4.3. Intranasal Insulin treatment and behavior:

Additional groups of young and aging rats were subdivided into cohorts: young saline (n = 5), young insulin (n = 6), aging saline (n = 8) and aging insulin (n = 10). Based on previous studies with intranasal insulin application in humans and animal models, we treated rats with insulin Humalog at a dose of 2IU/d for 21 d (Benedict et al. 2004; B.-H. Kim et al. 2019; Y. Chen et al. 2014). Young insulin and aging insulin rats received intranasal insulin of 2IU/20ul, divided into four 5 µl applications applied to the right naris using a fixed 5 µl pipette. Young saline and aging

saline control groups received 20 µl of 0.9% saline solution in four 5 µl application. All four cohorts were handled by the experimenter for 5 d prior to infusion. Insulin or saline treatment was applied for 21 consecutive days in the morning between 10 – 11 AM. Morris water maze testing was then performed on these four cohort groups as described above. Since it was previously shown that intranasal insulin peaks in the CSF within 30 min post-application (Born et al. 2002), during Day 0 through Day 5 (probe test) of the water maze task, each rat was given intranasal treatment 30 min prior to trial 1 of their 4 successive trials each day.

2.4.4. Hippocampal Slice Preparation:

Hippocampal dissection and slice preparation were performed as described previously (Underwood and Thompson 2016), with some modifications. Briefly, brains were extracted out of naïve young (n = 7) or aging (n = 8) anesthetized rats and placed in oxygenated ice-cold sucrose artificial cerebrospinal fluid (aCSF) (in mM: 3.0 KCl, 1.24 NaH2PO4·H20, 1.3 MgSO4, 2.4 CaCl2·2H2O, 26.0 NaHCO3, 220 sucrose and 10.0 D-glucose; pH 7.4) solution for 5 min. Hippocampi were dissected out from each hemisphere of the brain, and coronal hippocampal sections (400 µm) were obtained using a vibratome. Few sections were collected in 1.5 ml centrifuge tubes, washed 3 times with oxygenates ice-cold aCSF and homogenized in RIPA buffer for analysis on western blot. Sections were then transferred to a holding chamber with oxygenated room temperature aCSF (in mM: 3.0 KCl, 1.24 NaH2PO4·H20, 1.3 MgSO4, 2.4 CaCl2·2H2O, 26.0 NaHCO3, 124 NaCl and 10.0 D-glucose; pH 7.4.) and incubated for 45 min. Individual sections with similar hippocampal anatomy were selected and placed in 24-well plate with aCSF (one slice per well) for *ex-vivo* insulin stimulation.

2.4.5. Ex-vivo insulin stimulation:

Ex-vivo insulin stimulation of hippocampal sections was performed (as described by (Arnold et al. 2015), with modifications to insulin concentration and incubation time. Sections were washed 3 times with oxygenated room temperature aCSF and incubated with 24 nM Humalog insulin in aCSF for 10 min or with only aCSF for controls. Slices were replaced with ice-cold oxygenated aCSF to stop the biochemical reaction and washed 3 more times with ice-cold aCSF. Slices were then transferred to 1.5 ml centrifuge tubes with RIPA lysis buffer and homogenized for further analysis on western blot.

2.4.6. *In-vivo* insulin stimulation and tissue collection:

Both the young and aging cohorts were treated with intranasal insulin or saline for 21 d. Young saline (n = 3), young insulin (n = 4), aging saline (n = 3) or aging insulin (n = 3) rats were sacrificed 30 min after the intranasal application on day 21 (i.e. no behavioral testing occurred). The hippocampus from the right hemisphere was dissected out, and flash frozen in a super-cooled bath of 2-methylbutane (~ -150ºC) for 1.5 min. Hippocampus lysates were prepared from the flash frozen tissue to perform western blots.

2.4.7. Western Blotting and antibodies:

Hippocampus tissue lysates from naïve rats, from *ex-vivo* insulin stimulation and from *in-vivo* insulin stimulation were prepared in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1mM EDTA and 1% Triton x-100) containing protease inhibitor cocktail (Sigma P8340) and

phosphatase inhibitor cocktail 1 and 2 (Sigma P2850 and P5726). Protein quantification was performed using Pierce BCA Protein Assay Kit (Thermo Scientific) and samples were prepared in 4X Laemmli Sample buffer. Samples were loaded and separated on 12% SDS-PAGE gels (Bio-rad 161-0185), transferred to a PVDF membrane and blocked in 5% (w/v) milk in TBS (50mM Tris pH 7.4, 150mM NaCl) for 1 h at room temperature. Membranes were probed with primary antibody overnight at 4°C and with corresponding secondary antibody conjugated with HRP for 1 h at room temperature. Chemiluminescence signal was activated using Clarity Western ECL (Bio-rad 170-5061) and blots were imaged using a Bio-rad ChemiDoc XRS+ System. The intensity of protein bands was analyzed using NIH ImageJ software. Primary antibodies used for our studies were p-IRS1 Ser616 (Invitrogen 44-550G), IRS1 (Invitrogen, MA5-15072), pAKT Ser473 (Cell Signaling, 4060), pan AKT (Cell Signaling 2920), Hippocalcin (Abcam ab24560), Calmodulin (Abcam ab45689), KCCN2/KCa2.2/SK2 (Alomone Labs APC-028), and GAPDH (Proteintech 6004-1-Ig). For phosphorylated proteins such as p-IRS1 and p-AKT, the membranes were stripped with stripping buffer (0.1 M Glycine, 20 mM Mg $(CH3COO)_2$, 50 mM KCl, pH 2.2) and reprobed for total IRS1 and AKT respectively.

2.4.8. Statistical Analyses:

GraphPad Prism was used to perform statistical analyses and to generate graphs for both behavioral and western blot data. One-way and two-way ANOVAs were performed, and *post hoc* tests were performed using Bonferroni's correction and Tukey's correction respectively. Statistical significance was accepted at $p < 0.05$.

