MOLECULAR MECHANISMS UNDERLYING SYNAPTIC DYSFUNCTION IN AGING ALZHEIMER'S DISEASE AND DIABETES

by

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ABSTRACT

Alzheimer's disease (AD) and diabetes (DB) contribute to the development of early brain aging and cognitive dysfunction. Mechanistic basis of cognitive dysfunction in AD and DB is not well elucidated. Resistance to age related cognitive decline has been observed in growth hormone deficient, long-lived Ames dwarf mice. These mice show reduced circulating levels of growth hormone (GH) and insulin growth factor-1 (IGF-1); however, they have increased levels of these hormones in the hippocampus, a part of the brain where memory is encoded and consolidated. The hippocampal glutamatergic and cholinergic systems play a vital role in synaptic plasticity mechanisms of learning and memory. Therefore, we investigated the expression of glutamtergic synaptic markers and cholinergic and inflammatory enzymatic activity in the hippocampus of the Ames dwarf mice. We found an increase in postsynaptic glutamate receptor expression as well as increased cholineacetyl transferase and lactate dehydrogenase activity. We have also found elevated acetylcholine and enhanced postsynaptic glutamatergic activity in the hippocampus of these dwarf mice, which results in the development of improved synaptic plasticity and cognitive function. AD is associated with the early aging of the brain and results in the development of cognitive impairment. Amyloid beta (Aβ) is one of the major hallmarks of AD; the molecular mechanism by which Aβ alters glutamatergic system and impairs cognitive function in the early stages of AD is not well understood. To better understand the effects of Aβ upon the development of AD, we intracranially infused Aβ (1-42)

into wild type mice and found that diminished postsynaptic glutamate receptors that are crucial for learning and memory. Such finding implicates that Aβ, directly disrupts cognitive function and synaptic plasticity via attenuating postsynaptic glutamatergic transmission prior to development of neurodegeneration. Recent studies suggested direct link between AD and diabetes type 2. To better understand the mechanism of how insulin resistance impairs cognitive function, we utilized a transgenic type 2 diabetic mouse model (Leptin receptor knock out db/db mice). The nuclear receptor PPARγ plays a key role in metabolic regulation by modulating whole body glucose homeostasis and insulin sensitivity. Recently, PPARγ has also been implicated to have anti-inflammatory and neuroprotective effects in mouse models of AD. Consequently, we investigated the effects of PPARγ activation on diabetes-induced deficits in spatial and recognition memory in db/db mice. We found that pharmacological activation of PPARγ ameliorates diabetes-induced impairment of spatial and recognition memory and also synaptic transmission and LTP. These data can be explained by our findings that PPARγ activation enhanced the postsynaptic glutamate receptor expression in the db/db mouse model. In addition, we also found an increase in the insulin substrate-2 protein (IRS-2) and its effector proteins, which are involved in the insulin signaling cascade. Moreover, we found that synaptic plasticity master regulatory transcription factor CREB binding protein (CBP) also increased by PPARγ activation. These data offers a plausible hypothesis that PPARγ enhances synaptic plasticity and cognitive function by modulating insulin signaling pathway and synaptic plasticity associated transcription factors leading to improved glutamatergic synaptic transmission. Our findings suggest that molecular targets such as PPARγ may offer potential therapeutic targets for DB, AD and aging induced cognitive impairment.

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1. INTRODUCTION

Aging is a natural physiological process that leads to the development of cognitive impairment. Pathological conditions such as Alzheimer's disease (AD) and diabetes result in accelerated aging of the brain (Snwodon, 1997). Both of these conditions share common characteristics such as inflammation and oxidative stress (Butterfield et al., 2001). Although these pathological conditions share many similarities in the development of cognitive deficits, the specific molecular mechanisms remain unknown (Delamonte et al., 2006). Furthermore, AD and diabetes are at epidemic proportions and studies indicate possible mechanistic links between type 2 diabetes and AD pathology (Akter et al., 2011).

Animal models of aging have been utilized to elucidate the mechanisms of age-associated cognitive dysfunction (Gallagher, 1997; McKee and Squire, 1997; Pascalis and Bachevalier, 1999). Impaired insulin signaling, increased oxidative stress and inflammation have been implicated in the aging process (Ksiazek and Witowski, 2001). In this study, we used the long-lived Ames dwarf mouse model to investigate the molecular mechanisms of synaptic deficits and cognitive impairment in aging process. This animal has reduced circulating levels of growth hormone (GH) and insulin growth factor-1 (IGF-1) (Brown-Borg et al., 1996; Hauck et al., 2001). However, studies demonstrated an increase in the expression of GH and IGF-1 in the hippocampus of this animal model (Sun et al., 2005). It is believed that these characteristics lead to a delayed cognitive decline associated with the normal aging process. However, in this animal model mechanistic basis of resistance to age related cognitive decline is not well established. Reports indicate possible cholinergic and glutamatergic deficits in the aging and AD animal models (Kuhl et al., 1999).

AD is a progressive neurodegenerative disorder and it is growing exponentially in the US population (Hendrie, 1997; Lobo et al., 2000; Smith, 2006). Despite finding that Aβ plaques and hyperphosphorylated tau tangles are the major hallmarks of AD, their specific roles in the development of AD pathology remains elusive. Aβ (1-42) species is a potential neurotoxic peptide, which causes neurodegeneration in patients with AD (Kim et al., 2003; Behl et al., 1994). Several AD transgenic mouse models have shown an increase in Aβ production leading to the development of early cognitive impairment prior to severe neurodegeneration. To further understand the role of Aβ in the development of AD and cognitive impairment, we infused exogenous Aβ (1-42) intracranially into rodents and investigated the consequences on cognitive behavior such as spatial memory and recognition memory. We then carried out electrophysiological evaluations as well as assessed the molecular and biochemical changes in the hippocampus to better understand the mechanistic basis of alterations in cognitive behavior.

Diabetes is at epidemic proportions today and it leads to accelerated aging in many organ systems due to increased inflammation and oxidative stress. Type 2 diabetic animal models have been found to be associated with cognitive impairment due to altered synaptic transmission of glutamatergic system. We used a diabetic db/db mouse model (leptin receptor knockout) to further elucidate the molecular mechanisms of diabetes induced synaptic dysfunction that leads to cognitive deficits.

Thiazolidinediones (TZDs) are a class of insulin sensitizing drugs that activate the nuclear receptors; peroxisomal proliferator activating receptor gamma (PPARγ). These drugs transcriptionally regulate cellular energy metabolism and thus improve circulating levels of glucose and over all insulin sensitivity. Recently it has been demonstrated that PPARγ activation resulted in anti-inflammatory and neuroprotective effects in mouse models of AD. It has been

shown that several pathological characteristics associated with cognitive dysfunction, are shared by both AD and type 2 diabetes. Some of these include the decline of the insulin-signaling pathway in the brain resulting in increased Tau hyperphosphorylation and the ensuing loss of the synapse. Therefore, we hypothesized that PPARγ activation may ameliorate diabetes induced cognitive impairment by improving the insulin sensitivity and insulin signaling pathway in the hippocampus. Furthermore, we tested if rosiglitazone would improve the expression, and trafficking of glutamate receptors AMPAR and NMDAR in diabetic db/db mice.

Our current study provides insights into the development of cognitive dysfunction in the aging brain by studying three different animal models, which share common molecular mechanisms of altered synaptic plasticity and thus may provide a potential molecular targets for developing novel therapeutic options for cognitive decline associated with aging, AD and diabetes.

2. REVIEW OF LITERATURE

Aging and Molecular Mechanisms of Synaptic Dysfunction

Prevalence of Aging

Aging is a non-pathological, natural, constant, predictable physiological process that involves growth and development of living organisms. Aging rate varies person to person and depends on genes, environmental influences, life style and interactions between these factors. The life span is increased in the U.S. population due to the rapidly advancing medical and healthcare system. Aging population increases due to reduced mortality and increased life expectancy. The older age group (65+) is growing rapidly and a child born in 2002 could expect to live 77.3 years, 30 years longer than a child born in 1900 (Morgan and Kunkel, 2001). In the aging population, most of the older adults can expect to develop one or more chronic diseases. The most frequent pathological conditions among the elderly are hypertension – 49%; arthritis – 36%; heart disease – 31%; cancer – 20%; sinus problems – 15%; diabetes mellitus – 15% (Graf, 2006). Therefore, higher proportion of older adults will place increasing demands on healthcare systems and medical services.

Aging Theories and physiological changes

Aging is a complex molecular process and its mechanism is not well elucidated. Physiological aging refers to changes, with the passage of time, in the structure and processes of tissues, major organs and systems of the body that can ultimately affect our health, behavior, functional capacity, and survival. Physiological aging includes error theories, programmed theories and biological theories. Error theories of aging such as the free radical theory states,

deposits and accumulation of free radicals over time causing cell damage. Programmed theories of aging include telomerase shortening theory. Biological theories classify aging as genetic and non-genetic process. It is still not well understood why organisms age and why the aging process vary in speed and quality among different individuals (Frisard and Ravussin, 2006; Farley et al., 2006). Aging is not a pathological condition and it is vulnerable to various diseases and complications of medical treatment. Moreover, social, behavioral, physiological, morphological, cellular and molecular changes affect the aging process. Aging leads to continuous and irreversible decline in the efficiency of various physiological processes at the end of the reproductive phase (Balcombe and Sinclair, 2001). Aging involves steady decline of organ function and body systems leading to gradual systemic failure such as brain, heart, respiratory and muscular system dysfunction.

Aging and Cognitive Dysfunction

After the age of 25, neurodegeneration begins and gradually over time, causing reduced efficiency of neurotransmission affecting response time and coordination. It has been shown that decline in intellectual functioning associated with aging process (Anstey and Low, 2004) partly due to reduced efficiency of neurotransmission in the brain, resulting in slower information processing and greater loss of information during transmission. Most people experience a modest increase in memory problems, as they get older, particularly with regard to the ability to remember relatively recent experiences. There is impairment of the ability to accumulate new information and to retrieve existing information from memory. There is little decline in the ability to store new information once it is learned (Masunaga and Horn, 2001). Other factors

affecting cognitive performance are only indirectly related to the aging process (Henry and Phillips, 2006).

Synaptic deficits in Aging Brain

Aging of brain causes cognitive impairment by structural modifications, neurophysiological alterations such as less coordinated, delocalized activity, connectivity of brain regions in higher order cognitive functions, disruption of myelinated fibres that connect neurons in different cortical regions, altered expression of synaptic genes (Lu et al., 2004; Lee et al., 2000; Jiang et al., 2001) and imbalance between inhibitory and excitatory neurotransmission (Loerch et al., 2008). Deciphering the molecular mechanism of brain aging is crucial for treating and preventing age related cognitive deficits such as AD.

Long-term potentiation (LTP) is the synaptic plasticity mechanism and a well-established, cellular model of learning and memory. Age related alterations in synaptic plasticity that contributes to cognitive dysfunctions in the aged animals. Reduced synapse number leads to insufficient amount of cooperatively active functional synapses that are necessary for network modification required for learning and memory. In aged rats, there is a 27% decrease in axo-dendritic synapse number in the middle molecular layer of the dentate gyrus compared to young rats (Bondareff, W. & Geinisman; Geneisman et al., 1977). Spatial memory deficits have been shown to correlate with a reduction in perforated synapses at the medial perforant path–granule cell synapse (Geneisman et al., 1986). Total number of synaptic contacts per neuron was found to be diminished significantly in the dentate gyrus middle and inner molecular layer of aged rats relative to young adults. Both perforated and non-perforated axospinous synapses showed age-dependent decreases in numbers (Geneisman et al., 1992) and reduced field excitatory

postsynaptic potentiation (fEPSP) recorded in the dentate gyrus in the aged rat. This reduction is accompanied by a decrease in the presynaptic fibre potential amplitude at the perforant path–dentate gyrus (Barnes et al., 2000). Postsynaptic density area of axospinous synapses in CA1 region was compared between aged learning-impaired and learning-unimpaired rats, the impaired animals show a profound reduction in the postsynaptic density area of perforated synapses (Nicholson et al., 2004). These findings support the idea that many hippocampal perforated synapses become non-functional or silent in aged learning-impaired rats, and this loss of functional synapses might contribute to cognitive decline during normal aging.

The effects of morphological changes, alterations in biophysical properties and modified synaptic connections of aged neurons were assessed by long-term potentiation (LTP) and long-term depression (LTD). LTP can be divided into an induction phase (early phase LTP) and a maintenance phase (late-phase LTP). The induction phase involves the temporal association of presynaptic glutamate release with postsynaptic depolarization necessary to eject Mg^{2+} blockade from the pores of NMDA receptors, which results in an increase in intracellular Ca^{2+} (Bliss, T. V. & Collingridge, 1993). LTP maintenance is the continued expression of increased synaptic efficacy that persists after induction. It probably involves changes in gene expression and insertion of AMPA receptors into the postsynaptic membrane (Malinow, R. & Malenka, 2002). Aged rats have deficits in both LTP induction and maintenance in dentate gyrus and CA3 region (Barnes et al., 1980; Dieguez, D. Jr and Barea-Rodriguez, 2004). Aged CA1 neurons show weaker temporal summation of the multiple EPSPs induced by high-frequency stimulation (HFS). During high-frequency bursts, CA1 pyramidal cells are less depolarized, which explains the age-related LTP induction impairment in CA1 (Rosenzweig et al., 1997). Postsynaptic intracellular Ca^{2+} concentrations deregulations in aged animals (Thibault, O. & Landfield, 1996;

Foster, T. C. & Norris, 1997) could alter the probability of induction of either LTP or LTD. Alterations in Ca^{2+} channel conductance in CA1 pyramidal cells in the aged hippocampus that might lead to disruptions in Ca^{+2} ion homeostasis (Toescu et al., 2004). This impaired Ca^{+2} ion homeostasis leads to plasticity deficits in the aging process (Foster, T. C. & Norris; Landfield, P. W., 1988). Ca^{2+} associated plasticity impairments in aged rats supports that aged rats are more susceptible than young rats to LTD and to the reversal of LTP (Foster, T. C. & Norris, 1997). It has been shown recently that inhibition of Ca^{2+} release from intracellular Ca^{2+} stores attenuated LTD induction in aged CA1 neurons (Kumar, A. & Foster, 2005). Remodeling of neural network is crucial for behavioral impairment in ageing process. Certain properties of these networks are compromised during aging. Most of the age related changes can be linked to plasticity deficits, as blockade of NMDA receptors in young rats result in alterations in neural network patterns and functional dynamics that resemble those of aged rats (Kentros et al., 1998; Ekstrom et al., 2001). Therefore, aging causes impaired synaptic plasticity, and altered neural network pattern and dynamics thus lead to cognitive dysfunction.

Gene Expression Changes in Aging Brain

Maintenance of LTP requires gene expression and de novo protein synthesis and this process is altered in aged animals. Immediate-early genes (IEGs) such as c-fos play a crucial role in LTP induction and neural plasticity (Morgan et al., 1987; Cole et al., 1989). IEGs are dynamically regulated by specific forms of patterned synaptic activity, which is crucial for memory storage (Cole et al., 1989). IEGs are expressed by activated neurons during learning tasks such as spatial exploration (Guzowski et al., 1999). CREB (cAMP-responsive element-binding protein) phosphorylation plays a key role in the induction of IEGs expression.

Phosphorylated CREB promotes the transcription of IEGs. Based on the functional role of protein, IEGs can be grouped into two classes: inducible transcription factors and effector proteins. Inducible transcription factors are c-jun, c-fos and zif268. After c-jun and c-fos mRNAs are translated into proteins, their protein products can form a heterodimer called the activator protein 1 (AP1) complex. AP1 is a transcription factor that promotes the expression of late-response genes, some of which are important for the growth of new synapses or the modification of synaptic structure (Platenik et al., 2000; Clayton, 2000). The expression of zif268 is necessary for the maintenance of LTP and long-term memory (Jones et al., 2001). Effector IEGs are Narp (neuronal activity regulated pentraxin) and Arc (activity-regulated cytoskeletal gene). After transcription, Narp mRNA translocates to the synapse (Reti et al., 2002), where it is released and may act to cluster AMPA receptors on the postsynaptic membrane (O'Brien et al., 1999). After transcription, Arc mRNA localizes selectively to the region of the dendrite that receives the synaptic input that initiated transcription (Steward et al., 1998), and involved in the structural rearrangement of activated dendrites (Lyford et al., 1995). This involves AMPA receptor trafficking, as Arc protein has also been shown to reduce AMPA receptor currents (Guzowski et al., 2000). Arc expression is necessary for the maintenance, but not the induction, of LTP and long-term memory (Guzowski et al., 2000). Age-associated changes in gene expression in mice showed age-related alterations in the expression of hundreds of genes (Jiang et al., 2001; Lee et al., 2000). In behaviorally characterized rats, gene expression alterations in area CA1 were found to correlate with age-related cognitive decline. The behaviorally relevant up-regulated genes that are associated with inflammation and intracellular Ca^{2+} release signaling cascades. Whereas genes associated with energy metabolism, biosynthesis and activity-regulated synaptogenesis were downregulated. Arc and Narp were two of the genes that were shown to be down regulated

in aging process (Blalock et al., 2003). As many of the genes that are necessary for learning and memory are only highly expressed after synaptic activity, resting levels of expression might not capture an age difference that may occur in gene expression during behavioural tasks. There was no age-associated difference between young and aged rats in the basal level expression of c-fos, c-jun and AP1 and their activity (Smith et al., 2001). The expression levels of Arc, c-fos, c-jun, zif268 and Narp changed after LTP-inducing stimuli. In adult and aged memory-impaired rats, the induced expression levels of ARC, c-jun, junB, Zif268 and NARP mRNA are similar but they changed in expression after LTP induction (Lanahan et al., 1997). In aged rats, granule cells of the dentate gyrus, but not the pyramidal cells of areas CA1 and CA3, have a significantly smaller proportion of neurons that express Arc following spatial exploration task (Small et al., 2004). Young rats are found to have higher levels of c-fos mRNA compared with the old animals in the CA1 region. This indicates that although a similar number of pyramidal neurons express c-fos across different age groups, the individual cells from old animals transcribe less c-fos mRNA, which may lead to dysregulation of other synaptic related genes that depend on the AP1 transcription factor.

Signaling Cascades of Brain Aging

Brain is the master regulator in aging process and it regulates aging by conserved signaling pathways such as insulin/IGF signaling and oxidative metabolic activity. Insulin/IGF signaling can modulate cognitive decline in aging and pathological conditions such as AD and other neurodegenerative disorders. Neuroptrophic factors play a crucial role in neurogenesis, spine formation, synaptogenesis, enhanced synaptic plasticity and cognitive function. Moreover, they suppress neurodegeneration and shows neuroprotection. Insulin and IGF-1 are neurotrophic

factors and promote neuronal survival by inhibiting apoptosis (Van der Heide et al., 2006). Insulin and IGF-1 can also promote learning and memory (Van der Heide et al., 2006). By contrast, reduced signaling through the insulin/IGF-1 signaling pathway is a strongly conserved mechanism of lifespan extension (Broughton & Partridge, 2009). Reduced insulin and IGF signaling in the body causes caloric restriction and reduced oxidative stress thus result in extended life span and when insulin signaling increased in brain it supports neuroprotection and synaptic plasticity. Caloric restriction has beneficial effects on the function of the brain and its vulnerability to age-dependent pathology (Haigis et al., 2006). It reduces age-related brain atrophy (Colman et al., 2009), prevents age dependent alterations in gene expression (Lee et al., 2000; Park et al., 2009), and effectively resist age related cognitive impairment (Ingram et al., 1987) and also clears Aβ deposition and improve learning and memory in AD animal models (Halagappa et al., 2007).

Oxidative damage is a progressive conserved process and it plays a central role in age-related physiological functional decline in the organ system (Muller et al., 2007). Oxidative stress response genes are up regulated in aging (Yankner et al., 2008). Oxidative stress responses genes and DNA damage repair are the largest part of aging mechanism of human prefrontal cortex (Lu et al., 2004; Fraser et al., 2005). Dietary antioxidants suppress many age-related gene expression alterations in the mouse brain (Park et al., 2009) and can ameliorate cognitive decline and prevent oxidative damage to the brain in aging rats (Liu et al., 2002). In the aging human brain, oxidative damage to specific gene promoters results in gene silencing (Lu et al., 2004). Irreplaceable post-mitotic cells such as neurons, respond to unrepaired DNA damage by silencing expression of the affected genomic region by epigenetic mechanism such as more repressive transcriptional state (Lu et al., 2004; Loerch et al., 2008) rather than undergoing

apoptosis. Human brain ageing is accompanied by memory loss and reduced synaptic connectivity, but not significantly by neuronal loss reveals that loss of the ability to access stored memories underlies age-dependent memory deficits.

Animal Models of Aging

Several animal models were studied to decipher basic molecular mechanisms of the aging associated cognitive impairment in the brain. Human, Rhesus monkey, Rats, Mice, Drosophila fly, and C.elegan worms are used as animal models in aging studies and also including invitro cell culture models to decipher the molecular mechanism of aging related congnitive deficits. Transgenic animal models of accelerated aging and aging resistant mice models also have been studied. Understanding of the molecular basis of brain aging and the role of the brain in the aging body will allow us to rise to the challenge of treating and preventing cognitive decline and AD.

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Long-lived Ames Dwarf Mice

Ames dwarf mouse is the first mammalian mutant found to have an increased average and maximal lifespan (Bartke et al., 2001, 2003; Brown-Borg et al., 1996) and it is a murine model of circulating GH and IGF-1 deficiency, that exhibits dwarf phenotype characteristics and significantly extends lifespan by delaying physical aging and do not experience an age-related cognitive decline compared to their young counterpart. Therefore, this mice model is a potential animal model to study the molecular mechanisms of aging and aging associated cognitive

impairment. Mutated transcription factor Prop1 gene and Pit1 gene (homozygous for a loss-of-function mutation (df/df) at the Prop-1 locus) in Ames dwarf mice causes increased longevity (Krzisnik et al., 1999; Rosenbloom et al., 1999). The mutation impairs the development of the anterior pituitary resulting in defects of growth hormone (GH), Thyroid stimulating hormone (TSH), Insulin growth factor-1 (IGF-1) and Prolactin (PRL) producing cells in the adenohypophysis and primary deficiency of the corresponding hormones in the circulating levels (Bartke, 2000; Flurkey et al., 2001).This mice exhibit dwarf phenotype (one third of normal body size) and reduced growth rate (Bartke et al., 2001, 2003; Brown-Borg et al., 1996). A number of aging-related phenotypes are also delayed, including fatal neoplastic disease and declines in immune function, locomotor activity, learning and memory (Ikeno et al., 2003; Kinney et al., 2001).

Growth hormone (GH) plays a crucial role in growth, differentiation, and intermediary metabolism through mechanisms dependent on activation of the transmembrane GH receptor (Kopchick et al., 2000). GH has beneficial effects on certain functions of the central nervous system, including learning and memory, mental alertness, motivation, and working capacity (Nyberg, 2000). Decline of cognitive function with aging coincides with progressive decrease in circulating levels of GH and this decline can be ameliorated through GH treatment (Van Damm et al., 2000).