2.5. Results:

2.5.1. Age-related cognitive decline in FBN males:

FBN aging males demonstrated cognitive impairment on a Morris water maze task consequent to normal aging. On day 0, both young and aging males had similar latencies to reach the visible platform, demonstrating no motor or visual disability in aging rats (data not shown). From days 1 – 4, both young and aging rats exhibited acquisition of the spatial task, with learning curves demonstrating decreased time taken to find the hidden platform day-by-day. However, aging rats showed slower learning curves, taking significant longer times to reach the platform as compared to young rats (Fig. 2.1.A). The 2-way ANOVA analysis showed significant effect for days [$F_{(3,92)} = 33.14$, $p < 0.0001$], age [$F_{(1,92)} = 38.29$, $p < 0.0001$] and day x age interaction [$F_{(3,92)} = 6.604$, $p = 0.0005$]; *post hoc* analysis showed that aging rats performed better than young rats on day 1 ($p < 0.0001$) and day 2 ($p = 0.007$). Aging rats also showed a lower path efficiency to platform as compared to young rats (Fig. 2.1.C). The 2-way ANOVA analysis for path efficiency showed significant effect for days [$F_{(3,92)} = 36.13$, $p < 0.0001$] and age [$F_{(1,92)} = 52.58$, $p < 0.0001$]; with *post hoc* analysis indicating significant difference in path efficiency on day 1 ($p = 0.0003$), day 2 ($p < 0.0001$) and day 3 ($p = 0.0018$), We also analyzed path length and found that aging rats swam longer distance to reach the platform (Fig. 2.1.B). The 2-way ANOVA results showed significant effect for days [$F_{(3,92)} = 27.20$, $p < 0.0001$], age [$F_{(1,92)} = 28.14$, $p < 0.0001$] and day x age interaction [$F_{(3,92)} = 5.399$, $p = 0.002$]; while *post hoc* analysis showed aging rats swam longer distance as compared to young rats on day 1 ($p < 0.0001$) and day 2 ($p = 0.011$). Together, these demonstrate significant declines in hippocampus-dependent spatial memory and learning in

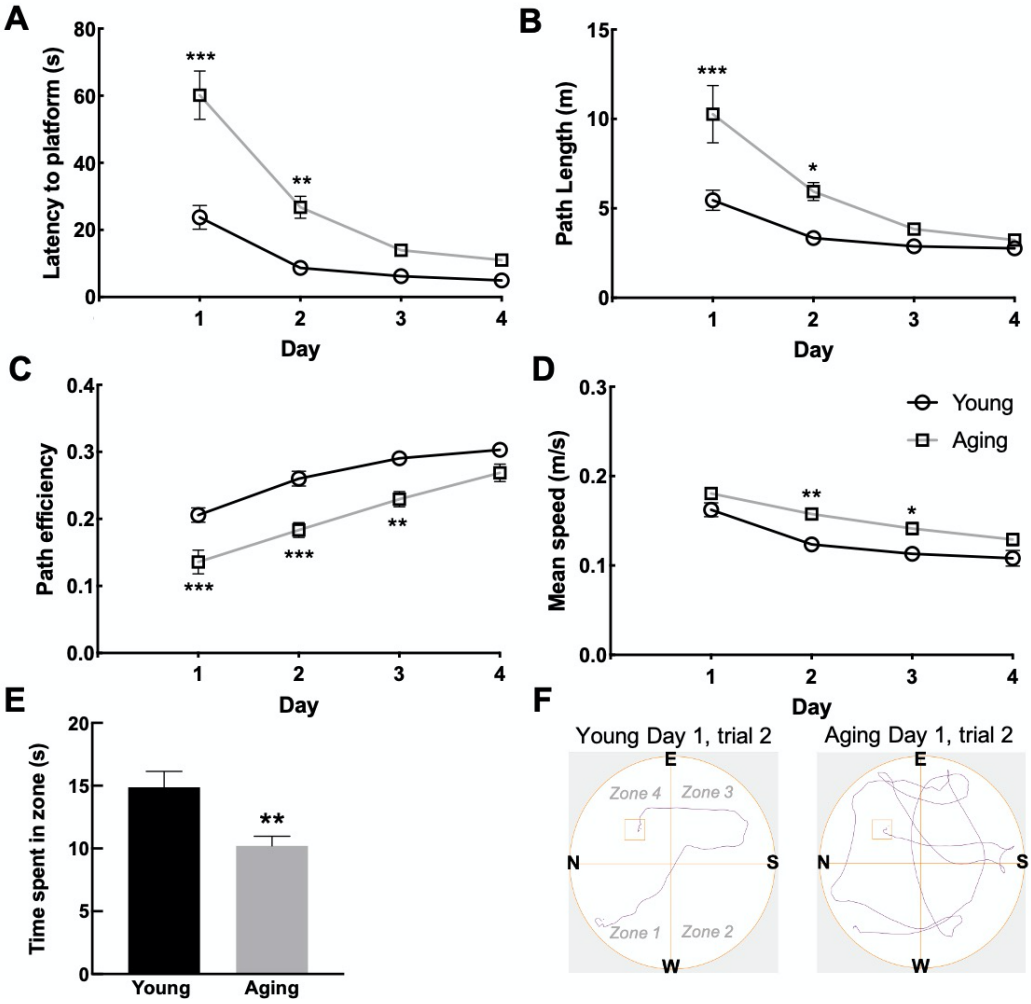

Fig 2.1. Age-related decline in spatial memory of FBN male rats in the Morris water maze. [A] Aging (22-24 mo) rats were significantly slower to find the hidden platform on days 1 and 2, with impaired learning curves compared to young (4-6 mo) rats. Each day's data represents an average of 4 learning trials/rat. [B-C] Aging rats showed significantly longer path length taken and impaired path efficiency to the platform across days 1-3 compared to young rats. [D] Mean swim speed was actually faster on days 2 and 3 in aging rats. [E] Probe testing for reference memory was performed on day 5, when rats explored the water maze for 30 s in the absence of the platform. Aging rats exhibited impaired memory recall, spending less time in the platform zone as compared to young rats. [F] Representative performance of young and aging rats on day 1 trial 2. Mean ± SEM shown. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. Young n = 10, aging n = 15.

FBN aging rats. An analysis of probe trials to analyze reference memory retention on day 5 in the absence of platform was also performed, indicating significant impairment in this relatively pure test of spatial memory. While young rats spent approximately 50% (15 s) of their exploration time (30 s) in the platform zone, aging rats spent significantly less time (< 10 s) in the platform zone (Fig. 2.1.E) [$p < 0.005$]. Mean speed was also analyzed across days 1 – 4. Post hoc analysis showed that aging rats swim speed was higher on day 2 ($p = 0.001$) and day 3 ($p = 0.014$) as compared to young rats (Fig. 2.1.D), however the difference was small and does not affect the overall results.

2.5.2. Intranasal insulin improves spatial memory and learning in aging rats:

Intranasal insulin treatment ameliorated the learning deficit and enhanced the spatial memory consolidation in aging rats. Aging rats treated with intranasal insulin showed significantly improved learning curve, taking significantly reduced time to reach platform on day 1 and 2 of acquisition phase (Fig. 2.2.B), as compared to aging rats treated with intranasal saline. When analyzed using a 2-way ANOVA, we found significant effect for days [$F_{(2,75)} = 53.34$, $p < 0.0001$], treatment [$F_{(3,75)} = 65.09$, $p < 0.0001$] and day x treatment interaction [$F_{(3,75)} = 16.4$, $p < 0.0001$]; *post hoc* analysis showed that insulin significantly improved performance in aging rats on day 1 and 2 ($p < 0.005$ for both days) as compared to aging saline group. During the acquisition phase, aging insulin group also exhibited better path efficiency as compared to aging saline group (Fig. 2.2.C). The 2-way ANOVA analysis showed significant effect on path efficiency for days [$F_{(2,75)} = 16.30$, $p < 0.0001$] and treatment [$F_{(3,75)} = 7.485$, $p = 0.0002$]; by *post hoc* analysis we found that aging insulin rats showed higher path efficiency only on day 1

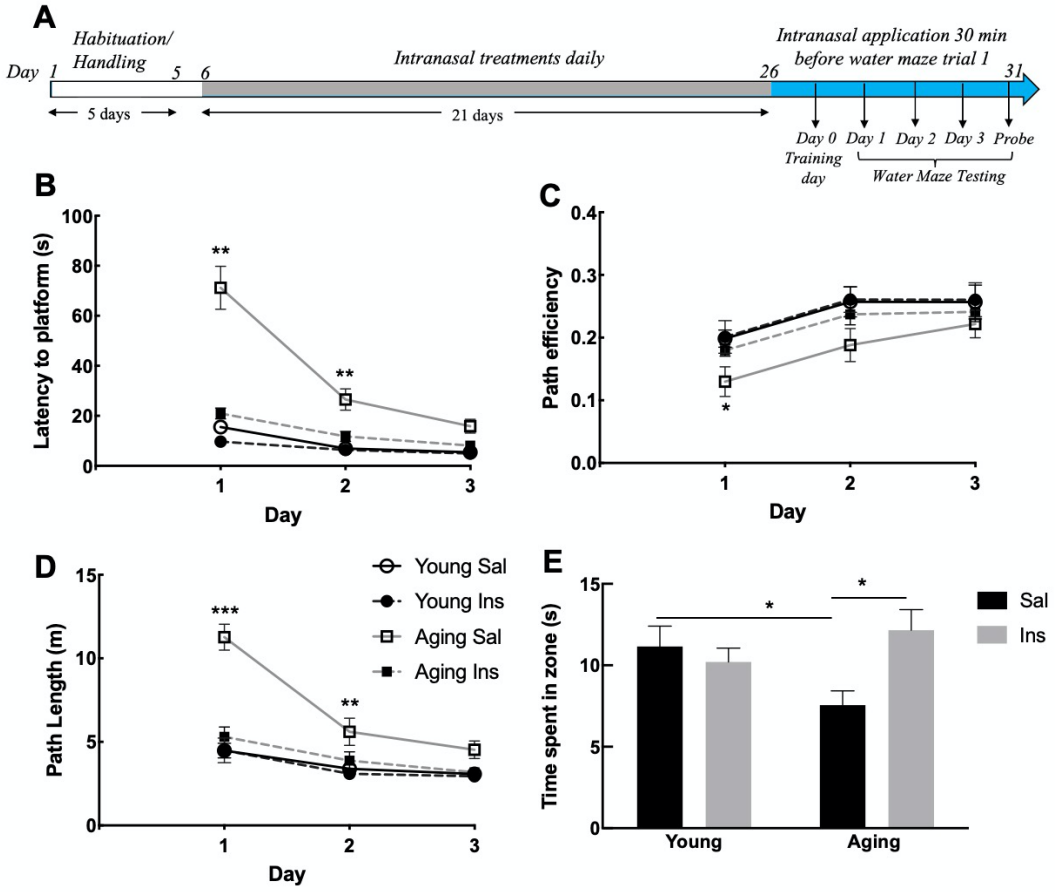

Fig 2.2. Intranasal insulin improves spatial memory in aging rats: [A] Morris water maze testing was performed after 21 d of intranasal saline or insulin treatment with both young and aging rats. Aging rats treated with intranasal insulin performed significantly better than aging rats treated with saline only; treatment essentially reversed the aging deficit in performance. Each day's data shown is an average of 4 learning trials. Insulin improved learning and spatial memory in aging but not in young rats. [B-C] Aging insulin-treated rats took less time and path length to find the platform and showed a [D] higher path efficiency as compared to aging saline controls. For probe testing, all 4 cohorts were allowed to explore the water maze tank for 30 s in the absence of the platform. Aging insulin-treated rats spent more time in the former platform zone [E], demonstrating the effect of insulin in memory retention. This insulin-dependent spatial memory improvement is only observed in aging but not in young rats. Mean ± SEM shown. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ shows significance between the groups. Young saline n = 5, young insulin n = 6, aging saline n = 8, aging insulin n = 10.

[p =0.041] as compared to aging saline rats. Next, we analyzed path length and found significant effect for days [$F_{(2,75)} = 29.39$, $p < 0.0001$] and treatment [$F_{(3,75)} = 29.67$, $p < 0.0001$]; *post hoc* analysis showed aging insulin rats swam shorter distance to the platform on day 1 ($p < 0.0001$) and day 2 ($p = 0.005$) as compared to aging saline rats (Fig. 2.2.D). Probe trial showed significantly improved reference memory retention in aging insulin group (Fig. 2.2.E). Aging rats treated with intranasal insulin spent more time exploring the platform zone as compared to aging rats treated with intranasal saline [$p = 0.011$]. The latency to platform, path efficiency, path length and time spent in zone of aging rats treated with insulin was similar to performance of young rats treated with intranasal saline and insulin. Notably, no effect of intranasal insulin was observed in young rats on latency, path efficiency, path length and time spend in the platform zone.