Insulin-like growth factor-1 is another neurotropic factor that stimulates neurogenesis, via a pattern of regulation across lifespan (Anderson et al. 2002). IGF-1 plays a key role in physical activity-induced neurogenesis (Trejo et al. 2001). IGF-1 mRNA expression is predominately located in neurons and astrocytes and, to a lesser extent, in oligodendrocytes and their precursors (Shinar and McMorris, 1995) and also be expressed by neural stem cells and early glial

progenitors. IGF-1 plays an important role in CNS development by acting in an autocrine and/or paracrine fashion. In addition, a variety of insults that result in CNS demyelination induce IGF-1 gene expression near or at the sites of injury (Gehrmann et al., 1994; Yao et al.,1995), suggesting a role for IGF-1 in protecting oligodendrocytes from injury and/or in promoting their regeneration following injury. Caloric restriction is well documented to delay aging and prolong life and improves cognitive function in laboratory animals. In caloric-restricted animals, plasma IGF-1 levels were reduced (Bartke et al., 2003). Besides the essential role of regulating somatic growth and development, insulin-like growth factor-1 is also important in neuronal function. During development of the nervous system, IGF-1 plays a prominent neurotrophic role, stimulating differentiation and survival of specific neuronal populations (Wilkins et al., 2001; Vicario-Abejon et al., 2003). In the adult central nervous system (CNS), IGF-1 is a neuromodulator and is involved in regulation of synaptic plasticity (Torres-Aleman 1999). IGF-1 levels are reduced with age. Restoring IGF-1 levels was reported to enhance neurogenesis and ameliorate the age-related cognitive malfunction in aged brain (Markowska et al., 1998). Furthermore, in transgenic mice with increased expression of IGF-1 in the brain, the weight and volume of the brain are increased substantially due to increases in neuron number and total myelin (D'Ercole et al., 2002). Potent neurotrophic actions of IGF-1 and IGF-2 in both neurons and glia including stimulation of DNA and RNA synthesis (Lenoir and Honegger, 1983), induction of neurite outgrowth (Recio-Pinto et al., 1986), regulation of neurotransmitter release (Kar et al., 1997) and synaptogenesis (Ishii, 1989) and neuroprotection against neurotoxic insults (Dore et al., 1997). However, behavioral studies revealed that while normal animals exhibit the expected decline of memory with age, old Ames dwarf mice do not show any deficits in memory retention when compared with young normal or young dwarf mice. Ames dwarf mice with

circulating GH/IGF-1 deficiency have normal or improved cognitive function due to higher expression of GH/IGF-1 in the hippocampus. Increased phosphorylation of Akt and cyclic AMP responsive element-binding protein (CREB) were detected in the hippocampus of aged Ames dwarf mice (Sun et al., 2005). Evidence of activation of the anti-apoptosis signal transduction cascade was also found in the hippocampus of old dwarf mice. These data support the hypothesis that increase in hippocampal GH and IGF-1 protein expression and subsequent activation of PI3K/Akt-CREB and activation of anti-apoptosis signals might contribute to the maintenance of cognitive function and is likely to be responsible for the integrity of neuronal structure and maintenance of youthful levels of cognitive function in these long-lived mice during aging.

Evidence of increased hippocampal neurogenesis was found in the young adult Ames dwarf mice (Sun et al., 2005). Increase in numbers of newly generated cells and newborn neurons in the dentate gyrus of dwarfs compared with normal mice at 3 months of age (Sun et al., 2005). Decline in hippocampal neurogenesis observed in normal aged mice but not aged dwarf mice. Newly generated neurons in old dwarf mice were significantly greater than in old normal mice. Significant increase in hippacampal IGF-1 protein expression and activation of an anti-apoptosis signal transduction cascade promote the increase in the fraction of newborn neurons and neuronal survival in aged dwarf mice. Aging influences the balance between neurogenesis and apoptosis in the mouse hippocampus as well as the process of the differentiation of the neuronal progenitors. Locally produced IGF-1 works primarily as a promoting factor to increase neurogenesis in the hippocampus in early adulthood and it functions mainly as a survival factor in the central nervous system to inhibit neuronal death during aging.

Brain synthesizes very little insulin (Baskin et al., 1987) and little circulating insulin can cross the blood–brain barrier (Reinhardt and Bondy, 1994). However, IGF-1 is abundant in the

developing brain, where it is concentrated in large projection neurons (Bondy, 1991). IGF-1 and insulin receptors are also homologous, with nearly identical signal transducing domains engaging many of the same intracellular pathways. Recent evidence demonstrated that that IGF-1signaling is involved in the development of AD (Carro and Torres-Aleman 2004; Carro et al., 2005). High brain Aβ levels are found at an early age in mutant mice with low circulating IGF-1, and Aβ burden can be reduced in aging rats by increasing serum IGF-1(Carro and Torres-Aleman 2004). IGF-1 can protect neurons against insults relevant to the pathogenesis of AD, stroke and other age-related neurological disorders and can enhance learning and memory. Even though higher level expression of IGF-1 in the hippocampus leads to better cognitive function in the dwarf mice, specific molecular mechanism by which glutamatergic system altered in dwarf mice is not well elucidated.

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Synaptic Plasticity and Glutamatergic System

Hippocampus and Synaptic plasticity

Hippocampus is a vital structure in the brain found in each of the cerebral hemispheres and responsible for formation of experience-based new memories, memory encoding and consolidation. The hippocampus is part of the forebrain, located in the medial temporal lobe and forms a part of the limbic system. Major excitatory neurotransmission in the hippocampus is mediated by glutamate receptors. The significance of glutamatergic neurotransmission in the hippocampus is exemplified in several neurological diseases with dementia. Moreover, hippocampus has been implicated in both spatial and contextual memories. Spatial navigation test such as Morris water maze and Y maze are sensitive to hippocampal damage (Morris et al., 1982) and fornix damage (Devan and White, 1999). The three major subfields (Trisynaptic loop)

in the hippocampus have an elegant laminar organization in which the cell bodies are tightly packed in an interlocking C‑shaped arrangement, with afferent fibres terminating on selective regions of the dendritic tree. The hippocampus is also home to a rich diversity of inhibitory neurons. Hippocampus is the major system for synaptic plasticity in the context of putative information-storage mechanisms in the brain. The much-studied model of synaptic plasticity was first identified in the hippocampus and has been extensively characterized (Bliss, & Lømo, 1973; Bliss, & Gardner-Medwin, 1973). Recent studies have detected LTP like synaptic changes in the hippocampus (Gruart et al., 2006; Whitlock et al., 2006) and the amygdala (Rumpel et al., 2005) following learning. Other forms of activity-dependent plasticity have been found, including long-term depression (LTD) (Dudek and Bear, 2005), EPSP-spike (E‑S) potentiation (Abraham et al., 1985). In addition, hippocampal neurons can be cultured (Banker, & Cowan, 1977; Gahwiler, 1981) either as transverse organotypic slices or as populations of dissociated neurons, for periods of months, facilitating molecular manipulations such as over expression or RNAi-based knockdown of specific proteins. The larger picture of how synaptic plasticity in extensive networks of cells leads to the storage and recall of information remains unclear. Hebb proposed that connections between co-active neurons are strengthened through mechanisms of synaptic plasticity, so that subsequent activation by incoming stimulation of only a sub-component of the assembly will lead to activation of the whole assembly, thereby recapitulating the activity elicited by the original event. Long-term potentiation (LTP) is a Hebbian process, since its induction requires coincident activity of the pre- and postsynaptic neurons.

The presumptive causal link between synaptic plasticity and memory has been formalized by Morris and colleagues as the synaptic plasticity and memory (SPM) hypothesis: Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and

is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed (Martin et al., 2000). Long-term potentiation (LTP) an activity-dependent synaptic plasticity plays a key part in the forms of memory mediated by hippocampus. LTP is the increase in the chemical strength of a synapse after a tetanus stimulation that lasts for over an hour. Experimentally, a series of short, high frequency electric stimulation to a nerve cell synapse can strengthen/ potentiate, that synapse for minutes to hours. Hippocampal LTP has been proposed to be a neurophysiological correlate of memory (Bliss and Collingridge, 1993). Studies that investigated the underlying molecular mechanisms of LTP have provided molecular tools for improving LTP and consequently memory. The laminated organization of the hippocampus lends itself perfectly to extracellular recording techniques, allowing selective pathways to be stimulated and the evoked synaptic responses generated by a population of target neurons to be monitored for prolonged periods of time. The middle panel shows typical synaptic responses recorded from the apical dendritic region of the CA1 subfield following stimulation of the Schaffer-commissural pathway. Two stimulating electrodes are placed on either side of the recording electrode to evoke responses in overlapping populations of pyramidal cells through different sets of synapses. Tetanus a brief, high-frequency train of electrical stimuli can be used to induce LTP lasting for many hours in the tetanized pathway; the second, control pathway receives only test stimulation and is not potentiated following the tetanus to the experimental pathway. This demonstrates an important property of LTP, in learning and memory. LTP has two phases such as early phase (E-LTP), which is short lasting and a late phase (L-LTP) that lasts longer and depends on gene transcription and protein synthesis (Pang and Lu, 2004; Voronin et al., 1995). LTP can be experimentally induced in the CA1 area of the hippocampus by applying a couple trains of

tetanic stimulation to the synaptic connection between two neurons. Induction of LTP involves the activation of NMDA receptors, a subclass of glutamate receptors. Post-synaptic depolarization after short tetanus stimulation removes the voltage-dependent magnesium block of NMDA receptors. This results in an influx of Ca^{+2} through the NMDA receptor in the post-synaptic neuron (Pang and Lu, 2004). Post-synaptic mechanisms suggested that insertion of post-synaptic AMPA receptors by activation of CaMKII (Lu and Hawkins, 2002). Maintenance of LTP for hours after induction requires activation of cAMP-dependent protein kinase (PKA) and results in synthesis of RNA and proteins (Lu and Hawkins, 2002). Several pathways might be involved in maintenance of LTP. Cyclic AMP response element binding protein (CREB) is thought to be the primary transcription factor in the cascade of gene expression that leads to permanent structural changes at the level of the synapse (Impey et al., 1996).

Glutamate Receptors

In the mammalian hippocampus, excitatory neurotransmission is mainly mediated by glutamate receptors. It acts via two classes of receptors, ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), mechanisms that are thought to underlie learning and memory. They are thus also potential targets for therapies for CNS disorders such as Epilepsy, AD and Psychiatric diseases. The ionotropic glutamate receptors are multimeric assemblies of four and are subdivided into three groups (AMPA, NMDA and Kainate receptors) based on their pharmacology structural similarities. In addition, further family of delta-receptor subunits have more recently been shown to be important in some aspects of

synaptic plasticity. All ionotropic glutamate receptor subunits share a common basic structure. Like other ligand gated ion channels, such as the GABA receptor, the ionotropic glutamate receptor subunits possess four hydrophobic regions within the central portion of the sequence (TM1 – 4). However, in contrast to other receptor subunits, the TM2 domain forms a re-entrant loop giving these receptor subunits an extra cellular N-terminus and intracellular C-terminus. In addition, the long loop between TM3 and TM4, which is intracellular in other ligand gated ion channel subunits, is exposed to the cell surface, and forms part of the binding domain with the C-terminal half of the N-terminus.

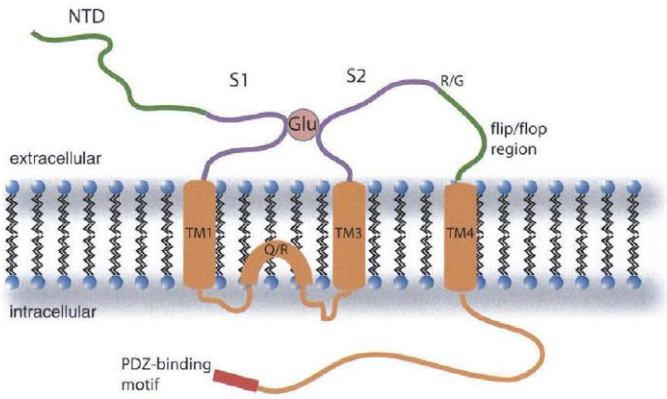
AMPAR

The roles of the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid AMPA type glutamate receptor in mediating basal synaptic transmission, synapse stabilization and synaptic plasticity in the central nervous system have been established. As a dynamic component of the postsynaptic membrane, AMPA receptors are capable of rapid translocation in response to changes in synaptic activity. The molecular and cellular mechanisms that control the assembly and synaptic targeting of individual AMPA receptors are beginning to be uncovered. Among different types of ionotropic glutamate receptors, the α-amino-3-hydroxyl-5- methyl-4-isoxazolepropionic acid (AMPA) type and the kainate (KA)-type receptors mediate the majority of fast synaptic transmission in the mammalian CNS, and the N-methyl-D-aspartate (NMDA)-type receptor mediate a slower component of synaptic transmission, which is important for modulating synaptic function. Kainite receptors (KAR) are non-NMDA ionotropic receptors. Openings of KARs are much shorter in duration than AMPA openings. Their permeability to Ca^{+2} is usually very slight but varies with subunits and RNA editing. KARs play a role in both

pre and post synaptic nueotransmission and compared to AMPAR its role is less significant and distribution of KARs in the brain is limited compared to AMPA and NMDA receptors, and their function is not well defined. Like most proteins, AMPA receptors are synthesized in the endoplasmic reticulum (ER) and trafficked to the Golgi apparatus. Neurons are highly polarized cells in which proteins are sorted and transported to target locations after exiting from the Golgi. Multiple pathways and mechanisms are involved in AMPA receptor membrane targeting. Four subunits, GluRl-4, contribute to the hetero-tetrameric assemblies of the AMPA receptor (Keinanen et al. 1990).

Various phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent kinase II (CaMKII) have been identified in the C-terminus of AMPA receptor subunits (Greengard et al. 1991). Phosphorylation of AMPA receptor subunits, GluRl in particular, potentiates channel activation, and increases single channel conductance, channel open frequency, and mean open time (Banke et al. 2000; Blackstone et al. 1994; Derkach et al.1999; Greengard et al. 1991; Oh and Derkach 2005). In addition, the C-terminal tails of the AMPA receptor subunits interact with a complex array of signaling and binding proteins.

Figure 2.1: Topology of AMPA receptor subunits.



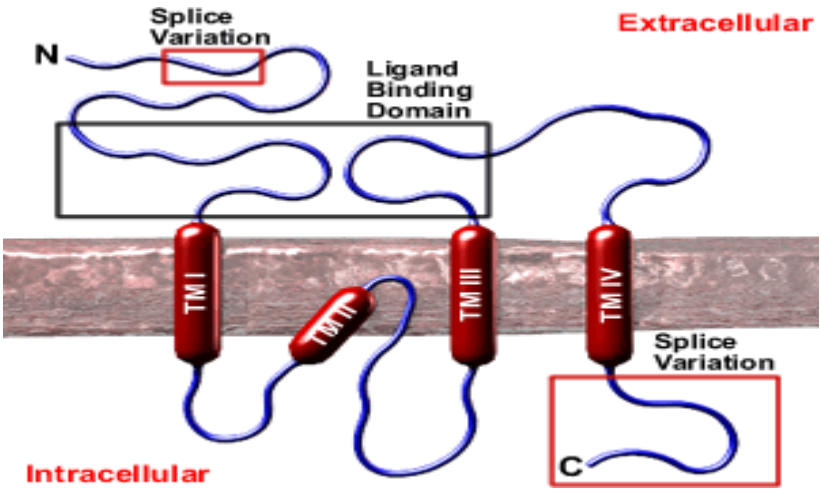
NMDAR

The N-methyl-D-aspartate receptor (NMDAR) is a member of the glutamate activated ion channel family. The receptor forms a cation-selective channel with high calcium permeability that is tightly regulated by oxidizing agents, protons, zinc, polyamines, protein kinases, calmodulin, and most notably magnesium (Dingledine et al. 1999; HoUmann and Heinemann 1994). At resting membrane potentials, the channel is blocked by physiological concentration of extracellular magnesium in manner a strongly dependent on voltage. Partial depolarization of the plasma membrane relieves the magnesium block and allows the flux of ions through the channel (Mayer et al. 1984; WoUmuth et al. 1998). This property allows the receptor to be a coincidence detector of pre and postsynaptic activity required in Hebbian models of plasticity (Bliss et al. 2003). Varied levels of synaptic NMDAR activation with corresponding degrees of calcium influx can result in multiple effects: low levels of NMDAR activation may produce depression of synaptic transmission, higher levels of activation with a larger influx of calcium may potentiate synaptic transmission (Cummings et al. 1996; Zucker 1999), and very high levels of calcium influx can result in cell death (Choi 1995). Thus, the number and properties of NMDA-R at a particular synapse must be well controlled in order to regulate the amount of calcium entry. It is now clear that the NMDA receptor is a tetrameric complex (Dingledine et al.1999; Rosenmund et al. 1998; WoUmuth and Sobolevsky 2004) and an obligate heteromultimer composed of NRl subunits and one or more NR2 subunits (Ishii et al. 1993; Meguro et al. 1992; Monyer et al. 1992). NRl subunits contain a binding site for glycine, and NR2 subunits are responsible for glutamate binding. The NRl subunit is ubiquitous and encoded by a single gene that gives rise to eight different splice variants (Zukin and Bennett 1995). Some properties, such as modulation by zinc, polyamines, and protein kinase C (PKC), are modified by incorporation of different splice

variants into the NMDA-R complex (Dingledine et al. 1999; Zukin and Bennett 1995). Four different genes encode the NR2 subunits (NR2A-D) and their expression is developmentally and regionally regulated (Dingledine et al. 1999; Monyer et al. 1994; Sheng et al. 1994). NR2 subunits modify the biophysical properties of the channel such as conductance, mean open time of the channel, and sensitivity to external magnesium (Dingledine et al. 1999; Ishii et al. 1993; Monyer et al. 1992). NR3 subunits have been shown to coassemble with NRl and NR2 subunits and modulate some channel properties (Ciabarra et al.1995; Das et al. 1998). Thus, a receptor complex assembled with different combinations of NRl, NR2, and NR3 subunits allows for considerable regulation of channel properties. The postsynaptic density is a proteinaceous specialization underneath the postsynaptic membrane of excitatory synapses that is composed of different ionotropic and metabotropic receptors, scaffolding proteins, and signaling complexes (Kennedy 1997; Walikonis et al. 2000). The NMDA-R is an integral part of the postsynaptic density and it is essential for synapse formation and maturation of neuronal connectivity. The targeting of the NMDA-R to synapses and its stabilization there depends on a series of interactions with other proteins. Transgenic animal studies showed that (Steigerwald et al. 2000) the carboxy termini domain of NMDA-R subunits control their trafficking and stabilization at synapses. The carboxy terminal domain of NMDA-R subunits are intracellular domains capable of interacting with different scaffolding and signaling proteins (Dingledine et al. 1999; Sheng and Sala 2001). In particular, the carboxy termini of NR2 subunits influence trafficking, localization, and internalization of the receptor. Despite the fact that the global homology among NR2 subunits is very high, at the carboxy terminal region it is only approximately 20 to 30 percent (Ishii et al. 1993; Meguro et al. 1992; Monyer et al. 1992), suggesting the potential for many interactions with regulatory proteins in a subunit specific manner. Thus, different subunits

not only regulate the properties of the receptor, but also regulate trafficking, surface expression, and insertion at synapses, as well as intracellular signaling by recruiting enzymes to the postsynaptic density.

Figure 2.2: Topology of NMDA receptor subunits.



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Alzheimer's Disease and Synaptic Dysfunction

Alzheimer's Disease (AD)

AD is an age dependent progressive neurodegenerative disorder that is characterized by memory impairment and loss of intellectual skills. AD is characterized by two major hallmarks in the brain; deposition of insoluble fibrillar Aβ peptide in extracellular plaques; aggregated hyperphosphorylated tau protein, which is found largely in the intracellular neurofibrillary tangles (Selkoe and Schenk, 2003). AD is the sixth leading cause of all deaths in the U.S and is the fifth leading cause of death in Americans aged 65 years.

Gene mutations in AD

Several genes known to be involved in AD are Familial genes APP, PSEN1, PSEN2, SorL1, risky genes APOE, GSK3b, DYRK1A, Tau, TOMM40, CLU, and PICALM. The amount of risk of AD that is attributable to genetics is estimated to be around 70%. Established genetic causes of AD include dominant mutations of the genes encoding amyloid precursor protein (APP) and presenilin1 (PSEN1) and presenilin2 (PSEN2) (Goate et al., 1991; Shellenberg et al., 1992; Levy- Lahad et al., 1995). The most consistently associated potential risk gene is ApoE. Individuals with two ApoEε4 alleles have a more than seven times increased risk of developing AD compared with those with ApoEε3 alleles. PSEN1 and PSEN2 mutations affect concentrations of Aβ1–42 because the presenilin proteins form part of γ secretase, which cleaves APP to produce Aβ. Several other genes that affect AD risk possibly have roles in the clearance or uptake of Aβ.

AD and Aβ Pathology

Aβ is generated by sequential proteolytic cleavage of amyloid precursor protein (APP) (De Strooper and Annaert, 2000) and is the primary constituent of senile plaques, which are hallmark to AD pathology (Masters et al., 1985). The nonamyloidogenic pathway involves cleavage by α-secretases, while the amyloidogenic pathway involves cleavage by β and γ-secretases (Jarrett et al., 1993; De Strooper et al., 2000). Aβ generated by γ-secretase activity can vary in length: the most common forms contain 38, 40 or 42 amino acids. Because of the two additional amino acids isoleucine and alanine, Aβ 1-42 aggregates more quickly than Aβ1-40 (Grimm et al., 2007) and is the major component of neuritic plaques in AD. The relevance of Aβ1-42 in AD is further supported by familial forms of AD. Most of the missense mutations in

the genes encoding APP and presenilin increase the production of Aβ1-42. Aβ42 polymerizes more readily, which may account for the increased toxicity compared to Aβ40 (Jarrett et al., 1993; Jarrett and Lansbury, 1993; Snyder et al., 1994). At normal concentrations Aβ is a soluble protein; however, in AD brains, increased concentrations of Aβ leads to peptide aggregation and the formation of less soluble forms such as oligomers, protofibrils and fibrils (Bitan et al., 2003). Abnormal processing of Aβ, as a result of altered production by β-secretase and γ-secretase cleavage of amyloid precursor protein (APP) or impaired Aβ cleavage mechanisms, leading to the accumulation of toxic aggregates, is a causal factor in AD (Hardy and Selkoe, 2002). Insoluble Aβ42 is markedly increased in the AD brain; nonetheless, AD patients consistently exhibit decreased levels of soluble Aβ42 in the CSF (Andreasen and Blennow, 2002), which is thought to reflect the increased deposition and decreased clearance in the brain (Wang et al., 1999). Presenilin mutations consistently result in increased production of Aβ42 strengthen the hypothesis that Aβ42 is the primary species responsible for the pathogenesis of AD (Borchelt et al., 1996; Ishii et al., 1997; Wisniewski et al., 1998).

Aβ and Synaptic Dysfunction

Aβ induces synaptic dysfunction. Synaptic loss in the hippocampus and neocortex is an early event and is the major structural correlate of cognitive dysfunction in AD (Gonatas et al., 1967; Davies et al., 1987; Scheff et al., 1990; Terry et al., 1991; DeKosky et al., 1996; Masliah, 1998; Arendt, 2001). Synaptic pathology is reflected by a loss of all major components of small synaptic vesicles and most peptides, accompanied by extensive aberrant changes of the synapse (Lassmann et al., 1993). The bulk of neocortical synaptic loss most likely derives from loss of cortico-cortical associational fibers (Morrison et al., 1990), rather than degeneration of

subcortical input (Arendt et al., 1995). Synapse and dendrite loss in AD exceeds that seen with normal aging (Terry et al., 1994). AD is a slowly progressing disorder including early synaptic degeneration. It is progressing from an initially reversible functionally responsive stage of down-regulation of synaptic function to stages irreversibly associated by marked synapse loss (Rapoport, 1999). Memory loss in AD may result from synaptic dysfunction that precedes large-scale neurodegeneration, where the synapse-to-neuron ratio is decreased by about 50% (Chapman et al., 1999; Chen et al., 2000). This is eventually accompanied by the loss of about 10-20% of cortical neurons (Masliah, 1998). AD dementia may be initiated before synapse degeneration by spine aberrations. Spine shape distortions are evident in other severe synaptic plasticity and physiology in cognitive diseases such as mental retardation. In PSAPP transgenic mouse models, synapse abnormalities as well as memory impairments correlate poorly with plaque burden and can occur before plaque formation (Holcomb et al., 1999; Hsia et al., 1999; Larson et al., 1999; Mucke et al., 2000; Jacobsen et al., 2006). Although Aβ antibodies prevent synaptic degeneration in transgenic mice (Buttini et al., 2005), memory impairment is reversed without plaque loss (Dodart et al., 2002; Kotilinek et al., 2002) suggesting that a toxin from Aβ, not present in plaques, may be the culprit behind synapse degeneration. AD brain (Gong et al., 2003; Kayed et al., 2003; Lacor et al., 2004) and cerebrospinal fluid (Georganopoulou et al., 2005; Haes et al., 2005) contain small neurotoxins that comprise soluble Aβ oligomers, termed Aβ derived diffusible ligands (ADDLs) (Lambert et al., 1998). Neuronal injury triggered by ADDLs is now viewed by many as a central feature of AD pathology (Standridge, 2006). ADDLs are gain-of-function ligands that target dendritic spines (Lacor et al., 2004) and disrupt synaptic plasticity (Lambert et al., 1998; Wang et al., 2002). ADDLs block LTP (Lambert et al., 1998; Wang et al., 2002) by binding directly to dendritic spines (Lacor et al., 2004).

Animal Models of AD

Strong support for the Aβ hypothesis came from a small proportion of familial AD clusters that are caused by mutations of APP, which lead to increased Aβ levels and the relatively early onset of dementia. Many groups have developed transgenic mice that overexpress these mutant forms of human APP. Most currently studied models show cognitive deficits and age-related disruption of synaptic markers and amyloid plaque deposition, but few strains show evidence of significant cell death (Janus et al. 2000; Ashe 2001; Chapman et al. 2001; Richardson & Burns 2002).The neurophysiological consequences of such mutations have been examined in the hippocampus of these mutant mice. This has allowed the investigation of the role of age related factors such as plaque deposition and synaptic loss in functional deficits. Most studies have reported, principally, either inhibition of LTP or reduction in baseline fast excitatory transmission prior to plaque deposition. The relative importance of these changes and apparent discrepancies still need to be resolved.

Exogenous Aβ induced Rodent model of AD

Several studies have shown that synthetic Aβ inhibits LTP induction in vitro. Thus, in hippocampal slices prepared from 20–30-day-old rats, soluble Aβ 1–42 (500 nM) was found to inhibit LTP induction by strong HFS of the medial perforant path in the dentate gyrus both of the population spike (Lambert et al., 1998) and EPSPs (Wang et al., 2002). Both early- and late-phase LTP were strongly inhibited in these studies, whereas basal AMPA receptor mediated synaptic transmission was not altered, although there was a reduction in paired-pulse depression at a short (20 ms) inter-pulse interval (Wang et al., 2002). In these studies, Aβ 1–42 was specially prepared to contain large metastable Aβ oligomeric assemblies (termed ADDLs),

providing evidence that non-fibrillar Aβ can selectively disrupt both short-term and long-term synaptic plasticity. Similarly, LTP of field EPSPs in rat CA1 and the medial perforant path of the dentate gyrus was inhibited by Aβ 1–40, Aβ 1–42 and the truncated Aβ fragment 25– 35 at concentrations of 200 nM or 1 mM. The N-terminal sequence of Aβ 25–35 was found to be necessary for inhibition of LTP induction (Chen et al., 2000). The inhibitory effects of Aβ on LTP occurred in the absence of changes in baseline transmission and thus do not appear to be caused by a toxic action of the Aβ resulting in rapid neurodegeneration. Moreover, truncated Aβ variants that were not lethal to cultured neurons also blocked LTP induction (Chen et al., 2000). Intriguingly, non-fibrillar Aβ 1–42 (Wang et al., 2002) and Aβ variants that did not form fibrils in vitro (Chen et al., 2000) inhibited LTP, pointing to a critical role for soluble peptide.

Aβ and Glutamatergic Dysfunction

Since ADDLs disrupt NMDA receptor-mediated CREB phosphorylation (Tong et al., 2001), it is not unexpected that surface glutamate receptor levels would be altered by ADDLs (Gong et al., 2003). Additionally, ADDLs induce abnormal expression of Arc (Lacor et al., 2004), a spine cytoskeletal protein that influences glutamate receptor trafficking (Mokin et al., 2006), and cause a major loss of surface NMDA receptors (Lacor et al., 2007). Loss of NMDA receptors has been seen in AD brain (Sze et al., 2001; Mishizen- Eberz et al., 2004) and in a transgenic AD mouse model (Snyder et al., 2005), and correlates with synaptic alterations and cognitive deficits (Terry et al., 1991; Sze et al., 1997; Counts et al., 2006). The large decrease in receptor expression reported by Lacor et al. (2007) occurred prior to changes in spine density, consistent with synaptic plasticity being compromised before onset of degeneration. In addition to affecting NMDA receptors, ADDLs promoted a rapid decrease in membrane expression of

EphB2. These two synaptic receptors physically interact via their extracellular domains (Dalva et al., 2000) and are functionally related to plasticity. NMDA receptors play a central role in the induction of LTP (Morris and Davis, 1994), and EphB2 exerts control over NMDA-dependent LTP (Matynia et al., 2002). Moreover, both receptors NMDAR and AMPAR influence dendritic spine morphology and maintenance (Carlisle and Kennedy, 2005). Taken together, the observed disruption of dendritic spines links ADDLs to a major facet of AD pathology and provides compelling evidence that ADDLs in AD brain cause neurophil damage believed to underlie dementia. Since many forms of hippocampal synaptic plasticity and toxicity are NMDA receptor-dependent, alterations in NMDA receptor-mediated synaptic transmission and related mechanisms may contribute to the effects of Aβ. The Aβ 1–42 has recently been reported to reduce NMDA receptor-mediated synaptic currents in the dentate gyrus (Chen et al., 2002). CREB phosphorylation has been implicated in late LTP and suppressed by sublethal concentration of Aβ 1–42. Fibrillary Aβ deposits are responsible for the eventual neuronal degeneration (Selkoe, 1991; Hardy and Higgins, 1992) and soluble Aβ oligomers disrupt glutamatergic synaptic function, which in turn leads to the characteristic cognitive deficits (Lambert et al., 1998; Hsia et al., 1999; Walsh and Selkoe, 2004). Together, these results suggest that impairment in synaptic function is an early event in the pathogenesis of AD before neurodegeneration starts. Uncovering the mechanisms whereby Aβ oligomers induce synaptic deficits is still at an early stage, and currently there is no consensus on the precise molecular pathways involved. A number of intracellular signaling pathways have been implicated in Aβ induced synaptic dysfunction, and different sources or assembly states of Aβ oligomers may have different effects on synaptic function. Moreover, the relative involvements of intracellular and extracellular Aβ oligomers remain to be defined.

Aβ over expression decreases spine density, partially occludes metabotropic glutamate receptor-dependent LTD, decreases synaptic AMPA receptor number, and requires second-messenger pathways implicated in LTD for its depressive effects. Expression of an AMPA receptor mutant that prevents its LTD-driven endocytosis blocks the morphological and synaptic depression induced by Aβ. Furthermore, Aβ can drive phosphorylation of AMPA receptor at a site important for AMPA receptor endocytosis during LTD, and mimicking this AMPA receptor phosphorylation produces the morphological and synaptic depression induced by Aβ. Together, the results show that Aβ generates structural and synaptic abnormalities via endocytosis of AMPA receptors. Additional questions to be examined include whether presynaptic or postsynaptic Aβ is responsible for the observed synaptic depression, whether there is a difference between acute and chronic exposure to elevated Aβ levels, and whether different Aβ oligomeric forms lead to different synaptic effects.

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Diabetes and Synaptic Dysfunction

Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects of insulin secretion or increased cellular resistance to insulin action or both. Marked hyperglycemic specific symptoms are polyuria, polydipsia, and weight loss and fatigue (Gavin et al., 1997). Chronic hyperglycemia and other metabolic disturbances of DM cause long-term tissue and organ damage as well as systemic dysfunction involving the eyes, kidneys, and nervous and vascular systems (Diabetes care 2009, American diabetes association 2009). Diabetes mellitus affects an estimated 24 million Americans (8.0%) and approximately 6 million of these Americans are undiagnosed and unaware of their disease (National diabetes statistics, 2007).

Pathophysiology of Diabetes

Type 1 diabetes, which results from destruction of beta cells in the pancreas, accounts for approximately 10% of all patients with diabetes in the United States. It leads to absolute insulin deficiency. Type 2 is the most common form of diabetes worldwide, accounting for approximately 90% of all cases of diabetes in the United States, and its prevalence is increasing. Its underlying defects can vary from predominant insulin resistance with relative insulin deficiency to a predominant insulin secretory defect with insulin resistance. A great deal of heterogeneity exists, and most patients with type 2 diabetes do not initially require insulin therapy. Type 2 diabetes occurs more frequently in adults than in children, and the incidence increases with age, especially after age of 40. A characteristic finding is darkening of the skin (acanthosis nigricans) and there is an increased incidence of insulin resistance (Fagot-Campagna et al., 2000; Diabtes care 2009). Many cognitive dysfunctions associated with metabolic syndrome and pathophysiologies of neurodegeneration are associated with diabetes. Mechanisms underlying the development of nervous system lesions and cognitive dysfunction in patients with disturbances in the insulin homeostasis have not been completely elucidated.

Type 2 diabetes and cognitive impairment

Type 2 diabetes patients showed cognitive deficits characterized by decreases in psychomotor speed (Gregg et al., 2000; Reaven et al., 1990), complex motor functioning (Reaven et al., 1990), executive functions (Reaven et al., 1990)], memory skills (Munshi et al., 2006; Perlmuter et al., 1984; Messier, 2005), processing speed (Messier, 2005), immediate and delayed recall (Grodstein.,2001), Rate of cognitive decline due to aging is increased 1·5-fold to

2·0-fold in individuals with Type 2 diabetes (Cukierman et al., 2005). Therefore, diabetes type 2 acts like accelerated aging.

Mechanism of Synaptic deficit in Type 2 Diabetes

Glucose is the main energy substrate of the human brain and glucose homeostasis is compromised in diabetes type 2. Eventhough brain constitutes only 2% of the human body weight, utilizes almost 25% of total body glucose (Magistretti and Pellerin, 1996). However, chronic hyperglycemia can be deleterious for the brain (Magistretti and Pellerin, 1996), has toxic effects, and can lead to slowly progressive functional and structural abnormalities in the brain (Arvanitakis et al., 2006). It can be one of the determinants of cognitive decline in people with abnormal glucose metabolism and impaired glucose homeostasis (Stewart and Liolitsa, 1999; Strachan et al., 1997). The glucose metabolism is used not only for energy substrate but also the breakdown of glucose provides important compounds for neurons, including neurotransmitters such as acetylcholine and glutamate (Shullingkamp et al., 2000). Glycemic control plays a key role by altering serum glycosylated hemoglobin -HbA1c level in preserving cognitive performance in Type 2 diabetes (Ryan and Geckle, 2000) such as working memory (Munshi et al., 2006), executive functioning and learning (Raeven et al., 1990). Moderate to severe hypoglycemia have been implicated as one possible etiology for long-term cognitive dysfunction in Type 2 diabetes (Sommerfield et al., 2003), and short-term memory disturbances due to hypoglycemic subject (Biessels et al., 2001; Schmidt et al., 2004). Increased ROS and AGEs are implicated in aging and also contribute to microvascular changes, what leads to microinfarcts and generalized brain atrophy; result in cognitive decline and dementia (Gold et al., 2005, Arvanitakis et al., 2004; White et al., 2000). Neuronal apoptosis and suppression of cell

proliferation/neurogenesis are observed in the hippocampus of diabetic rodents. Neurochemical changes contribute to cognitive dysfunction and insulin resistance impairs long-term potentiation (LTP), a fundamental mechanism for memory consolidation (Kamal et al., 2009). Neurotransmitter functions which are altered in diabetes mellitus include decreased acetylcholine production (Welsh and Wecker, 1991), decreased serotonin turnover, decreased dopamine activity, and increased norepinephrine (Ramakrishnan et al., 2004; Biessels et al., 1998). Change of glucose level is an important factor controlling learning and memory processes (Messier and Gagnon, 1996). Variation in blood glucose level may affect glutamatergic system integrity and function and the mechanism is poorly understood.

Insulin signaling and Synaptic plasticity

Insulin is a key neurotrophic factor other than its modulator role in food intake and energy homeostasis (Gerozissis, 2004; Ling et al., 2002; Banks et al., 2004; Steen et al., 2005). It regulates high glucose demanding brain activities such as cognitive function. AD patients were found to have lower than normal CSF levels of insulin (Steen et al., 2005). Insulin is neurotrophic at moderate concentrations; too much insulin in the brain may be associated with reduced Aβ clearance due to competition for the Insulin Degrading Enzyme (IDE) (Farris et al., 2003). Since IDE is much more selective for insulin than for Aβ, brain hyperinsulinism may deprive Aβ of its main clearance mechanism (Farris et al., 2003). Insulin crosses the blood-brain barrier, and might even be produced locally in the brain, exerting its effects on cells by binding to a specific cell surface receptor Insulin Receptor (IR) (Steen et al., 2005; Banks et al., 2004). Insulin receptors are distributed throughout the brain, being abundant in the hippocampus and the cortex (Bondy et al., 2004). Binding of insulin to its receptor activates the intrinsic tyrosine

kinase activity of the cytoplasmic domain of the insulin receptor. This leads to autophosphorylation of tyrosine residues, which initiates several intracellular signaling cascades (Kahn et al., 1988; White et al., 1998; Withers et al., 2000). In the brain, insulin influences the release and reuptake of neurotransmitters, and appears to improve learning and memory (Zhao et al., 2004). The initial components of the insulin receptor-signaling cascade in the brain are largely similar to those of the periphery (Zhao et al., 2004; saltiel et al., 2001). The downstream targets of the cascade are involving neuronal glutamate receptors (Zhao et al., 2004). Insulin receptor-mediated signal transduction controls the activity of several enzymes in a cascade-like manner. Phosphatidylinositol 3- kinase (PI3K) is insulin-regulated and activates protein kinase B (PKB/Akt) (Alessi et al., 1998; Vanhaesebroeck et al., 2000). PIP3 recruits PKB to the plasma membrane, where it is phosphorylated and activated by specific protein kinases (Lizcano and Alessi, 2002; Lizcano et al., 2002). PKB has many important cellular targets including glycogen synthase kinase 3 (GSK3). Phosphorylation of the N-terminal region of GSK3 by PKB causes inactivation of GSK3, reducing the phosphorylation of glycogen synthase (GS). Dephosphorylated GS, the active form of the enzyme increases the rate of conversion of glucose 6-phosphate to glycogen. This pathway links the insulin receptor at the cell surface with enzymes of glycogen metabolism within the cell (Cruz et al., 1995). GSK3 generally opposes the actions of insulin. Thus, GSK3 inhibits glycogen synthesis, glucose uptake, and also alters the expression of genes regulated by insulin (Skoog, 2000). GSK3 is highly expressed in all eukaryotic cells and is involved in a number of physiological processes ranging from glycogen metabolism to gene transcription (Woodget, 1990; Hansen et al., 2007). There are two isoforms of the enzyme that are ubiquitously expressed in mammals: GSK3α and GSK3β (Woodget, 1990). There is evidence that GSK3 plays a central role in AD, and that its deregulation accounts

for many of the pathological hallmarks of the disease in both sporadic and familial AD cases, leading to formulation of the hypothesis GSK3/AD (Hooper et al., 2008). Glycogen synthase kinase 3 is implicated in the hyperphosphorylation of tau, increased production of β-amyloid and in inflammatory responses. Glycogen synthase kinase 3 also reduces acetylcholine synthesis and is a key mediator of apoptosis. These findings are in accordance with alterations present in AD, including cholinergic deficit, memory impairment and neuronal loss (Hansen et al., 1998).

There is increasing evidence linking insulin resistance to cognitive decline and dementia in diabetes (De la monte, 2005). There are alterations in cerebral insulin receptor signaling, leading to a cerebral insulin resistant state. Cerebral insulin resistance has been implicated in accumulation of Aβ and tau protein, which are the main components of senile plaques and neurofibrillary tangles, respectively. GSK3 activity might be enhanced by insulin resistance, representing a possible link between insulin resistance and AD disease (Alvarez et al., 2004; Atwood et al., 1998). Several studies point to an intriguing relationship between diabetes mellitus and AD. Patients with AD have lower Cerebrospinal fluid insulin levels and reduced insulin-mediated glucose disposal when compared to healthy control subjects (Craft et al., 1998; Craft et al., 2003). While there is very little insulin mRNA in the brain, insulin mRNA can be detected in postmortem human brain, being reduced in AD brains. AD referred to as "Type 3 diabetes" due to CNS insulin resistance and a marked reduction in CNS insulin concentrations (Steen et al., 2005; Rivera et al., 2005). In addition, knockout of the insulin receptor gene is not sufficient to cause cognitive deficits or neurodegeneration even though some regions show enhanced GSK3β activity (Schubert et al., 2004). Impaired insulin receptor activation in the AD brain leads to insulin resistant brain state (Frolich et al., 1998). Another important link between insulin resistance and the amyloid cascade may be related to the IDE. Insulin degrading enzyme

is a metalloprotease enzyme responsible for insulin degradation and is also the main enzyme responsible for Aβ degradation (Farris et al., 2003). Insulin degrading enzyme is secreted to the extracellular space by microglial cells in the brain, where it degrades Aβ peptide, leading to reduced Aβ peptide concentration in the brain, thus reducing aggregation and plaque formation (Qiu et al., 1998). Insulin degrading enzyme levels have been reported to be decreased in the brains of AD patients (Perez et al., 2003; Cook et al., 2003), especially in the hippocampus (Caccamo et al., 2005). Hyperinsulinemia in people with pre-diabetes and T2DM effectively sequester IDE, reducing Aβ peptide degradation. This would increase levels of Aβ peptide, and promote many of the pathological features associated with AD. Supporting this model, the affinity for the binding of insulin to IDE is much greater than the one for the Aβ peptide (Qiu et al., 1997).

Animal Models of Diabetes induced Cognitive Dysfunction

Diabetic animal models can be divided into two classes, in which animals are rendered diabetic by specific experimental procedures, and those in which animals develop diabetes spontaneously (genetic predisposition/transgenic animal model). A variety of methods have been used to induce diabetes, such as pancreatectomy, exposure to viruses and administration of β-cell cytotoxic agents (alloxan), but intravenous or intraperitoneal injection of the β-cell cytotoxic agent streptozotocin (STZ) is used most often. STZ-diabetic rats develop end-organ damage that affects the eyes, kidneys, heart, blood vessels and nervous system. Advantages of the STZ-model are that it is well characterized and that diabetes can be induced at any given age, thus allowing studies of the interaction of diabetes and ageing. Recently a new model of intracerebroventricular-STZ (ic-STZ) attracted specific attention to study sporadic AD pathology

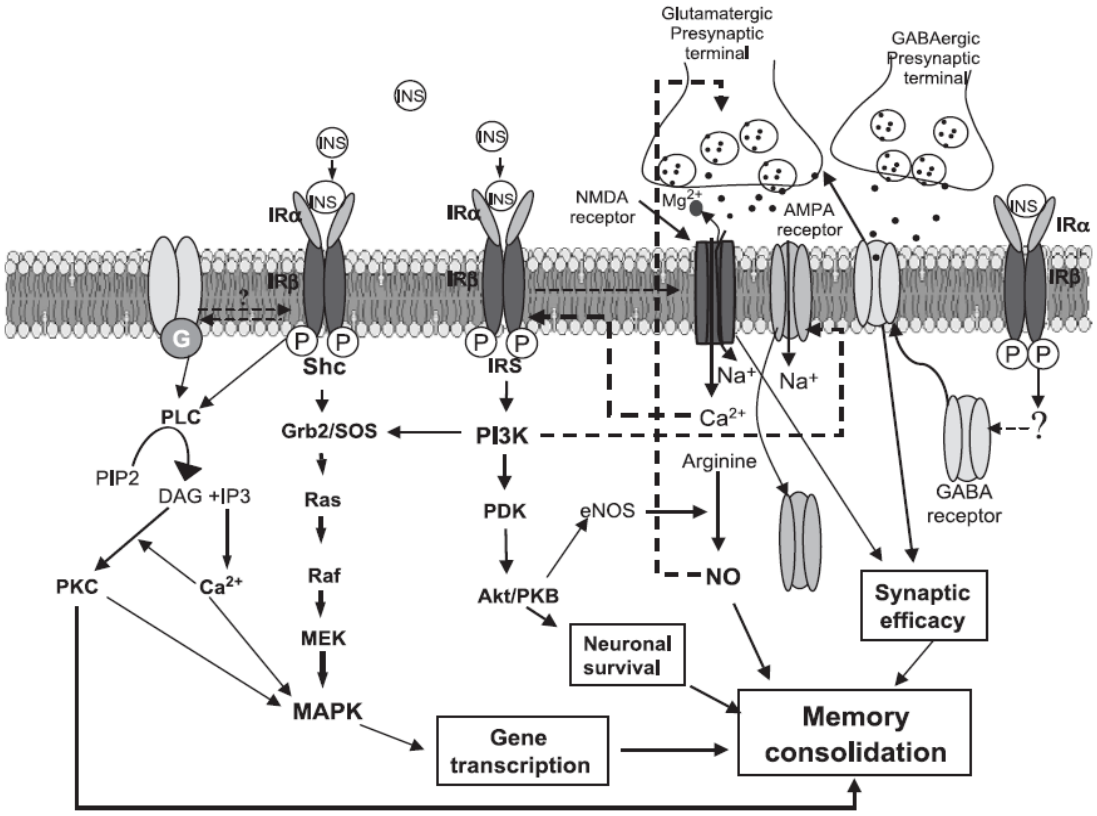
and link between diabetes and AD. This model exhibited only central insulin resistance but not peripheral insulin resistance. It has been shown that ic-STZ rodents exhibited impairment in basal synaptic transmission and LTP and behavioral deficits such as hippacampal spatial memory impairment. Moreover, this model showed impairment in glutamatergic system in the hippocampus (Shonessy et al., 2011; De la monte et al., 2005). To reveal molecular mechanism of central insulin resistance and its impact on synaptic dysfunction and relationship between AD and diabetes, this model has to be characterized further. With respect to type 2 diabetes, rodents with genetically determined diabetes and obesity have attracted specific attention (db/db mice, ob/ob mice, Zucker fa/fa rats and Zucker diabetic fa/fa rats) (Crisa et al., 1992). In general, these animals display obesity, insulin resistance and hyperinsulinaemia, with variable degrees of hyperglycaemia. Although these models mimic certain endocrinological aspects of type 2 diabetes reasonably well, most of them have not yet been sufficiently characterized. Still, as recent clinical studies indicate that the effect of diabetes on the brain might be most pronounced in elderly individuals with type 2 diabetes, these models need to be developed further.