2.5.3. Insulin signaling is downregulated in aging hippocampus:

Western blot analysis of hippocampal lysate from naïve young and aging rats, showed a downregulation of insulin signaling in aging hippocampus. The increase in phosphorylation of IRS1 at serine-616 (Fig. 2.3.A) [$p < 0.005$] and decrease in the phosphorylation of Akt at serine-473 (Fig. 2.3.B) [$p < 0.01$] in aging hippocampus compared to young hippocampus, indicates inhibition of insulin signaling in aging hippocampus. *Ex-vivo* insulin stimulation induces activation of insulin signaling in young and aging hippocampus. Insulin reduces the level of phosphorylated IRS-ser616 (Fig. 2.4.A) [$F_{(1,26)} = 7.290$, $p = 0.015$] in both age groups. *Post hoc* analysis indicated that insulin significantly reduced p-IRS level in aging hippocampus [$p = 0.04$], while effect of insulin in reducing p-IRS in young hippocampus was not significant [$p = 0.73$].

Ex-vivo insulin stimulation induces phosphorylation of Akt-ser473 (Fig. 2.4.B) [$F_{(1,26)} = 11.22$, $p = 0.001$] in young and aging hippocampal slices. Post hoc analysis showed that insulin significantly increased p-Akt levels in young [$p = 0.02$] and aging [$p = 0.009$] hippocampus. While there is a decrease in insulin and insulin receptors in aging brain (Zaia and Piantanelli 1996; Frölich et al. 1998), aging hippocampus retains insulin sensitivity.

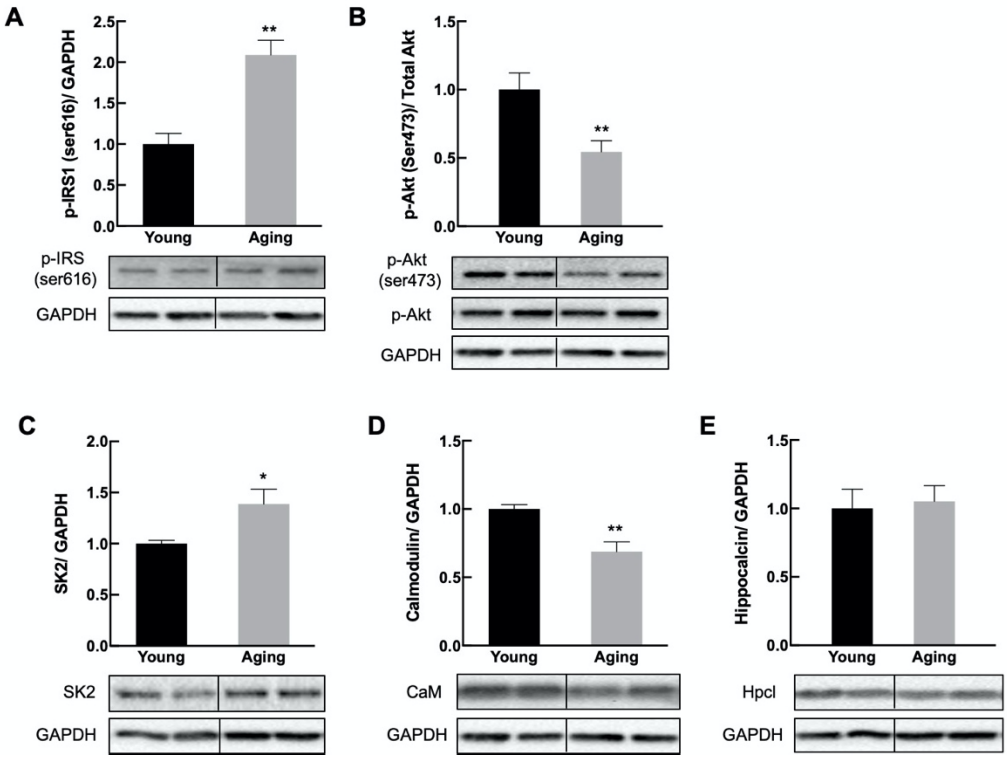

Fig 2.3. Aging inhibits hippocampal insulin signaling alters mAHP-related protein expression: Western blots of hippocampal lysates from naïve young or aging male rats show inhibition of insulin signaling in aging hippocampus, with [A] an increase in phosphorylation of IRS1 at serine 616, and [B] a decrease in phosphorylation of Akt at serine 473. Expression of the [C] SK2 channel protein mediating the mAHP current is increased in aging hippocampus, while expression of the associated calcium-sensor [D] Calmodulin is decreased. Expression of [E] Hippocalcin, the calcium-sensor modulating the sAHP current, exhibits no age-dependent changes. Mean SEM shown. *$p < 0.05$, **$p < 0.005$. Young n = 7, aging n = 8.

2.5.4. Insulin reverses age-related overexpression of SK2 and downregulates hippocalcin:

The expression level of SK2 channel mediating the potassium current underlying mAHP is significantly upregulated in aging hippocampus (Fig. 2.3.C) [$p = 0.02$]. While the calcium sensor calmodulin that gates mAHP, is downregulated aging hippocampus (Fig. 2.3.D) [$p = 0.001$]. There is no age effect on the expression level of hippocalcin, the calcium sensor that gates sAHP (Fig. 2.3.E) [$p = 0.78$]. We further analyzed the effect of insulin on expression level of Sk2, calmodulin and hippocalcin in young and aging hippocampal slices via *ex-vivo* insulin stimulation. Western blot results showed that insulin significantly reduced expression of SK2 channel in aging hippocampus, but not in young hippocampus (Fig. 2.4.C) [$F_{(1,26)} = 9.67$, $p < 0.005$]. *Post hoc* analysis showed insulin downregulates SK2 levels in aging hippocampus [$p = 0.008$], while has no effect on SK2 level in young hippocampus [$p = 0.8$]. Insulin had no effect on the expression of calmodulin in either young or aging hippocampus (Fig. 2.4.D) [$F_{(1,26)} = 0.117$, $p = 0.735$]. Notably, insulin significantly downregulated the expression level of hippocalcin in both age groups (Fig. 2.4.E) [$F_{(1,26)} = 10.32$, $p < 0.005$]. *Post hoc* test showed that insulin significantly reduces hippocalcin levels in young [$p = 0.021$] and in aging [$p = 0.042$] hippocampus; these results corroborate the insulin-dependent decrease in amplitude and duration of sAHP in young and aging neurons (Maimaiti et al. 2016; Underwood and Thompson 2016).