Diabetes and Glutamatergic Dysfunction

Relatively little is known about the effects of diabetes on postsynaptic glutamate receptors in the hippocampus. Further studies needed to characterize different diabetes type1 and type 2 models and how diabetes alters postsynaptic glutamate receptors their expression trafficking and functionality channel kinetics and subunit composition. In Sprague-Dawley rats, after six to eight weeks STZ-diabetes, the affinity of glutamate for AMPA, but not for NMDA receptors, was reported to be decreased, possibly owing to changes in the glutamate receptor GluR1 subunit expression (Gagne et al., 1997). Loss of LTP maintenance in diabetic rats was a result of

disruption of Ca^{+2} dependent processes (Biessels et al., 1996) that modulate postsynaptic AMPA receptors during synaptic potentiation (Chabot et al., 1997). The finding of reduced affinity of glutamate for AMPA receptors is consistent with a reduction of the baseline slope of the field EPSP. The phosphorylation of the NR2A/B subunits by Ca^{+2}/calmodulin-dependent protein kinase II was reduced in diabetes (Di Luca et al., 1999). It is suggested that these NMDA-receptor-related changes underlie the LTP deficits. Over expression of NR2B in the forebrains of non-diabetic transgenic mice leads to enhanced activation of NMDA receptors, thus facilitating induction of LTP and enhancing the learning abilities of these mice (Tang et al., 1999). When applied to brain slices, insulin inhibits the spontaneous firing rate of hippocampal pyramidal neurons and the frequency of AMPA-receptor mediated miniature EPSCs of cerebellar Purkinje neurons (Wang et al., 2000). In addition, insulin attenuates the amplitude of AMPA-receptor-mediated currents in cerebellar Purkinje neurons, through the stimulation of clathrin-dependent receptor internalization, a phenomenon that appears to have links with cerebellar LTD (Wang et al., 2000). In hippocampal slices insulin has been shown to increase NMDA-receptor mediated EPSPs (Liu et al., 1995). Insulin signalling varies in different brain regions (de la Fernandes et al., 1999). Insulin plays a modulatory role in synaptic transmission in the brain. However, studies of its involvement in behaviour and synaptic transmission have so far mainly examined its effects after local administration (intracerebroventricular) or ex vivo and also remodeling of postsynaptic glutamate receptors by insulin are not well understood.

Figure 2.3: Insulin signaling cascade in synaptic plasticity (Zhao et al., 2004).



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PPARγ and Synaptic Plasticity

PPARs

Peroxisome proliferators-activated receptors (PPARs) are ligand activated transcription factors belonging to the orphan nuclear receptor super family (Blanquart et al., 2003) whose activity is regulated by steroids and lipid metabolites (Feige et al., 2006; Michalik and Wahli, 2007). PPARs are involved in adipocyte differentiation, lipid storage and glucose homeostasis of the adipose tissue, brain, placenta and skin (Michalik et al. 2006; Kummer and Heneka 2008). Three different PPAR isoforms have been identified namely PPARα, PPARβ/δ, and PPARγ, with similar protein structure despite differences in coding genes (Nuclear Receptors Nomenclature Committee 1999) and display distinct physiological and pharmacological functions depending on their target genes and their tissue distribution (Bishop-Bailey, 2000;

Buchan and Hassall, 2000). PPARα and PPARγ are key regulators of cellular differentiation processes and anti-inflammatory regulation, whereas PPAR β/δ has also been associated with modulation of immune response (Wanger and Wanger, 2010). The natural ligands of these receptors include long chain fatty acids, eicosanoids, oxidized lipoproteins and lipids (Landreth et al., 2008). Activation of PPARα, by natural ligands such as fatty acids and eicosanoid derivates or by synthetic ligands (lipid lowering fibrates), regulates lipid and lipoprotein metabolism (Berger et al., 2005). Activation of PPARγ by prostaglandins or by synthetic ligands, such as anti-diabetic thiazolidinediones, regulates glucose metabolism by modulating insulin sensitivity (Berger et al., 2005). Non-steroidal anti-inflammatory drugs are also weak agonists of PPARγ and PPARα. PPARβ/δ is one of the most widely expressed members of the PPAR family. Funtction of PPARβ/δ is not well established. PPARβ/δ also plays a key role in lipid metabolism and regulates serum lipid profiles and fatty acid beta oxidation in muscle and adipose tissue. Synthetic ligands of PPARβ/δ are presently in preclinical trials (Luquet et al., 2004).

PPAR γ

PPARγ has at least two promoters and results in the production of two isoforms, 1 and 2. PPARγ1 isoform is abundantly expressed in white adipose tissue, pancreatic beta cells, spleen, intestines, brain and heart (Lehrke and Lazar 2005). PPARγ 2 isoform is expressed in white and brown fat and plays a pivotal role in fat cell differentiation and lipid storage (Tontonoz et al., 1994). The predominant function of PPARγ include lipid and carbohydrate metabolism (Pakala et al., 2004; Rangwala and Lazar 2004). The activation of these receptors by their ligands improves insulin sensitivity and hence PPARγ ligands, the thiazolidinediones, have emerged as a

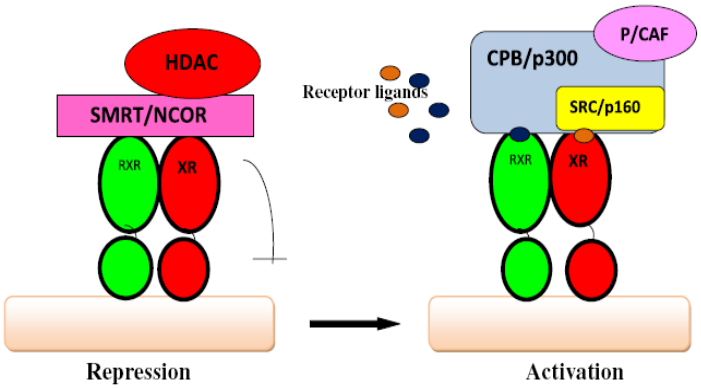
powerful class of anti diabetic drugs (Fiévet et al., 2006). The exogenous ligands for PPARγ include Rosiglitazone, Pioglitazone, ciglitazone, troglitazone, KRP-297, GW1929, GW 7845 and L-165041, respectively (Huang et al., 2009).

Molecular Mechanisms of PPAR γ activity

PPARγ acts positively on lipid metabolic enzymes (Chawla et al., 2001; Kleiver, 1999) and acts as a lipid sensor. The natural ligands of PPARγ are long-chain fatty acids, eicosanoids, and oxidised lipoproteins (Lehrke M and Lazar, 2005). Activation of this receptor results in enhanced insulin sensitivity of peripheral organs (Lehrke and Lazar, 2005). The ability of PPARγ agonists to act as insulin sensitizers results in the normalization of plasma glucose levels in diabetic patients (Picard and Auwex, 2002) and also this receptor exhibits potent anti-inflammatory activity, and receptor activation results in suppression of inflammatory gene expression (Daynes and Jones, 2002). Ligand-activated transcription factors PPARs bind to sequence-specific elements of its target genes and directly regulate their expression (Desvergne and Wahli 1999; Diradourian et al., 2005) by forming heterodimers with retinoid X receptors (RXRs). Target gene activation is controlled by specific PPAR response elements (PPRE) AGGTCA in the promoter region (Yu and Reddy 2007). Under the inactivated conditions, these heterodimers are associated with corepressors, like nuclear receptor corepressor-1 (NCoR), silencing mediator of retinoid and thyroid-responsive trancription (SMRT) and histone deacetylase (HDAC) complexes, which inhibit the gene expression. However, binding of the ligand induces conformational changes, which mechanistically facilitates the release of corepressors and HDAC complexes and promotes the recruitment of coactivators and histone acetyl transferase (HAT) complexes (Pratt and Toft 1997; Li et al., 2003; Rosenfeld et al., 2006)

and the coactivator–receptor complex finally initiates the gene transcription. PPARγ activation results in either transactivation or transrepression of target gene depend on target gene sequence.

Figure 2.4: Mechanism of PPARγ Activation and Repression



PPARs Expression in the Brain

PPARs are expressed in different regions of brain of adult mice. PPARβ/δ mRNA is preferentially present in the cerebellum, the brain stem and the cortex; PPARγ mRNA is enriched in the olfactory areas and the cortex (Braissant et al., 1996). It has been shown that expression of all the three isomers of PPARs in different levels in the hippocampus (Moreno et al., 2004). Ubiquitous expression pattern has been revealed for PPARα, whereas PPARβ/ δ was found in dentate gyrus/CA1region, and the expression of PPARγ was restricted to CA3 region (Woods et al., 2003; Gofflot et al., 2007). Pattern of this expression is isotype specific and regulated during development, suggesting that the PPARs may play a crucial role during the CNS development; their functions in brain are still not well understood.

Effect of PPAR γ in the brain

The anti-inflammatory actions of PPARγ agonists have been anticipated to account for the protective role in AD. Reports depict that the activation of PPARγ in the microglial cells suppresses the expression of proinflammatory genes (Landreth et al., 2008). The ability of PPARγ agonists to repress the proinflammatory genes evolves through the antagonism of the transcription factor NF-κB, AP-1, STATs, MMPs, COX-2 and iNOS (Chung et al., 2008). PPAR agonists have also been reported to suppress Aβ-mediated activation of microglial cells in vitro (Jiang et al., 2008). Studies have shown that pioglitazone acts to inhibit the iNOS and COX-2 expression in animal models of AD (Kaur et al., 2009). Aβ stimulated expression of inflammatory cytokines and cyclooxygenase are inhibited by PPARγ activation (Combs et al., 2000) and also clearance of Aβ peptide (Camacho et al., 2004). PPAR agonists produce a reduction in amyloid pathology may result as an outcome of PPARγ to affect the Aβ homeostasis (Landreth 2007). PPARγ agonist rosiglitazone treatment significantly reduces Aβ-42 levels and regulates Aβ synthesis, inflammation, energy utilization, lipid metabolism and insulin sensitivity, which may account for their protective role in AD (Landreth 2007; Jiang et al., 2008). Aβ toxicity reduced by Wnt/β-catenin signaling mechanism, which is involved in neuronal survival and devleopment by activation of PPARγ (Innestrosa et al., 2005). As insulin contributes to normal cognitive functioning and also plays a role in regulating the amyloid precursor protein (APP) and its derivate Aβ peptide. Oral treatment of PPARγ agonist pioglitazone for 7 days showed reduced glial activation and Aβ plaques in the hippocampus and cortex of transgenic mice model of AD. PPARγ activation improves mitochondrial functions, and this may be one of beneficial effects on memory and cognition (Mancuso et al., 2008; Swerdlow and Khan 2009; Swerdlow et al., 2010). PPARγ agonist rosiglitazone induces neuronal mitochondrial biogenesis

and potentiates glucose utilization, leading to improved cellular and cognitive functions in AD (Strum et al., 2007). Insulin receptors are localized in the hippocampus and cortex, the areas, which are associated with memory (Biessels et al., 2006). It has also been suggested that brain insulin receptor signaling is markedly reduced in AD brain (Frolich et al., 1999; Messier and Teutenberg 2005). Further reports illustrate that increasing plasma insulin levels in patients with insulin resistance causes a concurrent increase in brain levels of Aβ and inflammatory markers (Craft et al., 2003, Craft 2006; Pathan et al., 2008). Aβ build up during hyperinsulinemia may arise as an outcome of reduced Aβ degradation by insulin-degrading enzyme (IDE) (Farris et al., 2004, 2005). IDE levels have been reported to be reduced in AD patients. PPAR receptor activation may increase the insulin sensitivity and may also increase the amount of IDE in the AD brain (Farris et al., 2003; Hamilton et al., 2007). Enhancement of IDE levels may increase the Aβ clearance through proteolytic degradation by the enzyme (Du et al., 2009).

Thiazolidinediones (TZD)

Receptor-dependent and independent pathways are activated by TZD in the brain (Sundararajan et al., 2006; Feinstein et al., 2005). The receptor-dependent beneficial aspects of TZD use, including activation of Wnt signaling (Inestrosa et al., 2005), reduced inflammation in animal models of neurodegeneration (Heneka et al., 2005) and suppression of inducible nitric oxidase synthase (Heneka et al., 2000). Neuronal Ca^{+2} homeostasis is also influenced by PPARγ agonist. Enhanced Ca^{+2} transients present in AD models and in cells from AD patients (Hirashima et al., 1996). PPAR agonists rosiglitazone and troglitazone have prevented Aβ-induced cell death and morphological changes in rat hippocampal neurons, commitantly with restoration of cytoplasmic β-catenin levels and inhibition of glycogen synthase kinase 3β activity

in tau hyperphosphorylation (Inestrosa et al., 2005). Rosiglitazone protects the hippocampal and dorsal root ganglion neurons against Aβ-induced mitochondrial damage and nerve growth factor deprivation induced apoptosis by the upregulation of anti-apoptotic protein Bcl-2 (Fuenzalida et al., 2007). Direct binding of PPARγ ligands to mitochondrial proteins in a receptor independent manner could indirectly cause protective changes in the gene expression (Nicolakakis and Hamel, 2010). This would occur through a decrease in pyruvate driven respiration, leading to an increased in lactate production, free radical and oxidative stress, eliciting, in turn, a stress response characterised by increased expression of heat shock proteins and anti-inflammatory genes (Feinstein et al., 2005). Rosiglitazone was found in the brain after oral administration in submicromolar amounts (Landreth et al., 2008). Several clinical studies have evaluated thiazolidinedione therapy as a medical treatment for neurocognitive deficits. Treatment of rosiglitazone (4 mg/day) for 6 months in mild AD (Watson et al., 2005) showed enhanced memory and cognitive functions compared to the placebo-treated control group. The beneficial effects of PPARgamma in memory and cognition arise due to inhibition of inflammation, enhancement of mitochondrial and insulin actions in the brain alongwith protective effects on amyloid pathology. These central effects of PPARs together with the peripheral effects may synergistically contribute to the onset and progression of AD.

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3. MOLECULAR MECHANISMS OF MEMORY ENHANCEMENT IN LONG - LIVING AMES DWARF MICE

ABSTRACT

Aging is a natural physiological process thus leads to cognitive dysfunction. Despite the fact that many aging animal models have been studied, the mechanistic basis of aging associated cognitive impairment is not well elucidated. Growth hormone deficient Ames dwarf mice showed extended life span and resistance to age related cognitive decline. The mechanism by which memory enhanced in this animal model is not well understood. Glutamatergic and cholinergic systems play a crucial role in synaptic plasticity and cognitive function and these neurotransmitter systems impaired in aging animal models. Therefore, to decipher the mechanistic basis of enhanced memory in this animal model, we investigated the alterations in glutamatergic and cholinergic systems. We evaluated enzymatic activities of cholinergic system, inflammatory enzymes, glutamatergic synaptic markers and synaptic-associated auxiliary proteins expression in the hippocampus of this mouse model. We found that, there is no significant difference in NOs and AChE activity and interestingly ChAT and LDH activity increased in the dwarf mice brain. Since ChAT enhances the production of acetylcholine and LDH increases the ATP energy production, these would contribute to the enhanced synaptic transmission and synaptic plasticity lead to enhanced cognitive performance in the dwarf mice. Moreover, we found that inflammatory response and oxidative stress molecules decreased in hippocampus in dwarf mice such as TNF- α, TNFR1, SAPK/JNK and p38/MAPK. We also found increased expression of postsynaptic markers PSD-95, NMDAR subunit NR1, AMPAR subunit GluR2, and presynaptic synaptophysin supported enhanced basal synaptic transmission and LTP and better spatial memory observed in dwarf mice. Increased AKT phosphorylation,

increased expression of BDNF and ILK may contribute to reduced tau hyperphosphorylation and reduced Aβ accumulation. This finding supports that increased kinetic activity of glutamate ion channels and more functional glutamate receptor insertion to the postsynaptic sites and enhanced neurogenesis and synaptogenesis in dwarf mice lead to enhanced synaptic transmission and spatial memory. Therefore reduced oxidative stress, increased neurogenesis and enhanced glutamate receptors expression, trafficking and activity in the hippocampus of dwarf mice helps them to resist age related cognitive decline and synaptic deficit. This study will help us to develop potential novel drug therapy for age related cognitive decline and elucidate the common molecular mechanisms shared by aging and other neurodegenerative diseases such as AD.

INTRODUCTION

Rapidly advancing progress in medicine leads to increased aging population. Therefore, prevalence of age related cognitive impairment increases with the expansion of aging population. Despite aging not being a pathological condition, aging associated changes lead to organ dysfunction and systemic failure. There is strong evidence supporting the deleterious effects of aging in the brain, which leads to cognitive decline. There have been many *in vivo* animal models and *in vitro* cell culture models of aging studied. Still the mechanistic basis of aging related cognitive dysfunction is not well elucidated.

Age associated changes are observed in perceptual and procedural memory processing. Therefore normal aging results in impaired working memory and episodic memory. Moreover, retrieval of specific information declined in aging people. Slow mental activity, declining attention, unable to avoid unwanted information, and an overall reduction in cognitive control are found in the aging process (Luo L, Craik FI, 2008). Several human, primates and rodent

studies in aging showed alterations in hippocampal and medial temporal lobe and its associated decline in object recognition memory (McKee and Squire, 1993; Pascalis and Bachevalier, 1999). Present studies in cognitive aging models of rodents showed age related spatial memory impairment but not cued memory in water maze. Moreover, they showed age associated decline in recognition memory including impaired recollection and non-spatial recognition memory (Robistek et al., 2008).

Cholinergic system plays a crucial role in cognitive function. Release and synthesis of acetylcholine is reduced in aged rodents (Wu et al., 1988). Acetylcholine level increased in the early stages of AD and declined drastically in the later stages of AD. Acetylcholine degrading enzyme Acetylcholine Esterase (AChE) activity is not affected in normal aging but it is decreased in AD (Kuhl et al., 1999). Choline acetyltransferase (ChAT) plays a key role in learning and memory processes by synthesising acetylcholine, and its activity inhibited in striatum, cortex and hippocampus from aged brain and Aβ induced toxicity in AD (Zambrzycka et al., 2002). LDH activity decreases with age (Prathima et al., 1999) and ATP production in the brain reduced thus leading to impaired synaptic transmission and synaptogenesis. Chronic oxidative stress showed decreased activity of LDH in the cortex, striatum and hippocampus. Therefore, aging associated oxidative stress may reduce the activity of LDH and reduces energy production in the brain. Biological aging is associated with increased oxidative stress and decreased NOS activity (Harvey and Nel, 2003).

Ames dwarf mice showed resistance to age related cognitive decline. Therefore, it is a potential animal model to study the mechanism of aging related cognitive impairment and synaptic dysfunction. Ames dwarf mouse has a Prop-1 gene mutation resulting in impairment of anterior pituitary development that leads to a deficiency of circulating growth hormone, thyroid

stimulating hormone, and prolactin (Brown-Borg et al. 1996; Hauck et al., 2001). These mice are much smaller and live significantly longer (70% longer life span) than their normal siblings (Brown-Borg et al., 2005). The lack of plasma GH results in severely reduced plasma levels of insulin-like growth factor 1 (IGF-1). Therefore, they showed primary GH deficiency and secondary IGF-1 deficiency in the plasma. Despite decreased circulating levels of IGF-1, these animals have been shown to exhibit enhanced cognitive function and resistance to age related cognitive decline (Kinney et al. 2001). Interestingly recent studies showed that local expression of IGF-1 in the brain and local IGF-1 might be able to maintain central nervous system function independently of regulation by the hypothalamic-pituitary axis (Sun et al. 2005). Moreover, it has also been shown that old aged Ames dwarf mice have elevated levels of IGF-1 protein in the hippocampus, whereas the levels of the corresponding mRNAs are as high as in normal mice (Sun et al. 2005). Interestingly, they have enhanced insulin sensitivity, elevated antioxidative enzymes and the ability to withstand oxidative stressors compared to wild type animals (Brown-Borg and Rakoczy, 2005). Ames dwarf mice also have higher levels of hippocampal neurogenesis that might contribute to the delay of cognitive loss during aging (Sun and Bartke 2007; Sun et al. 2005). The Ames dwarf mice experience a delay in physical aging and have an increased life span with enhanced memory (BrownBorg et al., 1996; Bartke et al., 2001; Bartke, 2000). It has been demonstrated that the beneficial effect of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) on the development and function of the central nervous system (Werther et al., 1990; D'Ercole et al., 1996). IGF-1 is a neurotrophic factor, and plays a key role in insulin signaling in the brain and may contribute to neurogenesis and better cognitive function via enhancing synaptic transmission and synaptic plasticity. Recently, several behavioral studies showed that Ames dwarf mice have better hippocampal-based spatial memory compared with

age-matched wild-type mice. Ames mice are able to retain this memory capability following an oxidative stress induced by Kainic acid (KA) (Sharma et al. 2010).

The hippocampus is the major brain region where memory is encoded and consolidated and is implicated in spatial memory performance (Rolls 2000; O'Keefe and Nadel 1978). Studies on human subjects with hippocampal damage provide evidence that this brain region plays a critical role in spatial memory (Abrahams et al. 1997; Astur et al. 2002; Feigenbaum et al. 1996; Goldstein et al. 1989; Maguire et al. 1996; Rosenbaum et al. 2000). Hippocampus has been shown to be susceptible to AD. It has been shown that hippocampus of dwarf mice have increased neurogenesis and resistance to Aβ toxicity compared to normal mice (Sun et al., 2005; Sun et al., 2007; Schrag et al., 2008). Several factors are believed to give rise to the late onset sporadic form of AD. Scacchi et al (2008) have studied the variation of three enzymes of the cholinergic system: acetylcholinesterase, butyrylcholinesterase, and choline acetyltransferase and found a significant difference between patients and controls in ChAT expression and activity. This study and several other findings suggest the involvement of ChAT activity in the late onset sporadic form of AD and related cognitive deficit.

Glutamate is the major excitatory neurotransmitter in most of the excitatory synapses in the mammalian brain and plays a crucial role in synaptic plasticity and cognitive function. This neurotransmitter is associated with motor behaviour, cognition and emotion, all of which altered with normal aging process. Aging brain showed alterations in glutamatergic system including decrease of glutamate content in tissue from cerebral cortex and hippocampus, reduced glutamate uptake capacity, loss in the number of high affinity glutamate transporters in glutamatergic terminals. Interestingly it has been shown that decrease in the density of glutamatergic NMDA receptors with age and receptor mediated responses in cerebral structures

(Baskys et al., 1990; Gonzales et al., 1991; Cepeda et al., 1996). Moreover, an increase in the affinity of the NMDA receptor for glutamate (Cohen and Muller, 1992) as well as changes in the influence of modulatory sites on the NMDA receptor complex with age have also been reported (Miyoshi et al., 1990; Piggott et al., 1992). Decreases in glutamatergic AMPA receptor density has been reported in frontal and parietal cortices of mice (Magnusson and Cotman, 1993). In the hippocampus, different subregions seem to change differently with age and an age-related decrease in AMPA receptor density has been reported in certain areas of the hippocampus (Cimino et al., 1993; Magnusson and Cotman, 1993). In accordance with the decrease in AMPA receptor density described in the CA1 area of the hippocampus (Magnusson and Cotman, 1993), there is also a decrease in depolarization in response to AMPA application in this area of the brain (Barnes et al., 1992). Recent studies showing that the decrease of NMDA and AMPA receptors in the hippocampus was significantly correlated with age-related declines in learning (Magnusson, 1998). Regarding the glutamatergic kainate receptor, there have been reports of decreases (Magnusson and Cotman, 1993) in the density of kainate binding with age in the cerebral cortex and the hippocampus.

Long-term potentiation (LTP) is a well-represented cellular and molecular mechanistic model for learning and memory (deToledo-Morrell et al. 1988; Gallagher and Nicolle 1993; Landfield and Lynch 1977; Moore et al.1993; Shankar et al. 1998; Ward et al. 1999). Activation of NMDA receptor plays a key role in the acquisition of spatial memory (Morris et al. 1986; Tsien et al. 1996). Pharmacological antagonist blockade of NMDA receptors or deletion of NMDA receptor subunit 1 (NR1) leads to a substantial impairment of spatial memory (Danysz et al. 1995; Morris et al. 1990; Tsien et al. 1996). Similar to impairment of spatial memory, NR1knockout mice exhibit impairment of LTP (Nakazawa et al. 2003). NMDAR involves in the

formation of spatial memory and in the regulation of neurogenesis in adults (Arvidsson et al. 2001; Luk et al. 2003; Deisseroth et al. 2004; Joo et al. 2007). Newly formed neurons in the dentate gyrus are involved in learning and memory circuits (Zhao et al. 2006; Toni et al. 2007; Ge et al. 2007) and increased dentate neurogenesis has been positively correlated with spatial learning and memory performance (Drapeau et al. 2003; Kempermann et al. 2004; Shors et al. 2001).

Our collaborator Holly Brown-Borg's lab recently showed enhanced learning and memory in the Ames mice due to enhanced synaptic transmission and LTP, antioxidant status, enhanced neurogenesis, and differential expression of NMDAR subunits NR2A, NR2B and kainate receptor subunits KAR 1, KAR2, GluR5, GluR6/7 in Ames dwarf as compared to their wild-type counterpart.

Despite recent data, support the dwarf mice are resistant to age related cognitive decline, definite molecular mechanism underlying better cognitive function in dwarf mice is not well understood. Therefore, we proposed to study enzymatic activities of cholinergic system and inflammatory enzymes, and synaptic molecules of glutamatergic system, and neurotrophic factors in the hippocampus of the dwarf mice. We hypothesized that dwarf mice show resistance to age related cognitive dysfunction via altering cholinergic and glutamatergic system and related synaptic auxiliary proteins in the hippocampus. This study will help us to understand the mechanistic basis of age related cognitive deficit and impaired synaptic transmission. Our finding will provide significant contribution in identifying and validating potential molecular targets for developing novel therapeutic options and also it will help to elucidate the central mechanism of age related cognitive impairment shared by severe neurodenerative disorders such as AD.

MATERIALS AND METHODS

Chemicals:

BCA protein assay reagent kit and Coomassie plus protein assay reagent kits were purchased from Pierce Biotechnology Inc. (Rockford, IL). All other chemicals were purchased from Sigma chemicals, St. Louis, MO, USA, otherwise stated.

Animals:

A growth hormone deficient Ames dwarf mouse is a potential animal model for studying aging related alterations in cognitive function. Ames dwarf and age-matched wild type mice were maintained at the University of North Dakota (UND) facilities under controlled conditions of photoperiod (12 h light:12 h dark cycle) and temperature (22 ± 1 °C) with free access to food (Laboratory Rodent Diet, PMI Nutrition Intl., St. Louis, MO) and water. Ames dwarf (df/df) mice were derived from a closed colony with heterogeneous background (over 20 years). Dwarf mice were generated by mating either homozygous (df/df) or heterozygous (df/+) dwarf males with carrier females (df/+). The breeding stock for the Ames Dwarf colony was housed, genotyped and euthanized at university of North Dakota and entire brain were isolated and stored at -80 °C, and preserved in dry ice, and sent to auburn university by Brown-Berg. All mice were 9 months old males. Ames dwarf homozygous for Prop1df gene (Prop1df/Prop1df) (n=5) and similar age control group heterozygous for Prop1df gene (Prop1df/+) (n=5). All procedures involving animals were reviewed and approved by the UND Institutional Animal Care and Use Committee in accordance with the NIH guidelines for the care and use of laboratory animals. Nine months old, animals were sacrificed and the brains were homogenized for the biochemical

assays and hippocampus were isolated and homogenized at stored at -80°C for western immunoblot analysis.

Ames Dwarf Mice Breeding:

This mutant dwarf mouse carries a recessive mutation in the Prop1 gene (Prop1df) and homozygous mice are dwarf and exhibit extended lifespan. Heterozygotes are normal in size. In the mutant mouse-aging colony, the Ames Dwarf mice were bred from a heterozygous female crossed to a homozygous mutant male. Therefore, one half of the offspring were heterozygous and one-half was homozygous mutants. Normal sized control mice available from this colony were all heterozygous. These mice were not genotyped as the homozygotes were clearly distinguished from heterozygotes by size. Ames dwarf mice (Prop1df/Prop1df) have difficulty maintaining their body temperature so it was essential that they were housed with normal sized mice. They were housed with heterozygous (Prop1df/+) mice in the colony and also shipped with normal sized mice and housed with normal sized mice upon receipt. Therefore, when ordering Ames dwarf mice, at least one normal-sized control (heterozygote) was ordered for each dwarf mouse (homozygote) ordered. The fat diet (6%) was used as its better for the dwarf mice than a 4% fat diet and powdered food was placed in the bottom of the cage.

Enzymatic activity Assays:

Nitric oxide synthase activities were measured as described previously (Zhu et al. 2003; Luo et al. 2005). In brief, the brain was homogenized in ice-cold PBS, pH 7.4, and centrifuged at 10, 000 g for 20 min at 4°C. The supernatant was ultracentrifuged at 100, 000 g for 15 min at 4°C. NOS activity in the filtrates was measured using a commercially available kit (Calbiochem,

San Diego, CA, USA). Activities of acetylcholine esterase (AChE), cholineacetyl transferase (ChAT) and lactate dehydrogenase (LDH) were measured in brains of dwarf and wild type mice. Acetylcholine esterase (AChE) activity was measured spectrophotometrically at 412nm. Choline acetyl transferase (ChAT) activity was measured spectrophotometrically by reading the absorbance of acetylcholine yield at 324nm. Total tissue LDH activity was determined by following initial rate of pyruvate reduction to lactate (Simaga et al., 2008). Protein content was assayed using the Coomassie plus protein assay reagent kit. Bovine serum albumin was used as a standard.

Western Immunoblot:

Whole hippocampal homogenates were lysed in a chilled buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4, 1% NP- 40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na3VO4, and 1 mM NaF). The lysates were incubated for 15 minutes on ice and centrifuged for 15 minutes at 10,000 x g, at 4°C. The supernatants containing the protein extract were collected, and their protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Duplicate samples for individual mice were subjected to 10% SDS-PAGE and subsequently blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were saturated with 5% non-fat dry milk (Biorad) or 1% BSA and then incubated with anti-Akt (1:1000; Cell Signaling Technology), anti-phospho-Akt (1:1000;Cell Signaling Technology), anti-ILK (1:1000; Cell Signaling Technology), anti-GluR2 (1:1000; Millipore), anti-NR1 (1:1000; Millipore), anti-BDNF (1:500;Millipore), anti-synaptophysin (1:1000; Cell Signaling Technology), anti-PSD95 (1:1000; Cell Signaling Technology), anti- SAPK/JNK, anti-

p38/MAPK (1:1000; Cell Signaling Technology), anti- TNF-α (1:1000; Cell Signaling Technology) anti- TNFR1 (1:1000; Cell Signaling Technology) anti- TNFR2 (1:1000; Cell Signaling Technology) and anti-GAPDH (1:5000; Millipore) antibodies overnight at 4°C. The membranes were subsequently incubated at room temperature for 1 hour with the corresponding anti-rabbit or anti-mouse antibodies (1:5000 and 1:10,000 respectively; Millipore). The membranes were then incubated in Immobilon Western HRP Substrate (Millipore) for 5 minutes and imaged using the Molecular Imager ChemiDoc XRS system (Bio-Rad). Signals were subsequently quantified by densitometric analyses using Quantity One Analysis software (Bio-Rad). The densities of each band, which represented individual animals, were normalized to GAPDH and these normalized values were averaged for control and dwarf mice groups.

Statistical Analysis:

Experiments were performed with the investigator blinded to the treatment the animals received. Data were expressed as mean ± SEM. All results were normalized to the basal values and analyzed and significance was determined using a two-tailed unpaired Student's t-test. The level of significance was set for $p<0.05$. All statistical calculations were performed on a computer using commercially available statistical packages.

RESULTS

Evaluation of Enzymatic activity in Dwarf mice Hippocampus

Biochemical enzymatic activity assay was performed to evaluate the change of cholinergic enzyme activity and inflammatory enzyme activity in dwarf mice and same age control group (n=5). Nitric oxide synthase (NOS) enzymatic activity was measured in nano-

moles of nitoric oxide (NO) produced per mg of protein (enzyme) per hour and presented in Figure1.A. There was no significant difference between dwarf and normal control mice in NOS activity. Therefore, both animal groups produced same amount of NO in the hippocampus of the brain. Total tissue lactate dehydrogenase (LDH) enzyme activity was determined by following initial rate of pyruvate reduction to lactate (Simaga et al., 2008). Protein content was assayed using the Coomassie plus protein assay reagent kit and bovine serum albumin was used as a standard. Total tissue lactate dehydrogenase activity was significantly increased (127.523%) in the dwarf mice hippocampus compared to their wild type control counter parts (Fig.3.2.B, n=5, $p < 0.001$).

There are two key enzymes involved in neurotransmitter acetylcholine metabolism in the brain such as acetylcholine esterase (AChE) and choline acetyl transferase (ChAT). Using biochemical assays, the enzymatic activities of AChE and ChAT were measured. There was no significant difference between dwarf and normal control mice hippocampus in AChE activity (Change in absorbance at 412nm/min/mg protein) (Fig.3.2.A). Interestingly ChAT activity (Change in absorbance/min/mg protein) in dwarf mice hippocampus was significantly increased (138.01%) compared to their age matched control group (Fig.3.2.B, n=5, $p< 0.001$).

Western immunoblot

To investigate inflammation and oxidative stress in the hippocampus, protein expression levels of TNF-α, TNF-α receptor1 (TNFR1),TNFR2 and p38/MAPK and SAPK/JNK were measured from dwarf mice hippocampal homogenate and their same age control counterpart using western immunoblotting. There was a significant reduction in Inflammatory marker TNF-α (53.7%) and its receptor TNFR-1 (56.73%) protein expression in the dwarf mice hippocampus

compared to its same age control group counter part (Fig.3.3, n=5, $p < 0.001$). Interestingly there was no significant change in the protein expression level of TNFR-2 in the dwarf mice hippocampus compared to its control group (Figure.3.3). P38/MAPK (66.2%, $p<0.01$) and SAPK/JNK (61.8%, $p<0.001$) protein expression levels were significantly decreased in dwarf mice hippocampus compared to its control group (Figure.3.4, n=5).

Synaptic plasticity associated proteins, neurotrophic factor BDNF, kinases ILK, AKT and activity of AKT (phosphorylation of AKT) were measured by western immunoblotting. Total AKT ($p<0.001$, 128.43%) protein expression and AKT phosphorylation (AKT activity) ($p<0.01$, 145.1%) increased significantly in dwarf mice hippocampus compared to their control counter part (Fig.3.5, n=5). BDNF (138.4%) and ILK (143%) protein expression levels were significantly increased in dwarf mice hippocampus compared to same age control group.(Fig.3.6, n=5, $p<0.05$).

Synaptic proteins such as postsynaptic marker PSD-95, presynaptic vesicular protein Synaptophysin and glutamate receptor AMPAR subunit GluR2 and NMDAR subunit NR1 were measured by western blot. Synaptophysin (137.5%) and NR1 (133.8%, $p<0.05$) and PSD-95 (119.5%) and GluR2 (116.5%, $p<0.01$) protein expression levels were significantly increased in dwarf mice hippocampus compared to control group. (Fig.3.7, n=5).

FIGURES AND FIGURE LEGENDS

Fig.3.1

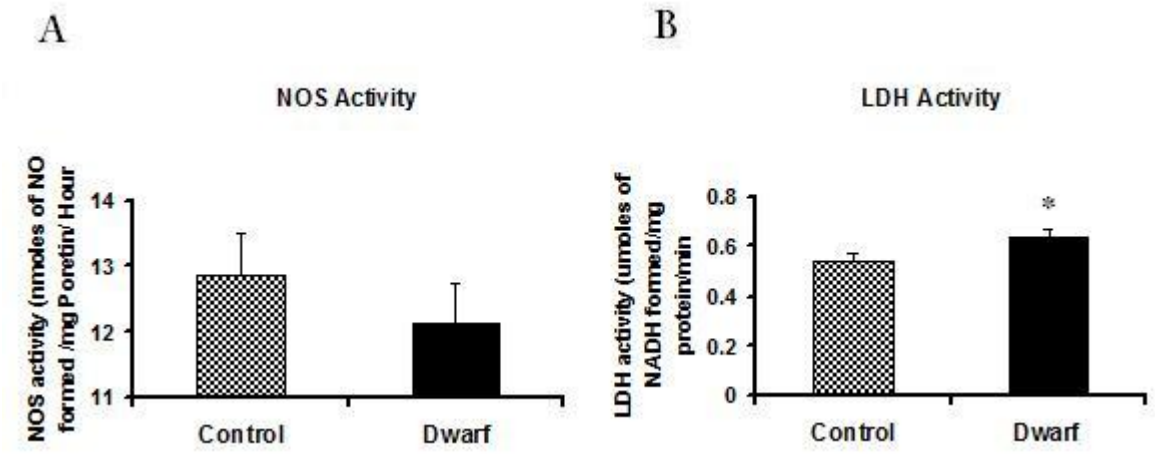

Fig.3.1: Increased Lactate dehydrogenase (LDH) activity in dwarf mice Hippocampus: Enzymatic activity of (B) Lactate dehydrogenase (LDH) increased significantly (* $p<0.001$) and (A) Nitric oxide synthase (NOS) activity did not change in Dwarf mice hippocampus compared to control group. Values are Mean±SEM. Two tailed student t-test unpaired, n=5, *$p < 0.001$.

Fig.3.2

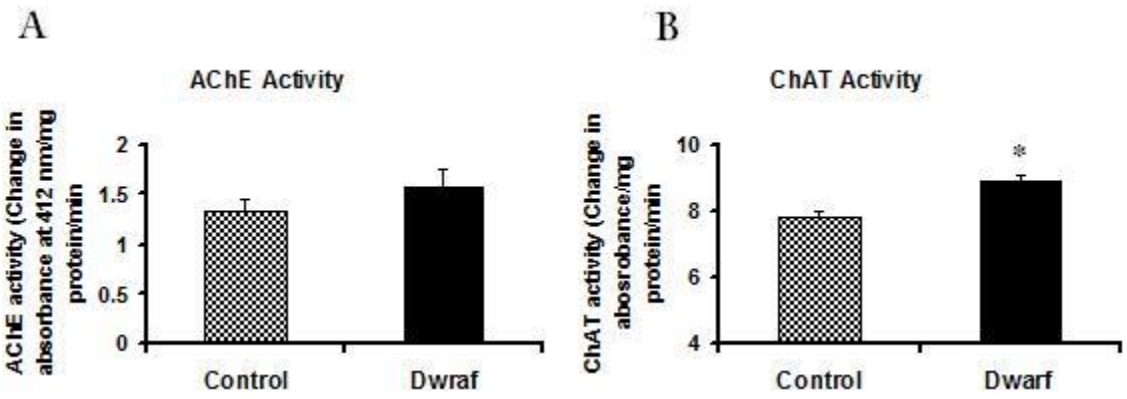

Fig.3.2: Choline acetyl transferase (ChAT) activity decreased in dwarf mice hippocampus. Enzymatic activity of (B) Choline acetyl transferase (ChAT) decreased significantly and (A)

Acetyl choline esterase (AChE) does not change in dwarf mice Hippocampus compared to control group. Values are Mean±SEM. Two tailed student t-test unpaired, n=5, * p < 0.001.

Fig.3.3

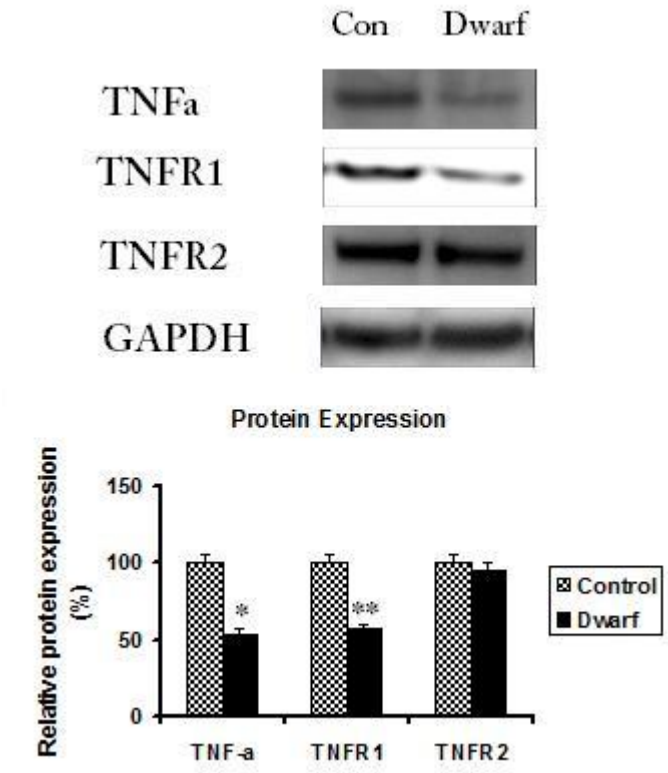

Fig.3.3: Inflammatory marker TNF- α and its receptor TNFR1 protein expression decreased in Dwarf hippocampus. Inflammatory marker TNF-α (* p<0.001) and its receptor TNFR1 (** p<0.01) protein expression significantly decreased and there is no change in TNFR2 protein expression in the dwarf mice Hippocampus compared to control. Values are Mean±SEM. Two tailed student t-test unpaired, n=5, * p<0.001, **p<0.01. Band density values are normalized to house keeping/loading control GAPDH.

Fig.3.4

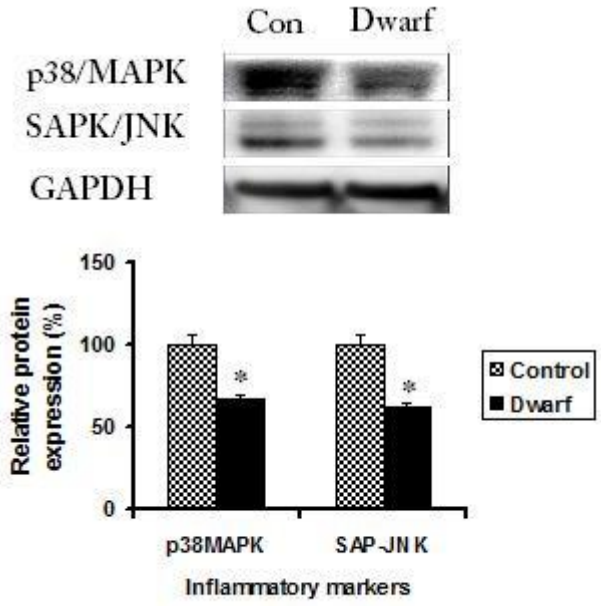

Fig.3.4: Inflammatory protein expression of p38MAPK and SAPK/JNK decreased in Dwarf mice hippocampus. Inflammatory markers p38/MAPK and SAPK/JNK protein expression significantly decreased in Dwarf mice hippocampus compared to same age control group. Values are Mean ± SEM. Two tailed student t-test unpaired, n= 5, * $p < 0.001$. Band density values are normalized to house keeping/loading control GAPDH.

Fig.3.5

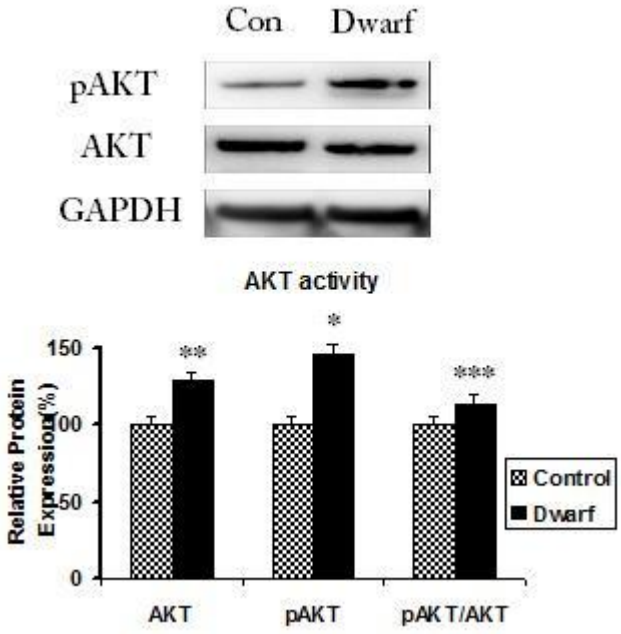

Fig.3.5: AKT protein expression and phosphorylation increased in Dwarf mice Hippocampus. Total protein expression of AKT and AKT phosphorylation significantly Increased dwarf mice hippocampus compared to control group. Values are Mean±SEM. Two tailed student t-test unpaired, n=5, * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$. Band density values are normalized to house keeping/loading control GAPDH.

Fig.3.6

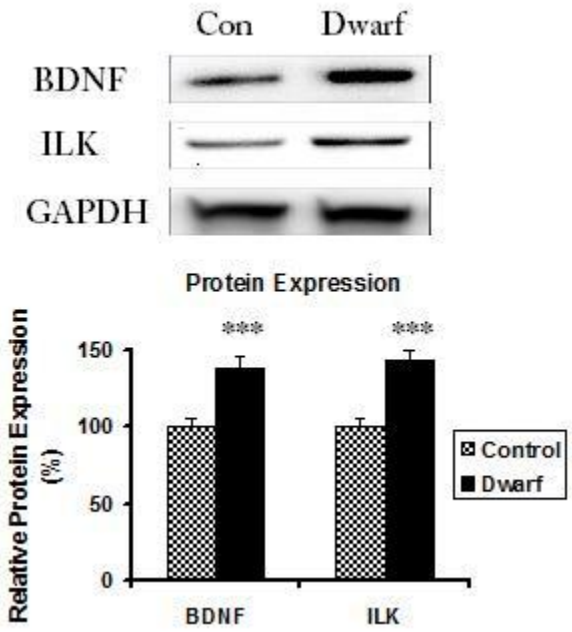

Fig.3.6: BDNF and ILK protein expression increased in dwarf mice hippocampus. Protein expression of BDNF and ILK significantly increased (*** $p<0.05$) in dwarf mice hippocampus compared to same age control group. Values are Mean±SEM. N= 5. Two tailed student t-test unpaired, *** $p < 0.05$. Band density values are normalized to house keeping loading control GAPDH.