2.5.5. Intranasal insulin potentially alters SK2 and hippocalcin *in-vivo*:

Given the effect of insulin on regulating expression of SK2 and hippocalcin in *ex-vivo* hippocalcin slices, we next assessed the effect of intranasal insulin *in-vivo*. We performed

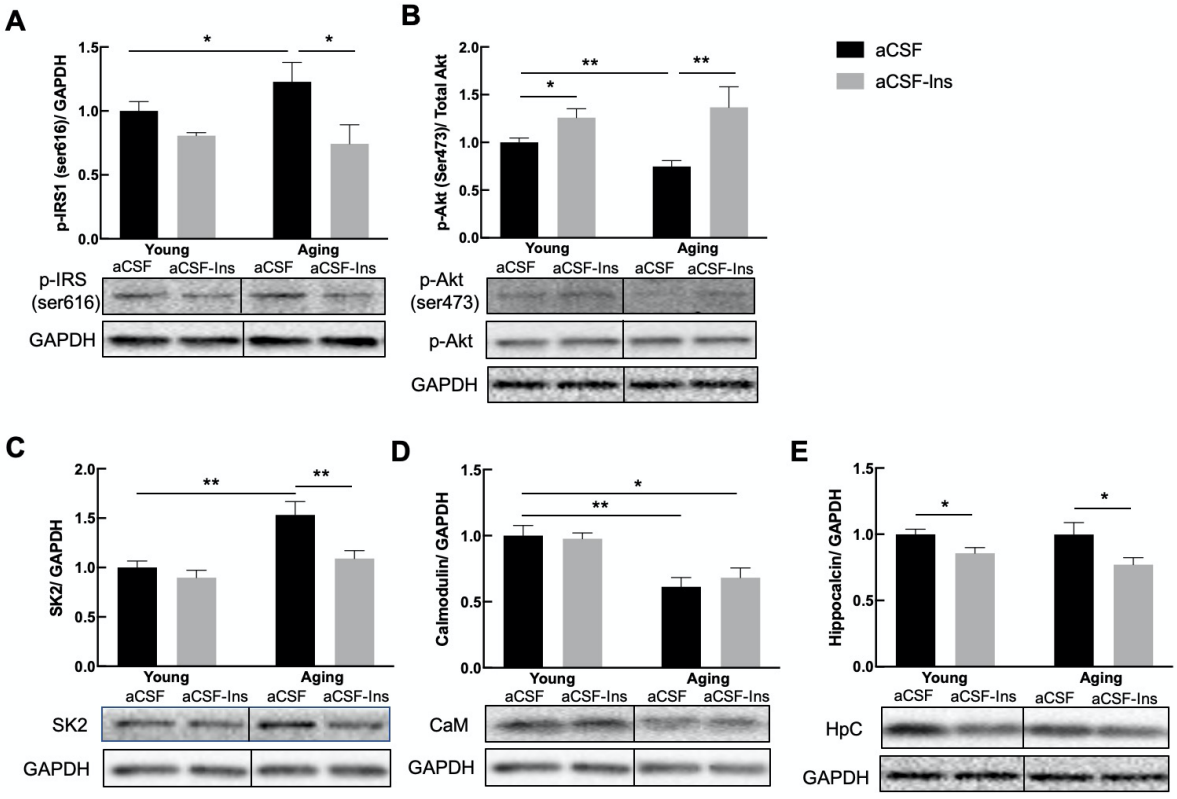

Fig 2.4. Potential molecular link between insulin signaling pathways and AHPs: *Ex-vivo* stimulation of aging hippocampal slices with insulin (24nM for 10 min) restored insulin-signaling [A, B] and SK2 expression [C] to levels similar to those seen in young slices, compared to aCSF controls. A. Aging enhanced p-IRS (ser616) levels, and insulin reduced phosphorylation of IRS at serine 616 in both young and aging hippocampus. B. Aging decreased p-Akt levels, and insulin increased phosphorylation of Akt at serine 473 in both young and aging hippocampus. [C] Insulin stimulation reduced the upregulated SK2 expression in aging hippocampus but had no significant effect in young hippocampus. D. Calmodulin expression was significantly reduced in aging, but insulin stimulation had no effect at either age. E. Hippocalcin expression was not altered by aging, but insulin reduced hippocalcin expression in young and aging hippocampus. Mean ± SEM shown. *$p < 0.05$, **$p < 0.005$. Young aCSF n = 7, young insulin n = 7, aging aCSF n = 8, aging insulin n = 8.

western blot analysis on hippocampal tissue collected from young and aging animals on day 21 of intranasal application. Intranasal insulin showed a trend of decrease in expression level of SK2 in aging hippocampus *in-vivo* (Fig. 2.5.A), although not significant [$p = 0.14$]. Intranasal

insulin did not alter the expression of hippocalcin in young or aging hippocampus *in-vivo.* It is important to note that number of animals in this set of data was small and further investigation is necessary.

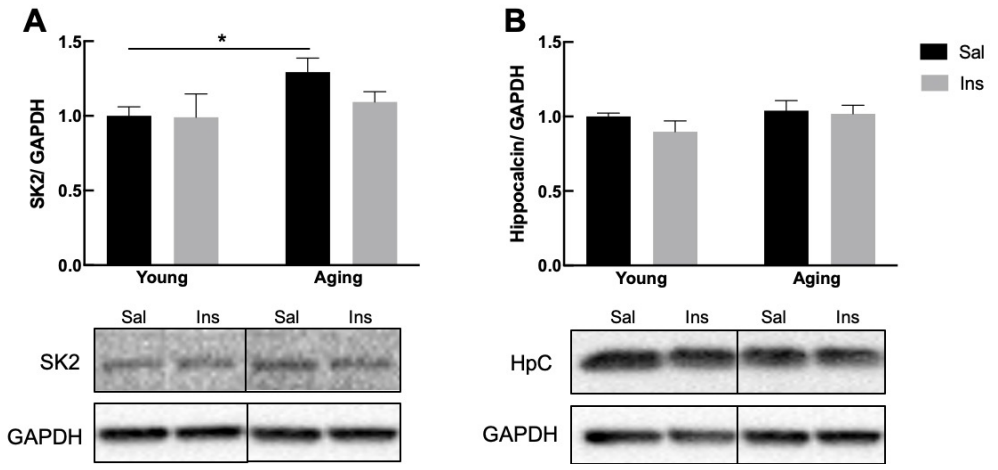

Fig 2.5. Effect of intranasal insulin on SK2 and hippocalcin expression *in-vivo*: Western blot analyses showing *in-vivo* effects of aging and of intranasal insulin on expression of [A] SK2 and [B] hippocalcin in young and aging hippocampus after 21 d of intranasal insulin application. Aging enhanced expression of SK2 protein, but not of hippocalcin. [A] Compared to *ex-vivo* insulin stimulation, intranasal insulin showed a trend toward reducing expression of SK2 in aging hippocampus; however, the effect was not significant. [B] Intranasal insulin showed no effect on hippocalcin expression in young or aging hippocampus. Mean ± SEM shown. *$p <$ 0.05. Young saline n = 3, young insulin n = 4, aging saline n = 3, aging insulin n = 3.

2.6. Discussion:

Reduced intrinsic excitability of hippocampal pyramidal neurons is a well-described factor in impaired spatial and other memory functions in aging (Tombaugh, Rowe, and Rose 2005; Lucien T. Thompson, Mover, and Disterhoft 1996), and treatments restoring this excitability to a more youthful state also reverse age-associated memory impairments (J F Disterhoft, Moyer, and Thompson 1994; John C Gant et al. 2015, 2018; Kronforst-Collins et al. 1997; Moyer et al. 1992;

M M Oh et al. 1999; L T Thompson and Disterhoft 1997). In our hands and as widely reported, aging rats exhibit significant age-associated spatial memory impairments assessed using water maze tasks (Fig. 2.1), and the intranasal insulin treatment given in the current study reverse this impairment (Fig. 2.2).

Increased amplitude and duration of post-burst AHPs reduces intrinsic neuronal excitability in aging hippocampus, which hinders hippocampal dependent-learning and spatial memory consolidation (Power et al. 2002; John F Disterhoft et al. 1996). Insulin has been shown to improve learning and memory in aging (Park et al. 2000; Kern et al. 2001), and also helps regulate the amplitude and duration of the post-burst AHP, specifically both the mAHP and sAHP (Underwood and Thompson 2016). Insulin or insulin-signaling plays a potential role in increasing neuronal excitability in aging hippocampus by reducing the amplitude and duration of AHP (Anderson et al. 2017; Maimaiti et al. 2016; Kamal et al. 2012). In the current study, we looked at whether insulin altered the expression of proteins mediating medium and slow components of post burst AHPs and compared the effects between young and aging rat hippocampus.

Age-related change in the expression of the calcium-dependent potassium channel SK2 and its constitutively bound calcium sensor calmodulin, has been previously reported (Ballesteros-Merino et al. 2012; Levine, Gosnell, and Morley 1986; J. C. Gant et al. 2014), which is consistent within our own data. The expression of total SK2 in hippocampus increases with aging (Fig. 2.3.C), while expression of total calmodulin in decreases with aging (Fig. 2.3.D). Our

data shows that the calcium-sensor hippocalcin, which may gate the sAHP, is not altered with aging in whole hippocampal lysate (Fig. 2.3.E). Previously it has been reported that hippocalcin decreases almost 20% in aging CA3 neurons while hippocalcin expression is maintained in aging CA1 neurons (Furuta et al. 1999). Notably, while calmodulin gating the SK2 current is constitutively bound (Keen et al. 1999; Xia et al. 1998; Schumacher et al. 2001), hippocalcin is postulated NOT to be constitutively bound (contributing to the slow gating kinetics of the sAHP), so a lack of change in expression of hippocalcin in aging does not directly address observed increases in the sAHP in aging (Tzingounis et al. 2007). Until the specific ion channel gating the sAHP is clearly and definitively isolated, the underlying molecular mechanism will remain obscure.

With aging, neurons exhibit reduced insulin signaling, due to diminished extracellular insulin and reductions in expression of insulin receptors in the brain (Zaia and Piantanelli 1996; Zhao et al. 2004; Frölich et al. 1998). In our study, aging produced an increase in p-IRS (Fig. 2.3.A) but a decrease in p-Akt (Fig. 2.3.B). Our experimental *ex-vivo* insulin stimulation of aging hippocampal slices produced a robust activation of IRS-PI3K/Akt insulin signaling pathway (see Fig. 2.4.A-B). We analyzed the insulin-dependent change in expression of SK2, calmodulin and hippocalcin in young and aging hippocampus via *ex-vivo* insulin stimulation of hippocampal slices. Insulin restored (reduced) the expression of SK2 channel in aging hippocampus (Fig. 2.4.C) to levels similar to that seen in young hippocampus. Insulin potentially regulates the gene transcription of SK2 channel via insulin dependent PKC (protein kinase C) activation in hippocampal neurons (Zhao et al. 2004). Calmodulin expression in the hippocampus is reduced

with aging (Fig. 2.3.D); our *ex-vivo* insulin stimulation experiments demonstrated is no effect of insulin on expression of calmodulin in either young or aging hippocampus. *Ex-vivo* insulin stimulation downregulated expression of hippocalcin in both young and aging hippocampus; the effects on the still unidentified sAHP channel protein remain unknown.

Intranasal insulin improves hippocampal-dependent learning and spatial memory consolidation, and *ex-vivo* insulin application reduces the amplitude and duration of mAHP and sAHP (Underwood & Thompson, 2016). In the current study, we have shown that insulin regulates these effects at a molecular level. Insulin decreases the expression of total SK2 (mAHP channel protein) in the aging hippocampus, reducing the number of channels available to mediate the enhanced mAHP current normally observed under control conditions in aging neurons. Insulin-dependent downregulation of hippocalcin in young and aging hippocampus is also consistent with observed insulin-dependent decreases in amplitude and duration of sAHP in young and aging CA1 neurons (Maimaiti et al. 2016), but still requires additional study.

Our data shows that intranasal insulin enhances performance in aging rats learning the Morris water maze, which is a hippocampal-dependent spatial memory task. Rats with hippocampal lesions show impairment in learning this water maze task (Morris et al. 1982). Therefore, we predict that insulin treatment would not improve spatial memory in rats with hippocampal lesions. On the other hand, the presence of insulin receptor antagonists in aging brain (such as glucagon, used to treat hypoglycemia, which is BBB permeable) would produce greater cognitive impairments. Also, pharmacological treatments altering Akt signaling, such as the common

diabetes clinical drug metformin (Rice et al. 2011) would alter the results reported here.

In summary, we report that chronic low dose intranasal insulin treatment can boost learning and enhance spatial memory consolidation of hippocampus-dependent tasks in aging. Intranasal insulin can reverse cognitive deficits induced by impaired insulin signaling in aging brain. Our study further links insulin signaling to hippocampal-intrinsic excitability. Our data suggests that activation of insulin signaling in hippocampus reduces AHP amplitude by (i) reversing the age-related overexpression of SK2 channel in aging, and (ii) downregulating hippocalcin expression in both young and aging hippocampus. The work presented here supports the use of intranasal insulin therapy to ameliorate age-related cognitive decline, and supports the need of further exploration of insulin-signaling pathways leading to other potential molecular changes underlying impaired intrinsic excitability in aging and other dementias such as AD.