Fig.3.7

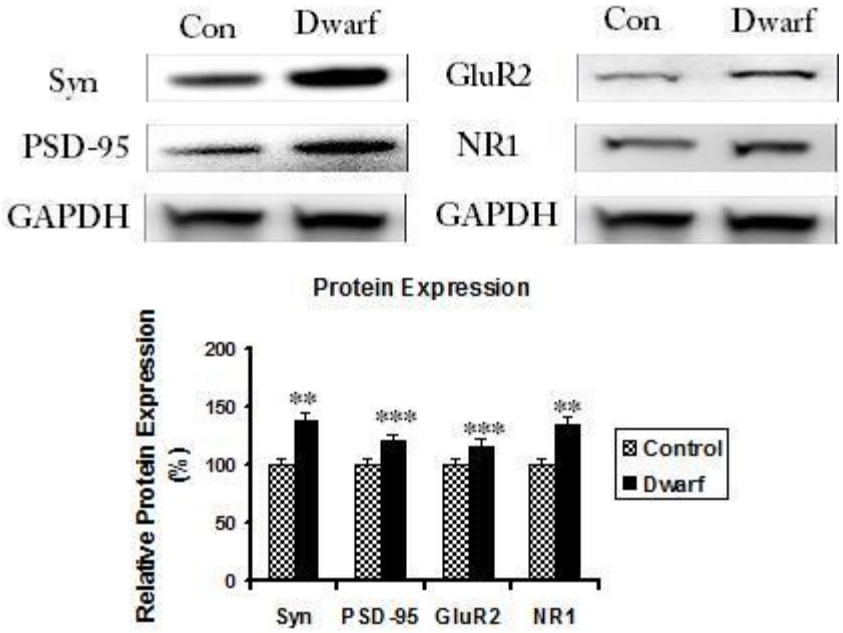

Fig.3.7: Change of protein expression of glutamate receptor subunits and post and Presynaptic markers in the dwarf hippocampus. Protein expression of presynaptic marker Synaptophysin (Syn) and postsynaptic marker PSD-95 and Glutamate receptor AMPAR subunit GluR2 and NMDAR subunit NR1 significantly Increase in dwarf mice hippocampus compared to same age control group. Values are Mean±SEM. Two tailed student t-test unpaired, n=5, *** $p < 0.05$, ** $p < 0.01$, Band density values are normalized to house keeping/loading control GAPDH.

DISCUSSION

Long-lived Ames dwarf mouse is resistant to age related cognitive dysfunction. This mouse model showed 70% longer life span (Brown-borg et al., 1996) and better cognitive performance compared to wild type of the same age group. It has been shown that peripheral

deficiency in GH and IGF-1 in this mouse model leads to caloric restriction and extended life span (Kinney et al., 2001). The molecular mechanistic basis of improved cognitive function in this dwarf mouse is not yet well understood. Increased expression of IGF-1 locally in the hippocampus increases neurogenesis and it may lead to better cognitive performance and resistance to age related cognitive decline (Sun et al., 2005; Suna and Bartke, 2007). Liver-specific IGF1- deficient mice exhibit 75% reduction in circulating IGF-1 levels; LTP was absent but was restored following treatment with IGF-1 (Trejo et al. 2007). Therefore, this finding supports that IGF-1-deficient Ames dwarf mice maintain considerable brain IGF-1 levels to induce and maintain long-term potentiation and synaptic plasticity during aging.

Behavioral studies showed enhanced hippocampal spatial memory in the dwarf mice compared to the wild type (Sharma et al., 2010). There are various reports that have linked different subunits of NMDA receptors with spatial memory, LTP, and neurogenesis (Cao et al. 2007; Clayton et al. 2002; Arvidsson et al. 2001; Nacher and McEwen 2006). Recently our collaborator Brown-Borg's laboratory found that extracellular field recording of basal synaptic transmission and LTP increased in the dwarf mice hippocampus compared to the same aged wild type group. Therefore, there may be other mechanisms to resist age related cognitive decline in the dwarf mice. Moreover, they found that gene expression levels of NR1 and NR2A and protein levels of NR2A and NR2B were higher in Ames dwarf mice hippocampus as compared to wild-type counterparts. This evidence supports that the elevated levels of NMDA receptors may contribute to the enhanced learning and memory observed in dwarf mice. Moreover, increased expression of kainate receptor subunit protein (KAR2) in the hippocampus also supports better cognitive performance in the aged dwarf mice. Ames dwarf mice showed a larger pool of newly formed neurons in the hippocampus to integrate synaptically, providing a cellular resource for

improved cognitive function. Ames dwarf mice showed enhanced neurogenesis following KA induced neurotoxic challenge in the hippocampus thus potentially forming new synaptic connections to preserve memory, a response that was not observed in the wild-type mice. Further investigation is needed to understand the mechanism by which cognitive function is improved by increased number of dendritic spines and synaptogenesis in the hippocampus. Integration of newly formed neurons in the hippocampal synapses and identification of regulatory factors involved in synaptic integration should be investigated further. To validate and support these findings and elucidate glutamatergic based mechanisms to explain resistance of age related cognitive decline in dwarf mice, we studied biochemical assays for neurotransmitters, inflammatory and oxidative stress, glutamatergic receptors NMDAR and AMPAR expression, and pre and post synaptic protein markers, synaptic plasticity associated kinases and neurotrophic factors which enhance neurogenesis and synaptogenesis.

Our studies found that no change in the activity of nitric oxide synthase and TNF receptor 2 and we found that inflammatory markers TNF-α, TNFR1, SAPK/JNK, and p38/MAPK decreased in expression in the dwarf mice hippocampus compared to wild type group. This finding suggested that reduced inflammatory responses and oxidative stress during aging process in dwarf mice hippocampus. Reduced oxidative stress in the hippocampus leads to increased synaptogenesis and neurogenesis and resistance to oxidative stress induced neurodegeneration. Therefore, this mouse model showed enhanced synaptic transmission and synaptic plasticity and cognitive performance than their control counter part. Moreover, increased activation of lactate dehydrogenase enzyme in the hippocampus results in higher energy/ATP production in the brain. Increased energy formation in the brain supports increased neuronal activity and neurogenesis and enhanced synaptic transmission and plasticity. In the study of cholinergic system, we found

increased enzymatic activity of choline acetyl transferase and no change in the acetylcholine esterase activity inferred increased acetylcholine formation in the hippocampus and enhanced memory. Acetylcholine enhances short-term memory process. In the area of memory, cholinergic neurotransmission may enhance memory consolidation, especially of affectively important events; facilitate switching from online attentive processing to off-line memory consolidation; and prevent interference from previously stored patterns during the learning of new relationships. Acetylcholine enhances the activity of many cortical neurons, causing suppression of membrane potassium currents and thereby causing depolarization and suppression of adaptation and this could certainly enhance memory function. Acetylcholine mediated selective suppression of synaptic transmission in intrinsic synapses (piriform cortex and CA1 hippocampal subregion) can prevent recall of previously stored information by interfering with the learning of new information (Hasselmo and Bower, 1992; Hasselmo and Schnell, 1994; Hasselmo and Bower, 1993; Hasselmo, 1993). The cholinergic and glutamatergic neurotransmitter systems, which share a close functional relationship in memory consolidation. Several studies with AD mice models showed decreased level of acetylcholine in the brain. Cholinergic system regulates glutamatergic system and impaired acetylcholine synthesis affects glutamatergic system and results in cognitive dysfunction. Therefore, enhanced memory in the dwarf mice may be explained at least in part by the improved cholinergic function and the increased energy production in the neuronal cellular respiration. Previously it has been shown that dwarf mice exhibit increased neurogenesis and increased insulin like growth factor 1 (IGF-1) compared to normal mice (Sun et al., 2007) and also neurons of Dwarf mice display resistance to beta-amyloid toxicity compared to normal mice (Schrag et al., 2008). In addition to all these findings

our study provides evidences for the alterations in the enzyme activities and second messenger systems contribute to the improved brain health in dwarf mice.

Our study demonstrated increased expression of presynaptic vesicular protein marker synaptophysin and postsynaptic marker PSD-95 and also NMDAR receptor subunit NR1 and AMPAR subunit GluR2 in the dwarf hippocampus compared to control group. Interestingly phosphorylation of AKT, ILK and BDNF expression increased in the dwarf mice hippocampus. Previous studies showed that NMDAR subunit NR1 supports NMDAR dependent synaptic plasticity and neuronal proliferation, migration and neurite outgrowth (Le Greves et al., 2002, 2006). Increased expression of BDNF also enhanced NR1 expression thus leads to increased number of NMDAR receptors inserted to postsynaptic sites result in enhanced synaptic transmission and LTP. Holly Brown-Borg's laboratory recently showed increased NR1 expression (But not statistically significant) in the 3 months old dwarf mice hippocampus compared to the same age of the wild type group. Interestingly our 9 months old dwarf mice group showed significant increase in NR1 subunits expression in the hippocampus compared to the same age of the wild type group. This finding supports that NR1 subunit expression does not show age related decline in the dwarf hippocampus and supports better cognitive function during the aging process.

Increased expression of AMPAR subunit GluR2 was found in the dwarf hippocampus. The AMPA receptor subunit GluR2 dictates the critical biophysical properties of the receptor, strongly influences receptor assembly and trafficking, and plays pivotal roles in a number of forms of long-term synaptic plasticity. Transgenic mouse strains that lack GluR2 exhibit a wide variety of profound detrimental phenotypes in synaptic function, development, and behavior (Brusa et al., 1995; Feldmeyer et al., 1999; Gerlai et al., 1998; Hartmann et al., 2004; Higuchi et

al., 1993, 2000; Jia et al., 1996; Shimshek et al., 2006a, 2006b; Yan et al., 2002). Moreover, there is considerable evidence that disruption in GluR2 function is associated with a number of neurological disorders such as cerebral ischemia, amyotrophic lateral sclerosis, pain, and epilepsy (Cull-Candy et al., 2006). Therefore, increased GluR2 expression supports the resistance to age related cognitive dysfunction in the dwarf mice. Increased postsynaptic densities PSD95 enhance large number of glutamate receptor incorporation to the postsynaptic sites, and stabilize them and enhance their ion channel kinetics such as conductance and channel open probability. Increased expression of synaptophysin may increase the presynaptic neurotransmitter release and increases synaptic transmission. Increased expression of ILK and increased AKT phosphorylation not only increase BDNF expression but also phosphorylate GSK-3beta. Phosphorylated GSK-3beta binds with AMPAR subunit GluR1 and enhances their trafficking to post synaptic sites. BDNF also phosphorylates GluR1 and increases its insertion to post synaptic membrane. Recent studies found that dwarf mice showed increased phosphorylation of CREB in the hippocampus compared to control counter part (Sun et al., 2005). CREB is the master transcription factor for the regulation of LTP and synaptic plasticity. Therefore, increased activation of CREB in the dwarf hippocampus increases BDNF expression and enhance glutamate receptors insertion to the postsynaptic sites. Increased ILK expression in the dwarf hippocampus increases integrin mediated cytoskeleton dynamics (Blattner and Kretzler, 2005), BDNF expression, and increasing AMPAR receptor trafficking, spine morphology changes and improves synaptic transmission and synaptic plasticity. Ames dwarf mice showed reduction in tau hyperphosphorylation and reduced Aβ accumulation. Increased AKT phosphorylation and ILK expression may reduce tau hyperphosphorylation via phosphorylation of GSK3β and inhibits its activity of tau phosphorylation. Therefore, our

findings strongly support the resistance to age related cognitive impairment in the dwarf mice via enhanced synaptic glutamatergic activity in the hippocampus and will be helpful in the future to develop potential novel therapeutic option for age related cognitive decline and age related pathological disorders such as AD by enhancing neurogenesis and inhibiting pathological stress and neurodegeneration.

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4. AMYLOID BETA INDUCED COGNITIVE DYSFUNCTION

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia and a progressive neurodegenerative disorder associated with aging. Aβ peptides are one of the major hallmarks of AD and they are overproduced and accumulated in AD. The molecular mechanism by which Aβ induces cognitive impairment in the early stage of AD before neuronal loss is not well established. Aβ overexpressing animal models showed impaired glutamatergic synaptic transmission at the early stage and further investigation is needed to establish the mechanistic basis. Aβ species 1-42 is the potential neurotoxic peptide in cognitive impairment. Therefore, we hypothesized that intracerebroventricular (icv) administration of exogenous Aβ (1-42) impairs cognitive function in the early stage of AD via altering glutamatergic synaptic transmission before severe neurodegeneration. To test this hypothesis we investigated Aβ (1-42) icv infused rats for their hippocampal spatial memory and recognition memory by cognitive behavioral tasks and found that Aβ impaired the spatial memory and recognition memory. Electrophysiological extracellular field recordings of hippocampal CA1-CA3 regions of Aβ infused mice showed no significant change in short term plasticity and presynaptic neurotransmitter release. This animal model showed impaired basal synaptic transmission and long-term potentiation (LTP). Moreover, postsynaptic glutamatergic receptors NMDAR subunits (NR1 and NR2B) and AMPAR subunits (GluR1) downregulated and GluR1 phosphorylation reduced. Presynaptic marker synaptophysin and postsynaptic PSD-95 and AMPAR auxiliary protein stargazin also reduced in expression. We found reduced expression of BDNF and ILK in the hippocampus and reduced phosphorylation of AKT. These findings strongly support the

impairment of postsynaptic glutamatergic receptor trafficking to functional postsynaptic sites and their altered ion channel kinetics thus lead to impaired basal synaptic transmission, LTP and synaptic plasticity. Interestingly there was no oxidative stress or acetylcholine metabolic alteration in this animal model at the early stages of Aβ infusion supports our hypothesis that Aβ impairs cognitive process in the early stage of AD via altering glutamatergic system prior to neurodegeneration. Our data further support characterizing this animal model and helps us to identify potential molecular targets for developing novel therapeutic options. Moreover, it may help us to elucidate the molecular link between AD, aging and diabetes induced cognitive dysfunction.

INTRODUCTION

AD is a progressive neurodegenerative disorder and the most common form of dementia (Hendrie 1997; Lobo et al., 2000; Koo et al., 1999; Woo et al., 1998). It is characterized by accumulation of Aβ plaques and Tau tangles (Terry, 1963; Braak and Braak, 1997) in the brain and associated with aging. It is common in elderly population and about 4.5 million AD patients in US and yearly 4.6 million new cases added worldwide (Yaari and Corey-bloom, 2007; Smith, 2006). Rapid advancement of basic science and technology in medicine results in increased aged population and prevalence of AD increased dramatically. Therefore, interms of economy and public health concern AD is an important issue in the elderly population and needs to be addressed intensively. Therefore, understanding the molecular mechanisms of AD pathophysiology, and identifying and validating potential molecular targets of AD will be a great breakthrough in the development of AD drug therapy. Since AD is a complex disorder associated with multiple factors and its molecular mechanism is not well established yet, symptomatic

treatment is the only effective therapeutic option currently available. Many groups developed transgenic mice that over express human APP and Aβ. Most currently studied models show cognitive deficits and age-related disruption of synaptic markers and amyloid plaque deposition, but few strains show evidence of significant cell death (Janus et al. 2000; Ashe 2001; Chapman et al. 2001; Richardson & Burns 2002).The neurophysiological consequences of such mutations have been examined in the hippocampus of these mutant mice. Several other related transgenic models have examined the effects of modifying APP and PS expression on synaptic transmission and plasticity and therefore may help to elucidate their normal and/or pathological roles.

Oxidative stress and inflammation induced in the brain by Aβ, which have been postulated to play an important role in the pathogenesis of AD (Behl, 1999; McGeer and McGeer, 1999). Oxidative stress is a cause of neurodegeneration during aging (Leutner et al., 2001; Floyd and Hensley, 2002), and cause of impaired synaptic transmission and synaptic remodeling thus leading to cognitive decline. Indeed, the intracerebroventricular (i.c.v.) administration of Aβ (25-35) peptide into rodent brain induced histological and biochemical changes, cognitive deficits, and oxidative stress (Stepanichev et al., 1998) within 1 or 2 weeks of administration. Interms of the pathology of AD, many active substances produced by Aβ peptides stimulate inflammatory responses (Griffin et al., 1998).

Continuous infusion of Aβ (1-40) into the cerebral ventricle in rats results in learning and memory deficits that were accompanied by a reduction of cholineacetyl transferase activity, suggesting that accumulation of Aβ is related to cognitive impairments in AD (Nabeshima et al., 1998; Nitta et al., 1994, 1998). In rats treated with Aβ (1–40), dysfunction of cholinergic and dopaminergic neuronal systems are observed, as evidenced by the decrease in the nicotine and KCl-induced stimulation of acetylcholine and dopamine release *in vivo* (Itoh et al., 1996).

Furthermore, long-term potentiation is impaired in the CA1 field of the hippocampal slices prepared from the rat brain after continuous i.c.v. infusion of Aβ (1-40) (Itoh et al., 1999). Continuous infusion of Aβ (1-40) into the cerebral ventricle induced a time-dependent expression of inducible nitric oxide synthase and an overproduction of nitric oxide in the hippocampus although Aβ (42-1) had no effect (Tran et al., 2001). Aβ form senile plaque leading to cognitive dysfunction by impairing the signal cascade of the phosphatidylinositol-3-OH kinase (PI-3K) pathway (Kubo et al., 2002). The Aβ-induced overproduction of nitric oxide, which reacts rapidly with superoxide radical to yield highly reactive peroxynitrite, caused an increase in tyrosine nitration of a synaptic protein synaptophysin in the hippocampus (Tran et al., 2003). It has been demonstrated that the prolonged infusion of Aβ (1-42) results in a significant reduction of the immunoreactivity of antioxidant enzymes in the rat brain areas, although the same treatment with Aβ (1-40) had little effect (Kim et al., 2003). Evidence suggests that oxidative stresses are involved in the mechanism of Aβ-induced neurotoxicity (Butterfield et al., 1994; Butterfield and Stadtman, 1997; Butterfield and Lauderback, 2002), and AD pathogenesis (Markesbery, 1997; Yankner, 1996). Exposure to Aβ increases lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide in cultured cells (Behl et al., 1994). Similarly, increases in lipid peroxidation, protein carbonyl and oxidation of mitochondrial DNA have been observed in the brains of AD patients (Lyras et al., 1997).Yamada et al. (1999) demonstrated that treatment with antioxidants, such as idebenone and α-tocopherol prevents the learning and memory deficits induced by Aβ(1-42). However, they did not find increased lipid peroxidation in the brains of the Aβ (1-42) infused rats.

Oligomeric Aβ is potent neurotoxic compound leads to cognitive deficit by interrupting synaptic transmission and synaptic plasticity in various assembly forms (Cleary et al., 2005;

Townsend et al., 2006). It is considered as the major etiological agent for AD pathology. Several *in vivo* animal models, organotypic hippocampal tissue culture and *in vitro* neuronal cell culture studies showed evidence for abeta induced cognitive impairment due to synaptic dysfunction and synaptic loss. Alteration in APP processing by γ secretase leads to 38% of synaptic loss in AD brains (Yao et al., 2003). Earliest manifestation of AD pathology showed deficit in hippocampal Shaffer-collateral synapses, precedes accumulation of abeta plaques and neuronal atrophy (Larson et al., 1999; Yoshiyama, 2007). Most of the present studies showed that excitatory synaptic transmission and plasticity in the hippocampus are compromised in AD pathology and cholinergic and glutamatergic excitatory neurotransmitter systems are affected thus leads to synaptic dysfunction and neurodegeneration. Eventhough effect of Aβ (1-40) mediated synaptic deficit is well studied it is not clear how Aβ (1-42) affects cognitive impairment by altering glutamatergic synaptic transmission in the hippocampus in the early stage of AD before starting severe neurodegeneration.

Therefore, we hypothesized that exogenous icv administration of Aβ (1-42) species on rat hippocampus impairs cognitive function via altering glutamatergic synaptic transmission and synaptic plasticity before starting severe neurodegeneration resembles early stage of AD associated dementia. To test this hypothesis we administered exogenous Aβ (1-42) species to rat brain ventricles via intra-cerebroventricular stereotaxic injection (icv). We studied hippocampal spatial memory and recognition memory by Y maze test and novel object recognition test. Glutamatergic synaptic transmission and synaptic plasticity were studied by extracellular field recording of basal synaptic transmission and LTP. Moreover, we studied oxidative stress and cholinergic acetylcholine production in the hippocampus by biochemical enzyme activity assays and we evaluated glutamatergic synaptic receptors AMPAR and NMDAR subunit expression

and other neurotrophic factors and kinases in the hippocampus by employing various biochemical and molecular techniques.

MATERIALS AND METHODS

Chemicals

BCA protein assay reagent kit and Coomassie plus protein assay reagent kits were purchased from Pierce Biotechnology Inc. (Rockford, IL). All other chemical were purchased from Sigma chemicals, St. Louis, MO, USA, otherwise stated.

Animals

Two months old male Sprague Dawly rats (Charles River, Wilmington, MA) weighing 200–225g were used for behavioral study, electrophysiological experiments and all other biochemical assays and western immunoblotting. They were housed in the animal facility of Auburn University and given food pellet and water *ad libitum* and maintained in the 12 hours dark-light cycles.

Stereotaxic Surgery

Aβ (1-42, AnaSpec, CA) was infused intracerebroventricularly (icv), 4 nmol, to Sprague Dawley rats. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding IACUC committee at the Auburn University.

Behavioral Experiments

Rats were randomly divided into two experimental groups (10 rats per group) as follows: Control (infused with vehicle); Aβ (infused with Aβ); Changes in behavioral memory were tested by employing Object Recognition Task (ORT) and two trial Y Maze task. Rats were intensively handled (5 d by ~3 min per rat) and weighed once a week before behavioral testing.

Object Recognition Task (ORT)

The apparatus consisted of a Plexiglas open-field box (52×52×40cm). Half of the 40cm high wall was covered with blue paper and the other half was left transparent. Two transparent glass bottles (diameter 2.5cm, height 6.5cm) filled with sand were placed symmetrically about 5cm away from the blue wall. During the second trial, a gray color plastic cube (2.5×2.5×6cm) was placed for one of the glass bottle. The objects could not be displaced by a rat. A testing session comprised of two trials (Rutten et al., 2005, 2008). The duration was 3 minutes and 5 minutes for first (exploration) and the second (retention test) trial respectively. Inter trial interval used in this experiment was 24 hours. Rat was put back in the apparatus for the second trial and one of two identical objects was replaced by a novel object. Before each trial, the surface of the box was sprayed with a diluted ethyl alcohol solution to erase any scent cues. Behavior was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. Times spent exploring each object during T1 and T2 was recorded manually using a personal computer and a timer. In order to avoid the presence of olfactory cues the objects was always thoroughly cleaned using diluted ethyl alcohol before each trial. Discrimination index was calculated for each animal using the time spent exploring each object.

Two Trial Y Maze Test

Two trial Y Maze was employed to analyze spatial recognition memory using the previously described procedure (Ma et al., 2007). The Y-maze test consisted of two trials separated by a time interval. In the first (acquisition) trial, one arm of the maze (subsequently called novel arm) was closed with a guillotine door. Rat was placed at the distal end of one arm (starting arm), head pointing away from the center of the maze, and was allowed to visit the two accessible arms of the maze for 3 minutes. During the second (Retention test) trial rat has free access to the three arms and was allowed to explore the maze for 5 minutes. Behavior of rat in maze was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. This test is based on the interest of rat for novelty; hence, they explore preferentially unknown territories. The number of explorations of each arm was counted every min and the percentages of visits in the novel arm with respect to the total number of visits in the three arms during each min of the test was calculated.

Electrophysiological Recordings

Slice Preparation

The control rats and Aβ infused rats were analyzed in extra cellular field electrophysiology to determine the mechanism behind the cognitive behavioral changes. At the end of 10 days, in vitro brain slice experiments were performed following the methods described previously (Bukalo et al., 2004). Rats were deeply anaesthetized and decapitated, the brain was quickly extracted and immediately placed into ice-cold cutting solution (in mM): 250 sucrose, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) that was oxygenated with

95% O_2 and 5% CO_2. The brain was sliced (350-400 μm transverse slices) using a Vibratome (Warner instruments, Hamden, CT) and incubated in oxygenated artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1.5 $MgCl_2$) at room temperature for 1 hour.