2.7. Acknowledgements:

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CHAPTER 3

CONCLUSION

3.1. Summary of results and future directions

In this dissertation we aimed to (i) study the efficacy of a chronic low dose intranasal insulin therapy on cognitive deficits in aging rats and (ii) study the molecular mechanism underlying the insulin-dependent regulation of AHP in young and aging hippocampus.

Intranasal insulin dose of 2IU Humalog for 21 days, showed a pronounced effect in improving spatial memory consolidation and learning in aging rats. Both young and aging rats were tested on Morris water maze, which is a hippocampus-dependent spatial memory task. Intranasal treatment significantly improved performance of aging rats but had no effect on performance of young rats. One of the future experiments I propose is to test the rats on probe trial 1, 2 and 3 weeks following the intranasal insulin treatment and successfully learning the water maze task. I hypothesize that insulin aids in long-term memory consolidation in aging; and learning a task during or following a chronic intranasal insulin therapy could help retain the memory for a longer period of time. Second, we should look at effect of intranasal insulin treatment on other hippocampus-dependent behavior tasks such as trace eye blink conditioning (Disterhoft et al. 1996) and on tasks that are not hippocampus based such as inhibitory avoidance, in which basolateral amygdala contributes to fear-motivated memory consolidation (Wilensky, Schafe, and LeDoux 2000).

Next, we analyzed age-dependent changes in key proteins involved in insulin signaling and AHPs. Western blot analysis of young and aging hippocampus tissue showed increase in p-IRS_{ser616} and decease in p-Akt_{ser473} in aging, indicating downregulation of insulin signaling. We also report age-dependent (i) upregulation of SK2 channel that mediates mAHP and (ii) downregulation of calcium sensor calmodulin that gates mAHP, while there was (iii) no change in protein levels of calcium sensor hippocalcin that gates sAHP.

To determine the molecular effect of insulin on AHP, we performed ex-vivo insulin stimulation of hippocampal slices and analyzed them on western blot. Insulin stimulation activated the insulin signaling in young and aging hippocampus, as shown by insulin-dependent decrease in p-IRS_{ser616} levels and increase in p-Akt_{ser473} levels via western blot. Insulin stimulation also demonstrated that insulin promoted (i) downregulation of SK2 channel expression in aging hippocampus and (ii) downregulation of hippocalcin expression in both young and aging hippocampus. Insulin did not alter expression level of calmodulin in either young or aging hippocampus. Insulin-dependent regulation of protein expression of SK2 and hippocalcin might be one of the potential mechanisms involved in insulin-dependent decrease in mAHP and sAHP amplitudes, respectively.

SK2 channels are translated in the cytosol and translocated to plasma membrane, along with bound calcium sensor calmodulin (Lee et al. 2003). SK2 channel cell-surface expression is regulated by kinase PKA, where phosphorylation by PKA promotes endocytosis of SK2

channels (Pedarzani and Storm 1993). It is important to study the effect of insulin on activity of PKA and phosphorylation levels of SK2.

The insulin mediated downregulation of hippocalcin expression, is consistent with insulin-dependent decrease in sAHP amplitude in in young and aging hippocampus (Underwood and Thompson 2016; Maimaiti et al. 2016). Also, the amplitude of sAHP is significantly reduced in hippocampal pyramidal neurons of hippocalcin knockout mice (Tzingounis et al. 2007). However, hippocalcin expression does not change with age, and the increase in sAHP in aging hippocampus cannot be fully explained based on hippocalcin expression level. The identity and mechanism of the channel mediating sAHP is crucial.

Moreover, we performed ex-vivo insulin stimulation of hippocampal slices at 24 nM insulin for 10 mins. Ex-vivo insulin stimulation at different concentration of insulin and incubation time (i.e. 24nM for 20 min, 36 nM for 10 min, 36 nM for 20 min) will verify the insulin-dependent changes in SK2 and hippocalcin protein levels in young and aging hippocampus.

The effect of intranasal insulin on in-vivo expression levels of SK2 and hippocalcin was almost negligible. Insulin concentration peaks at around 30 mins after intranasal application (Born et al. 2002). We extracted the brains 30 mins after insulin application, which was probably not sufficient time to induce change in expression level of SK2 and hippocalcin. Protein expression analysis of hippocampus tissue at different time points after intranasal insulin should be performed.

This study was conducted only on FBN male rats, it is important to study the effect of intranasal insulin and insulin-dependent molecular changes in AHP in FBN female rats.

3.2. Potential Therapeutic application

In this study, we demonstrate that intranasal insulin therapy ameliorates cognitive decline associated with aging in animal model. Intranasal insulin has also shown to improve memory function in MCI and AD clinical trials (Reger et al. 2006, 2008). Along with memory benefits, intranasal insulin also improved mood, self-confidence and reduced depression in young adults (Benedict et al. 2004). At molecular level, intranasal insulin application in AD mice model reduces amyloid beta (Aβ) plaques (Chen et al. 2014) and reduces tau hyperphosphorylation in type 2 diabetic rats (Yang et al. 2013). Intranasal insulin restores insulin concentration and insulin signaling in CNS; and it prevents cognitive decline and pathology associated with downregulation of insulin signaling as seen in aging, AD and diabetes mellitus.

Apart from the benefits of intranasal insulin therapy, it is crucial to study the side-effects, if any, of continuous insulin elevation in the brain. One of the possible side-effects could be desensitization of insulin receptors in the brain (Kamal et al. 2012). Second, disrupting the insulin and leptin balance in the CNS could have possible side-effects on food intake and body weight (Kamal et al. 2012; Air et al. 2002). Moreover, most human insulin is synthesized in zinc formulations. Zinc is known to be neurotoxic and studies have shown intranasal zinc to cause loss-of-function of olfactory sensory neurons leading to long-term loss of smell (Hamidovic

2015; Lim et al. 2009). One of the zinc-free formulation of insulin is Apidra, which showed activation of insulin signaling in hippocampus after ICV application, however, did not improve cognition in aging rats on water maze task (Anderson et al. 2017). These are some of the important factors to be considered and needs further investigation.