Extra-Cellular Field Recordings of fEPSPs

Brain slices were transferred to submerged type recording chamber and continuously perfused (2ml/min) with oxygenated ACSF. Evoked EPSPs were elicited by stimulating the hippocampal Schaeffer-collateral fibers with a bipolar Teflon-coated platinum electrode placed in the CA3 sub field. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum using glass microelectrodes (2–4 MΩ) filled with ACSF placed ~300μm apart from recording electrode. Test pulses were set at an intensity that evoked 50% of maximum EPSP amplitude before intrusion of a population spike. Field excitatory postsynaptic potentials (fEPSPs) in CA1-CA3 synapses were recorded on a computerized stimulating and recording unit. Response to different stimulation intensities was recorded; the slope of fEPSPs and the amplitude of fiber volley were analyzed. In order to study the changes in presynaptic function, paired pulse facilitation was analyzed. Ratio of the slope of first response and the subsequent response against different inter-stimulus intervals were analyzed. Basal synaptic transmission was monitored for 15 minutes before inducing long-term potentiation.

Analysis of Synaptic Transmission and Synaptic Plasticity

Long-term potentiation (LTP) was induced by a theeta burst (TBS) protocol consisting of five TBS delivered at 20 seconds interval. TBS consisted 10 bursts delivered at 5 Hz. Each burst

consisted four pulses delivered at 100Hz. Data were acquired and fEPSPs were measured using LTP program software and analyzed offline. Post-tetanic potentiation (PTP) and LTP were defined as the percentage change from baseline for the 7 minutes immediately after TBS and for the last 5 minutes of the 60 minutes post-TBS recording period, respectively.

Biochemical Analysis

The effect of sildenafil and abeta infusion on oxidative stress and mitochondrial function as seen in superoxide dismutase activity, glutathione, and lipid peroxides was analyzed. Glutathione (GSH) content was estimated by employing OPT-condensation reaction with the tripeptide to yield a fluorescent product. Readings were taken at the activation/emission wavelengths of 337/423 nm spectrofluorimeter. Catalase activity (CAT) was assayed by rate of decrease in absorbance of H_2O_2 and measured by spectrophotometrically at 240nm. Acetylcholine esterase (AChE) activity was measured spectrophotometrically at 412nm. Choline acetyl transferase (ChAT) activity was measured spectrophotometrically by reading the absorbance of acetylcholine yield at 324nm. Superoxide dismutase (SOD) activity was measured spectrophotometrically using pyrogallol as substrate at 420nm. Protein was assayed using the Coomassie plus protein assay reagent kit. Bovine serum albumin was used as standard.

Western Immunoblot

Whole hippocampal homogenates were lysed in a chilled buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4, 1% NP- 40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). The lysates were incubated for 15 minutes on ice and centrifuged for

15 minutes at 10,000 x g, at 4°C. The supernatants containing the protein extract were collected, and their protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Duplicate samples for individual rats were subjected to 10% SDS-PAGE and subsequently blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were saturated with 5% non-fat dry milk (Biorad) or 1% BSA and then incubated with anti-Akt (1:1000; Cell Signaling Technology), anti-phosphoAkt (1:1000; Cell Signaling Technology), anti-ILK (1:1000; Cell Signaling Technology), anti-GluR1(1:1000; Millipore), anti-phospho GluR1 (1:1000; Millipore), anti-NR1 (1:1000; Millipore), anti- NR2A (1:2000; Santa Cruz), anti-NR2B (1:500; Santa Cruz), anti-BDNF (1:500; Millipore), anti-stargazin (1:1000; Cell Signaling Technology), anti-synaptophysin, anti- PSD95 (1:1000; Cell Signaling Technology) and anti-GAPDH (1:5000;Millipore) antibodies overnight at 4°C. The membranes were subsequently incubated at room temperature for 1 hour with the corresponding anti-rabbit or anti-mouse antibodies (1:5000 and 1:10,000 respectively; Millipore). The membranes were then incubated in Immobilon Western HRP Substrate (Millipore) for 5 minutes and imaged using the Molecular Imager ChemiDoc XRS system (Bio-Rad). Signals were subsequently quantified by densitometric analyses using Quantity One Analysis software (Bio-Rad). The densities of each band, which represented individual animals, were normalized to GAPDH and these normalized values were averaged for control and abeta groups. The data is presented as mean ±SEM. Significance was determined using a two-tailed unpaired Student's t-test.

Statistical Analysis

Experiments were performed with the investigator blinded to the treatment the animals received. Data were expressed as mean ± SEM. Electrophysiological results were normalized to

the basal values and analyzed by ANOVA with Tukey's post-hoc analysis. The level of significance was set for $P<0.05$. All statistical calculations were performed on a computer using a commercially available statistical package.

RESULTS

Behavioral Tasks (Object recognition and Spatial Memory)

We studied hippocampal spatial memory and recognition memory by employing Y maze test and object recognition test (ORT) on abeta infused Sprague dawley rats and control groups (n=10). We found that infusion of Aβ significantly impaired object recognition memory. Post-hoc analysis of object discrimination index (DI) showed significant reduction of DI in the Aβ infused rodent group compared to their control counter part (Fig.4.1.A, 34%, $p<0.05$). Moreover we found that infusion of Aβ significantly impaired spatial memory in the Y maze task ($p<0.05$, 81.12%, Fig.4.1.B) Novel arm exploration frequency and time spent was analyzed after 4 hour time interval after training session. Impairment of hippocampal object recognition memory and spatial memory in the abeta infused rodent model resembles cognitive deficit and neuronal dysfunction in AD pathology and this animal model can be considered as a potential candidate for studying the mechanisms of AD pathology.

Electrophysiological Analysis – Extracellular Field Recording

We investigated basal synaptic transmission in this abeta infused rat model by measuring presynaptic fiber volley (FV) amplitude and field excitatory postsynaptic potential (fEPSP) from CA1 region of the hippocampal Schaffer-collateral neuronal projections while stimulating CA3 region. FV amplitude was significantly decreased (Fig.4.3.A) by infusion of Aβ compared to

control counterpart. FV amplitude represents a measure of recruited number of presynaptic neurons. Therefore, Aβ infusion impairs ability to transfer the presynaptic stimulus into an axonal depolarization. Slope of fEPSP was measured by input output curve gradient and it was significantly decreased by abeta infusion (Fig.4.3.B). Therefore, decreased FV amplitude and fEPSP gradient showed possible post basal synaptic functional impairment in Aβ infused rat model (Fig.4.3.A & B). Paired pulse facilitation (PPF) is a measure of presynaptic dependent short-term plasticity and correlates with probability of glutamate neurotransmitter release (Chan et al., 2006). There was no significant change in PPF (Fig.4.3.C) in the abeta infused rats compared to control group.

We studied the effect of Aβ infusion on synaptic plasticity, by measuring the magnitude of post-tetanic potentiation (PTP) and long-term potentiation (LTP). LTP was induced by theta burst tetanization (5 trains with 20s inter-train interval). Basal synaptic transmission and PTP (short term plasticity) were significantly reduced (Fig.4.2.A & B) by abeta infusion (Control 172.74%, Aβ infused 145.41%, n=5, 6 slice recordings, **$p<0.01$). Moreover LTP was significantly impaired (Fig.4.2.A & C) by infusion of Aβ (Control 147.12%, Abeta infused 102.67%, n=5, 6 slice recordings, **$p<0.01$).

Western Immunoblotting

To investigate the effect of Aβ intracranial infusion in the rodent brain and characterize this AD animal model we measured postsynaptic glutamate receptor subunit expression and synaptic auxiliary protein expression levels in the abeta infused hippacampal homogenate and control counterpart. Neurotrophic factor BDNF and kinase ILK were significantly decreased in the Abeta infused rodent hippocampus compared to control group. (Fig.4.4, BDNF 78.56%, ILK

70.67%, n=5, p<0.05).There was no significant change in total protein expression of protein kinase AKT in abeta infused rodent hippocampus compared to control group. Phosphorylation of AKT (AKT kinase activity) decreased significantly in abeta infused rodent hippocampus compared to control group. (Fig.4.6, pAKT 77.45%, pAKT/AKT 70.67%, n=5, p<0.05)

Postsynaptic auxiliary proteins PSD-95, Stargazin and presynaptic vesicular protein synaptophysin were decreased significantly in abeta infused rat hippocampus compared to control group. (Fig.4.5, PSD-95 78.45%, Stargazin 82.77%, Synaptophysin 74.1%, n=5, p<0.01). Glutamate receptor AMPAR subunit GluR1 total protein expression and phosphorylation of GluR1 decreased significantly in abeta infused rat hippocampus compared to control group. (Fig.4.7, GluR1 83.67%, pGluR1 64.11%, pGluR1/GluR1 73.48%, n=5, p<0.05). NMDA receptor sub units NR1 and NR2B decreased significantly and there was no significant change in NR2A expression in the abeta infused rat hippocampus homogenate compared to same age control counterpart. (Fig.4.8, n=5, p<0.05).

Biochemical Assays – Oxidative Stress

We studied earlier the effect of Aβ infusion on oxidative stress and mitochondrial function using known standard markers such as Glutathione content, catalase activity, superoxide dismutase and glutathione peroxidase (Table 4.1). They were not significantly changed by Aβ infusion. It has been shown that antioxidants treatment (idebenone and α-tocopherol) rescued learning and memory deficits induced by Aβ (1-42) and interestingly they did not find increased lipid peroxidation in the brains of the Aβ (1-42) infused rats (Yamada et al., 1999).

We investigated the activity of the enzymes in the cholinergic system cholineacetyl transferase and acetylcholine esterase and we found that there was no significant difference among treatment groups of abeta and their control counter part in the activities of cholineacetyl

transferase and acetylcholine esterase (Table 4.1). Study of these enzyme activities reveal the effect of abeta on cholinergic neurotransmission system and act as early cause of markers of AD pathology.

We found that Aβ (1-42) infusion alters pre synaptic neuronal recruitment and synaptic plasticity (LTP) and decreases post synaptic glutamate receptors and their auxiliary proteins. There is no inflammatory response or oxidative stress in the hippocampus in our investigation period after abeta infusion intracranially. This date indicated that synaptic plasticity and cognitive function impaired by exogenous Aβ (1-42) intracranial infusion before starting oxidative stress induced neurodegeneration. This finding supports direct effect of Aβ on postsynaptic glutamatergic alterations thus lead to impaired synaptic transmission and cognitive dysfunction in this animal model. Our present results and previous findings strongly support to validate and characterize this Aβ infused rodent model to represent AD pathology of neuronal impairment and cognitive deficit.

FIGURES AND FIGURE LEGENDS

Fig.4.1

Fig.4.1.A

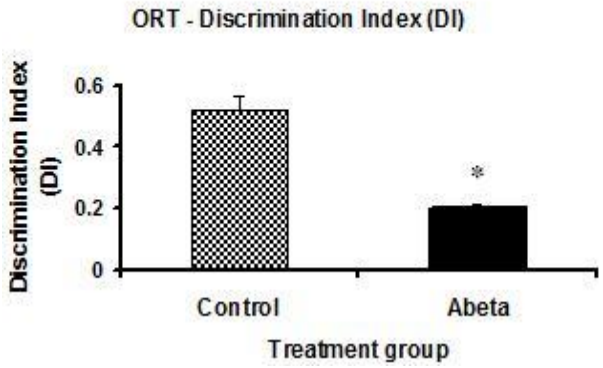

Fig.4.1.B

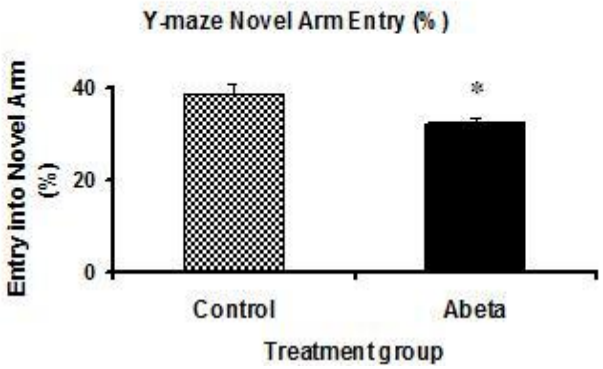

Fig.4.1: Effect of exogenous icv- Aβ infusion in Hippacampal Spatial memory and Object recognition memory: Aβ icv infusion in rats significantly reduces hippacampal object recognition memory (A) tasks in ORT test (decreased novel object discrimination index DI) and reduced hippacampal spatial memory (B) tasks in Y maze test (decreased novel arm entry frequency) compared to control group. N=10, Two tailed student's t-test unpaired, * $p<0.05$, Values are Mean ± SEM

Fig.4.2

Fig.4.2.A

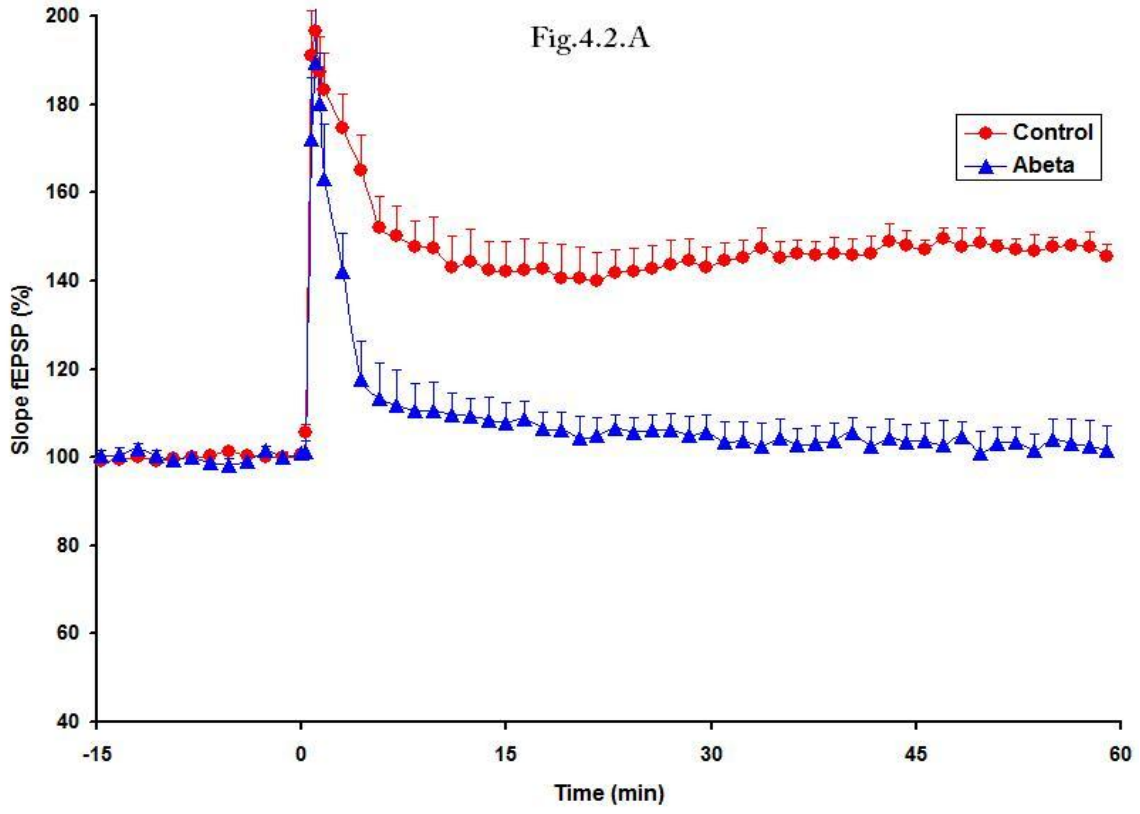

Fig.4.2.B

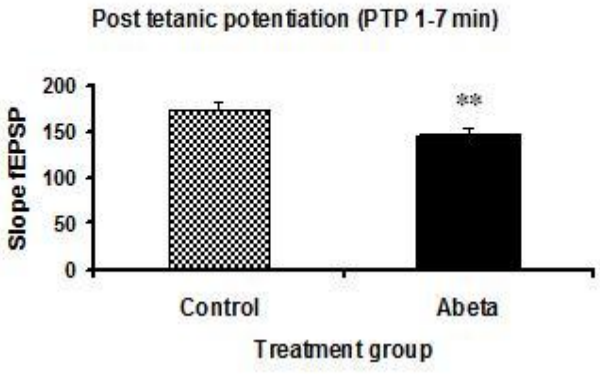

Fig.4.2.C

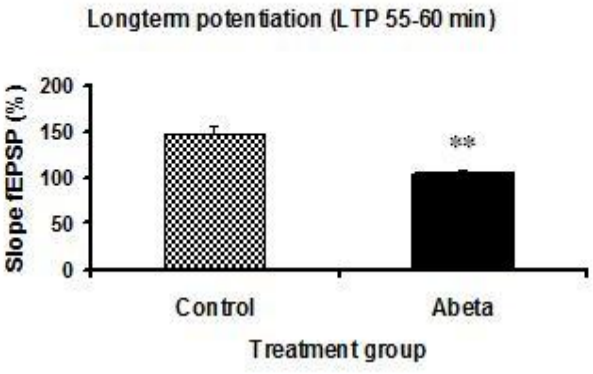

Fig.4.2: Effect of icv- Aβ in the slope of fEPSP percentage of baseline for PTP and LTP: icv Aβ infusion significantly reduces Long-term potentiation LTP (55-60 minutes after high frequency stimulation) (A&C) and PTP (A&B) (1-7 minutes after high frequency stimulation) in the hippacampal CA1-CA3 region of Shaffer-collateral pyramidal neurons in the rat brain slices compared to control slices. n=5, 6 slices, Two tailed student's t-test unpaired ** $p<0.01$, Values are Mean ± SEM.

Fig.4.3

Fig.4.3.A

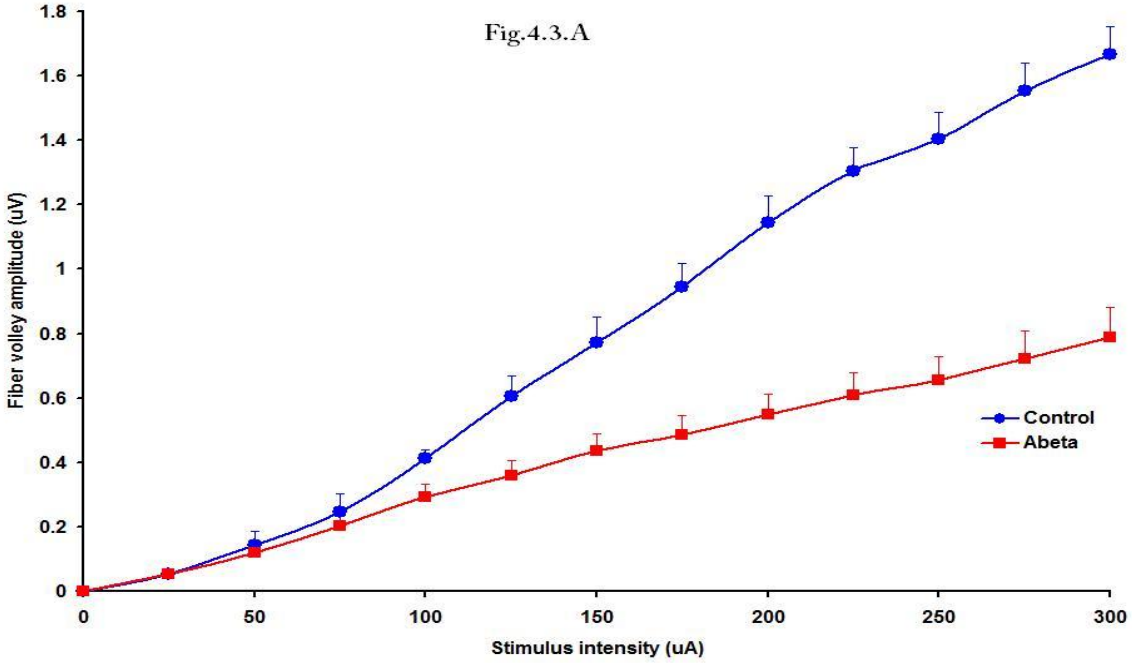

Fig.4.3.B

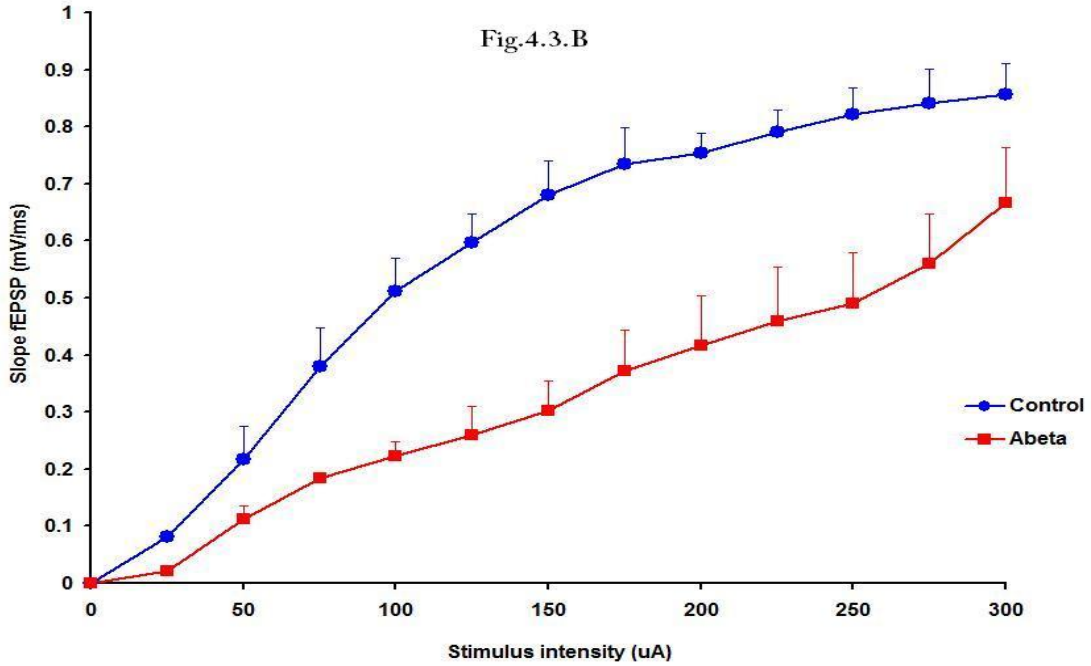

Fig.4.3.C

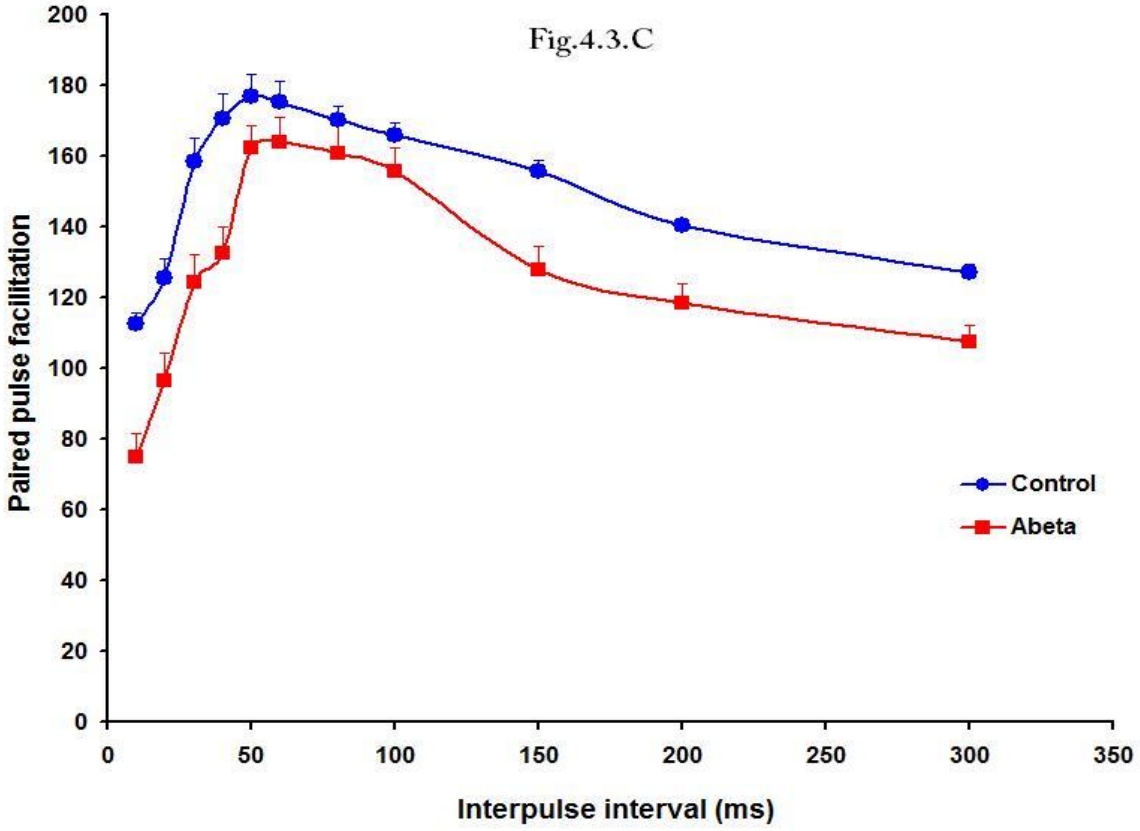

Fig.4.3: Effect of icv- Aβ infusion in basal synaptic transmission in the hippocampus: Fiber volley Amplitude (A) and slope of field excitatory post synaptic potentiation (fEPSP) (B) significantly decreased in abeta treated rats compared to control group. Paired pulse facilitation (PPF) (C) was not altered in Aβ treated rodents compared to control. n= 5, 6 slices, Two tailed student t-test unpaired ** $p<0.01$, Values are Mean ± SEM.