Intranasal insulin improves learning and spatial memory consolidation in aging rats. Frazier et al. showed that expression of constitutively active IR beta subunit improved learning and memory recall in aging animals. In a recent paper, Maimaiti et al. suggested that insulin reduces Ca^{2+}-dependent mAHP and sAHP by regulating intracellular Ca^{2+} concentration. They reported that insulin reduces voltage-gated calcium currents and calcium transients via ryanodine receptor (Maimaiti et al. 2017). These and our results demonstrate that manipulating insulin signaling could enhance hippocampal-dependent spatial memory formation and learning in aging. Targeting the molecular mechanism underlying insulin-dependent enhancement in cognition, by using a readily available and affordable drug, could benefit memory and learning in aging and other dementias.

3.3. Concluding note

Our study showed that chronic low dose intranasal insulin therapy is better at enhancing learning and memory formation compared to acute intranasal insulin therapy or ICV method in aging animal models. This is the first study, as per our knowledge, that reports the effect of insulin in altering expression of SK2 and hippocalcin; suggesting it as one of the potential mechanisms by

which insulin regulates mAHP and sAHP in the hippocampus and maintains intrinsic excitability.

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APPENDIX

CHAPTER 2 SUPPLEMENTAL FIGURES

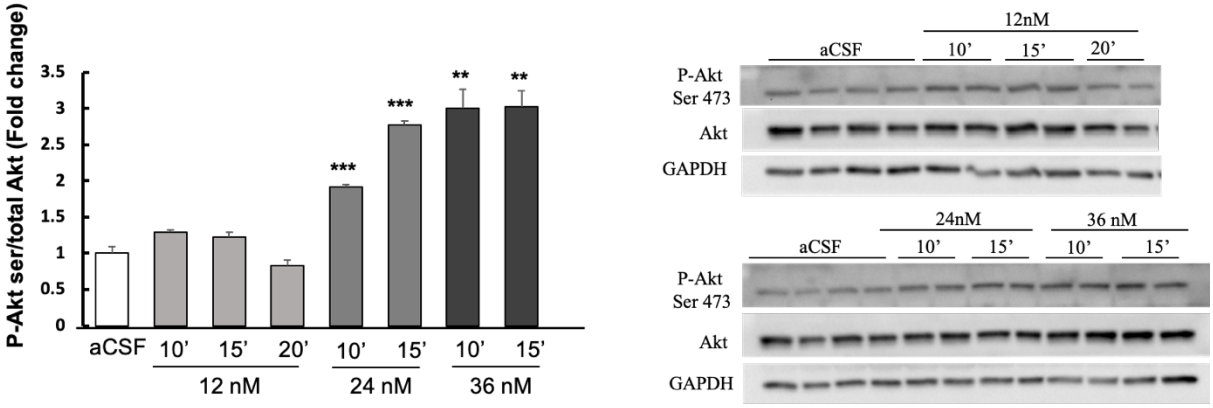

Fig A.1. Dose response of ex-vivo insulin stimulation on p-Akt in young hippocampus: Young hippocampal slices were incubated with insulin with different concentrations and time. Insulin stimulated phosphorylation of Akt at ser 473 in young hippocampus at 24 nM insulin for 10 min and increased dose- and time-dependently. n = 3. $^{**}p < 0.005$, $^{***}p < 0.0005$.

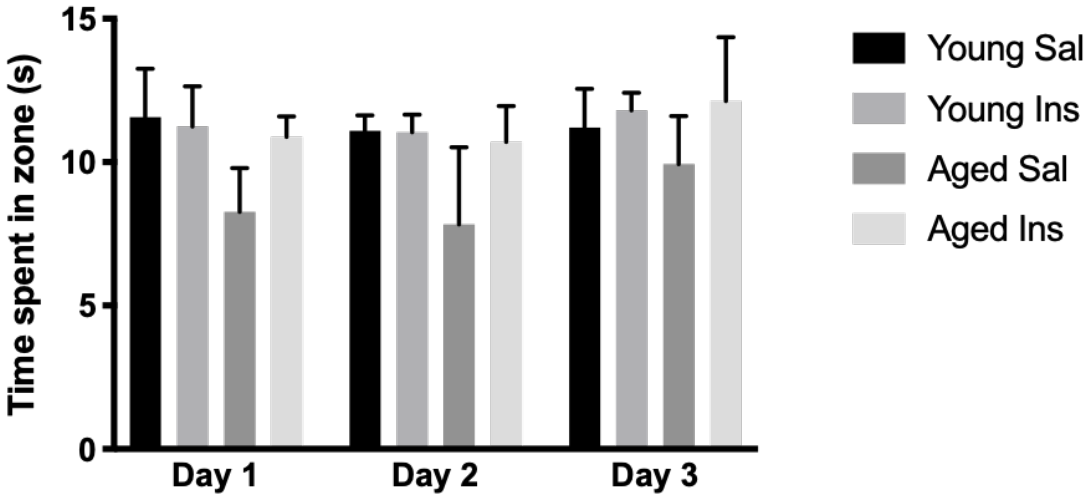

Fig A.2. Intranasal insulin effect on working memory in aging rats: Probe trial was performed on days 1 – 3 of acquisition phase, 30 mins after trial 4, to assess the effect of intranasal insulin on working memory (short-term memory retention) in aging. Aging rats treated with intranasal insulin spent more time in platform zone as compared to aging rats treated with intranasal saline. However, the difference was not significant. n = 3 each group.

BIOGRAPHICAL SKETCH

Neha Ramesh Tandon was born in a small town in India in November 1989. She graduated from St. Xavier's High School in 2007 and received a BS in Biotechnology from Christ College in 2010. Thereafter, she completed a post-graduate diploma program in Sophisticated Analytical Techniques in 2011. During her undergraduate program, Neha worked on many research projects and assisted her professors in conducting experiments. During her post-graduate diploma program, she successfully completed and wrote a thesis on "Estimation of Levofloxacin Hemihydrate as bulk drug and in pharmaceutical formulation by High Performance Thin Layer Chromatography (HPTLC)". She worked as a middle school teacher for 1 year before moving to the United States to pursue her graduate education. Neha was admitted to The University of Texas at Dallas in Fall 2012, in the MS degree program for Molecular and Cell Biology. She started volunteering as a research assistant in Dr. Heng Du's lab in Summer 2013. Later, she got admitted into a PhD program in Spring 2014 and continued her doctoral research in Dr. Lucien Thompson's lab. She received her MS in Molecular and Cell Biology in 2017. Neha has worked as a teaching assistant at UTD from 2013 to 2020.

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Poster Presentations:

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