Fig.4.4

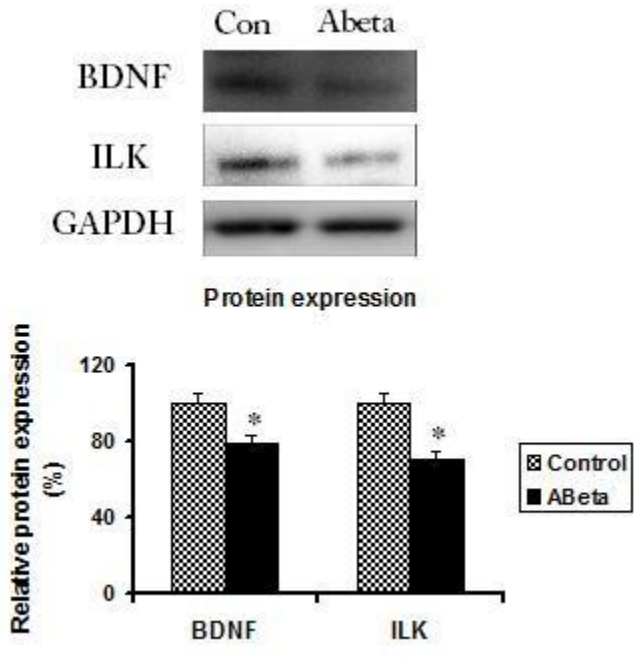

Fig.4.4: Effect of Aβ on protein expression of BDNF and ILK: Intracerebroventricular (icv) Aβ significantly decreased protein expression of BDNF and ILK in the hippocampus of the rats compared to their control group. n= 5, Values are Mean ±SEM, Two tailed student's t-test unpaired, * $p < 0.05$. Band density values are normalized to house keeping/loading control GAPDH.

Fig.4.5

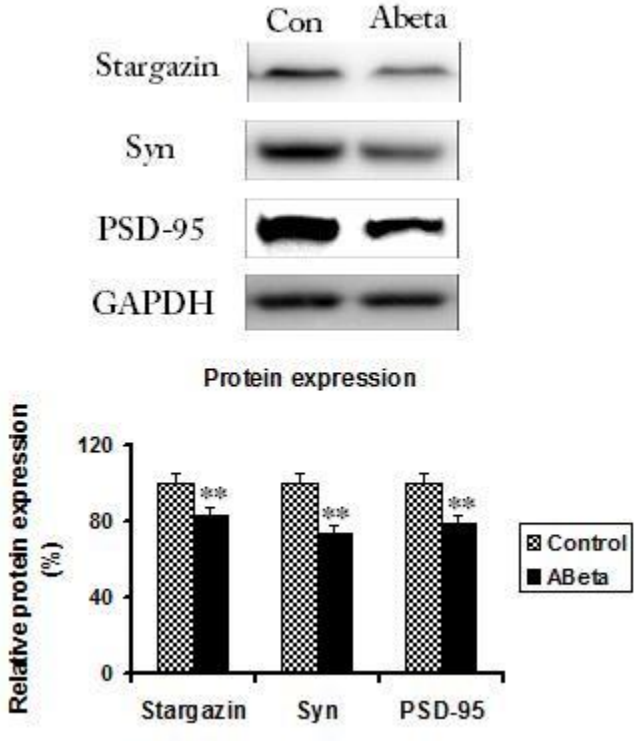

Fig.4.5: Change in protein expression of Stargazin, Synaptophysin (Syn) and PSD-95 by Aβ infusion: Post-synaptic Stargazin, pre-synaptic Synaptophysin and post-synaptic PSD-95 significantly decreased in icv Aβ infused rat hippocampus compared to control group (n=5). Values are Mean ±SEM. Two tailed student's t-test unpaired, ** $p < 0.01$. Band density values are normalized to house keeping/loading control GAPDH.

Fig.4.6

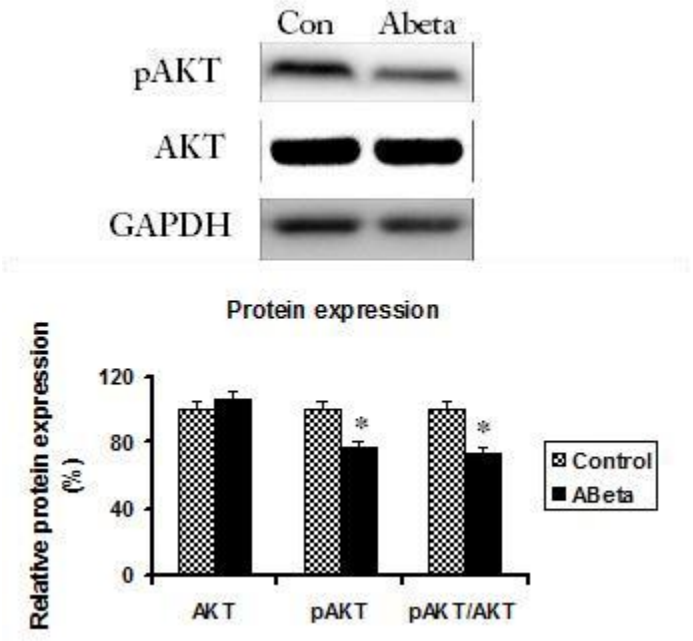

Fig.4.6: Effect of Aβ on AKT activity in the hippocampus: Total Protein expression of AKT doesn't change significantly in icv Aβ infused rat hippocampus compared to control group (n=5). AKT phosphorylation decreased significantly in icv Aβ infused rat hippocampus compared to their control group (n=5). Values are ±SEM. Two tailed student's t-test unpaired, * $p < 0.05$. Band density values are normalized to housekeeping/loading control GAPDH.

Fig.4.7

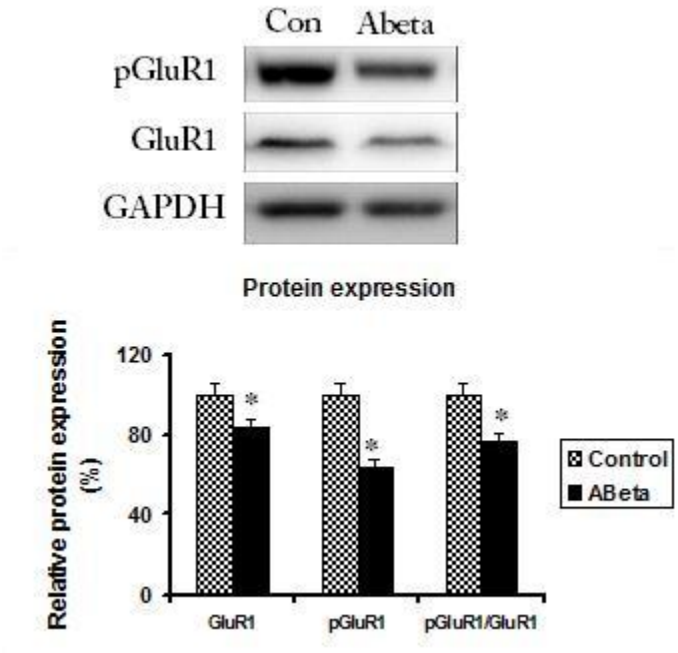

Fig.4.7: Change of AMPAR sub unit GluR1 protein expression and its phosphorylation by icv- Aβ infusion: Total Protein expression of Glutamate receptor AMPAR subunit GluR1and its phosphorylation significantly decreased in icv Aβ infused rat hippocampus compared to control group (n=5). Values are Mean ±SEM. Two tailed student t-test unpaired,* $p < 0.05$. Band density values are normalized to house keeping/loading control GAPDH.

Fig.4.8

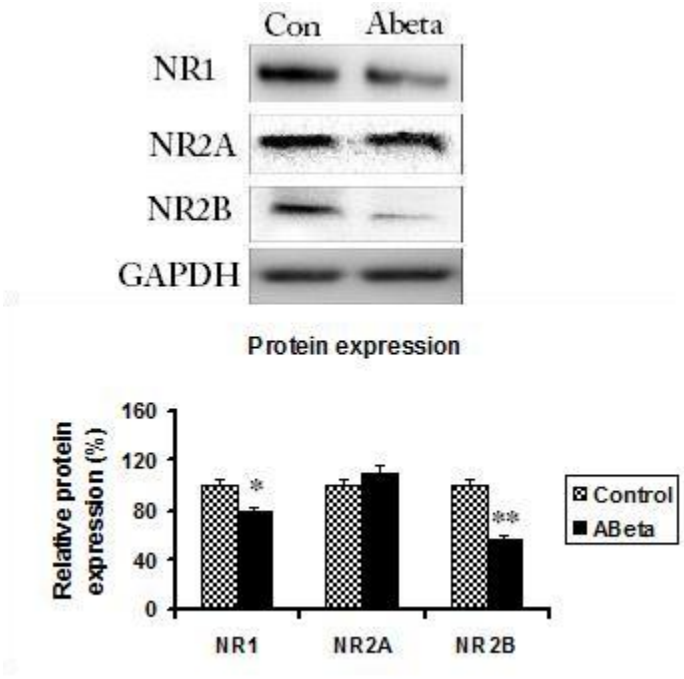

Fig.4.8: Change of Protein expression in NMDAR sub units NR1 and NR2B by icv-Aβ administration: NMDAR sub units NR1 and NR2B protein expression decreased significantly and there is no significant difference in NR2A protein expression in the icv ABeta infused rat hippocampus compared to control group (n=5). Values are Mean ±SEM. Two tailed student t- test unpaired, * $p < 0.05$, ** $p<0.01$. Band density values are normalized to house keeping/loading control GAPDH.

TABLES

Table 4.1: Change of inflammatory and cholinergic enzymatic activity in icv- Aβ infused rodent hippocampus.

Biochemical Assay - Enzymatic activity	Abeta infused Rat hippocampus compared to control (n=5)
Super oxide mutase (SOD)	No significant change
Catalase	No significant change
Glutathione content (GSH)	No significant change
GSH peroxidase (GSH-px)	No significant change
Acetylcholine esterase (AChE)	No significant change
Choline acetyl transferase (ChAT)	No significant change

Table 4.1: Effect of icv- abeta infusion in inflammatory and cholinergic enzymatic Activity in the hippocampus: There was no change in enzymatic activity of cholinergic enzymes and inflammatory enzymes. N=5, Values are Mean ± SEM.

DISCUSSION

Aβ pathology is one of the major hallmarks of AD. Molecular mechanism by which abeta amyloid impairs synaptic transmission and synaptic plasticity is not well elucidated. Cognitive deficit occurs in the early stage of AD before starting severe neurodegeneration that may be due to alterations in the glutamatergic synaptic transmission. Glutamatergic system is a fast excitatory synaptic transmission system in the mammalian brain and plays a crucial role in synaptic plasticity and long-term potentiation. Growing evidences suggested that beta amyloid directly alters glutamatergic synaptic transmission and impairs cognitive process and synaptic plasticity in the AD pathology. Mechanistic basis of this synaptic remodeling by direct effect of Aβ is not well established. Therefore, we studied exogenous Aβ (1-42) infused (icv) Rats for their cognitive function, basal synaptic transmission and synaptic plasticity, alteration in the postsynaptic glutamatergic system and oxidative stress in the hippocampus.

We found that hippacampal object recognition memory and spatial memory were impaired by Aβ (1-42) intracranial infusion in the brain. Several studies found the behavioral deficits related to memory impairment after intracerebral injections of amyloid peptides (Delobette et al., 1997; O'Hare et al., 1999; Harkany et al., 2001; Malin et al., 2001; Nakamura et al., 2001; Ammassari-Teule et al., 2002; Christensen et al., 2008). Behavioral effects depend on sequence of the peptide. Therefore, injection of amyloid fragments with scrambled or inverse sequences do not make any changes in the cognitive process (Ammassari-Teule et al., 2002; Malin et al., 2001). Therefore, potential neuro toxicity varies among different isoforms of Aβ species. Aβ (1-42) isoform showed most potential toxicity. Therefore, effect of toxicity and synaptic deficit depend on the different Aβ fragment composition, ratio and level.

Interestingly there were no significant changes in the oxidative stress and inflammatory markers such as SOD, catalase, GSH-px activity and GSH content after abeta infusion in our early investigation period. Therefore, in this model cognitive dysfunction occurs before inflammatory responses and oxidative stress start. Cholinergic neurotransmitter metabolic enzymes AChE and ChAT activity were not altered in this model. Therefore, there may be no alterations in the acetylcholine synthesis or degradation in the hippocampus at the beginning of Aβ infusion.

Moreover, electrophysiological experiments of extracellular field recording of hippacampal CA1-CA3 schaffer collateral pyramidal neurons showed significant changes in basal synaptic transmission and synaptic plasticity. FV amplitude was significantly impaired by infusion of Aβ leads to reduction in recruitment number of presynaptic neurons to functional synapses. Therefore, Aβ infusion impairs ability to transfer the presynaptic stimulus into an axonal depolarization. Paired pulse facilitation (PPF) was not altered by Aβ infusion suggested

that presynaptic dependent short term plasticity is intact and correlate with probability of glutamate neurotransmitter release (Chan et al., 2006). Therefore, glutamate release in the presynaptic terminal is not altered in this model. Reduction in fEPSP slope and FV amplitude showed possible post basal synaptic functional impairment in Aβ infused rat model.

We investigated the effect of Aβ infusion on synaptic plasticity, by analyzing the magnitude of post tetanic potentiation (PTP) and long-term potentiation (LTP). Basal synaptic transmission was impaired by Aβ infusion. Moreover, LTP (long-term plasticity) and PTP (short-term plasticity) were significantly impaired. It is interesting that glutamate can potentiate the inhibitory effect of Aβ 1–42 on LTP (Nakagami & Oda 2002). Decreased longterm potentiation (LTP) in this animal model supports longterm deficit in synaptic plasticity and altered hippacampal spatial and recognition memory. Interestingly there was a significant alteration in the post-tetanic potentiation and short-term plasticity in this animal model.

Aβ 1–42 has recently been reported to reduce NMDA receptor-mediated synaptic currents in the dentate gyrus (Chen et al. 2002) and Aβ 1–42 strongly suppressed a NMDA-evoked/depolarization in cultured cortical neurons, Both effects were blocked by the NMDA receptor antagonist, indicating their NMDA receptor dependence (Kim et al. 2001). Therefore, reduced expression levels of glutamate receptor NMDAR subunits NR1 and NR2B in our studies correlates with previous cell culture studies and transgenic AD Mouse model. In addition, we found that reduced AMPAR subunit GluR1 and reduction in GluR1 phosphorylation in this model support our finding of postsynaptic impairment in the synaptic transmission. Dephosphorylation of GluR1 internalizes AMPA receptors from postsynaptic sites and reduces number of AMPARs in the postsynaptic surface. Therefore, decreased GluR1 expression and its

reduced phosphorylation may decrease the number of AMPAR receptors in functional postsynaptic sites and thus leads to impaired synaptic transmission and cognitive dysfunction.

Postsynaptic density PSD-95 also reduced in expression suggested that impaired postsynaptic recruitment of NMDAR and AMPAR receptors from trans-golgi. Moreover, reduction in AMPAR anchoring auxiliary protein stargazin and decreased phosphorylation of GluR1 support reduced AMPAR incorporation to the functional postsynaptic membrane sites. Reduced expression of transmembrane regulatory protein stargazin not only impairs trafficking of AMPARs to the post synaptic sites but also reduces AMPARs ion channel kinetics such as channel open probability and channel open and close mean times and the conductance of the ion channels (Priel et al., 2005; Bats et al., 2007). Presynaptic vesicular protein marker synaptophysin also decreased in expression in this model. Interestingly paired pulse facilitation was not altered by Aβ. Therefore, further investigation needed to derive a conclusion about the alteration of presynaptic glutamate neurotransmitter release. Further studies including measuring glutamate level, calcium homeostasis, and alteration in presynaptic metabotrophic glutamate receptors needed to support our finding about abeta effect on presynaptic physiology in the synaptic transmission.

Reduction in the BDNF level in the hippocampus supports our finding of postsynaptic deficit. Reduced phosphorylation of AKT down regulates BDNF expression and increases the activity of GSK3β by phosphorylation. GSK-3β activation reduces BDNF level. BDNF directly involved with enhancing LTP and synaptic plasticity (Kang et al., 1997). Therefore, GSKβ activation decreased BDNF expression leads to impaired LTP. GSK3β activity also modulates BDNF expression (Mai et al., 2002; Bachmann et al., 2005). BDNF phosphorylates AMPAR subunit GluR1 and enhances its trafficking to the postsynaptic sites. We found reduced

phosphorylation of GluR1 and reduced expression of GluR1 subunits in this model. Therefore, reduced BDNF expression downregulate GluR1 expression and its phosphorylation and inhibits GluR1 –AMPAR incorporation to the postsynaptic sites. Recent studies found that GSK-3beta co precipitated with GluR1 and reduced GSK3β activity enhances GluR1 insertion to the postsynaptic surface during LTP induction. Therefore, it is clear that increased GSK3β activity after Aβ infusion reduces surface expression of GluR1 and reduces the number of AMPAR receptors to functional postsynaptic sites and impairs LTP and synaptic plasticity. GluR1 regulates calcium permeability and have inward rectifying voltage-current relationship. Influx of calcium and calcium homeostasis play a crucial role in late LTP and synaptic plasticity by activating kinases. Therefore, these findings strongly support deficit in the LTP in this Aβ infused rodent model.

ILK expression also decreased. ILK also phosphorylates GSK3β and decreases its activity. Interestingly ILK increases the expression of BDNF and enhances LTP. Moreover, ILK connected with integrins and regulates actin cytoskeleton dynamics (Blattner and Kretzler, 2005) and regulates spine morphology change during LTP induction. ILK also supports AMPAR trafficking to the postsynaptic sites via actin dynamics and enhances synaptic transmission (Kim and Lisman, 1999). Therefore reduced expression level of ILK and BDNF suppress postsynaptic glutamatergic receptor trafficking, neurogenesis, spine dendritogenesis and synaptogenesis and result in impaired synaptic transmission and synaptic plasticity.

Growing evidence supports the crucial role of insulin signaling in AD (Rivera et al., 2005). Abeta directly interacts with insulin receptor (IR) and induces its internalization. Therefore down regulation of IR reduces AKT phosphorylation and leads to reduced expression

of ILK and increased activity of GSK3β. Therefore, our findings support Aβ associated impairment in synaptic transmission and synaptic plasticity via glutamatergic innervations.

Studies with transgenic AD models such as Amyloid precursor protein (APP) over expression and PS-1 and PS-2 promoter deletion models of Aβ accumulation and Aβ infused *in vitro* neuronal cell culture and organotypic hippocampal tissue culture support Aβ hypothesis in the AD associated cognitive dysfunction. Recent studies from other labs and our findings suggest that synaptic dysfunction and synaptic loss begins at the beginning of the AD before apoptosis and neuronal loss take place. Our collective findings support the specific effect of the Aβ (1–42) peptide in the hippocampus associated memory deficit via altering postsynaptic glutamatergic system before severe neurodegeneration starts and further investigation needed to validate this molecular mechanistic model.

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5. PPARγ ACTIVATION AMELIORATES DIABETES INDUCED COGNITIVE DYSFUNCTION

ABSTRACT

The type 2 diabetes and AD shares common pathological characteristics of cognitive dysfunction. Although hyperglycemia, hyperlipidemia, oxidative stress and inflammation have been shown to be central mediators of type 2 diabetes induced cognitive deficits, the specific molecular mechanism involved in the development of diabetes induced cognitive impairment remains unknown. PPARγ (Peroxisomal Proliferator Activating Receptor gamma) is a member of the nuclear receptor class transcription factor that plays a key role in lipid metabolism and glucose homeostasis. In the body, it is mostly found to be expressed in adipose tissue; however, it is also abundantly expressed in the brain, liver, skeletal muscle and the heart. In the brain, it has been shown to be down regulated in patients and animal models of AD. Recent studies have shown that PPARγ ameliorates some of the pathological conditions associated with AD via its anti-inflammatory and neuroprotective properties. However, the molecular mechanism by which PPARγ activation enhances synaptic plasticity and cognitive function has not been established. Therefore, we hypothesized that diabetes induced synaptic dysfunction is ameliorated by PPARγ activation by modulating glutamatergic system. To test this hypothesis we used 8 weeks old male db/db leptin receptor knockout mice; a model for type 2 diabetes and orally administered them with the PPARγ agonist rosiglitazone for two weeks and investigated hippocampal recognition memory and spatial memory by performing object recognition tasks and Y maze test. Our findings from these cognitive behavioral studies were supported by extra cellular field recordings such as basal synaptic transmission and long-term potentiation (synaptic plasticity). To further investigate the beneficial effects of PPARγ upon the glutamatergic system under diabetogenic

stress (db/db), we studied the expression levels of postsynaptic glutamate receptors AMPAR and NMDAR. In addition, neurotrophic factors, which regulate the expression and trafficking of glutamate receptors, were also investigated to help better explain our findings associated with changes in receptor expression and trafficking. Lastly, because PPARγ activation improves whole body insulin sensitivity, we investigated the central and peripheral effect of PPARγ mediated alterations in insulin signaling pathway in diabetes induced cognitive impairment.

Our results indicate that the pharmacological activation of PPARγ ameliorated diabetes induced hippocampal recognition memory and spatial memory impairment. This may be explained by the PPARγ agonist enhancing the basal synaptic transmission and LTP. Furthermore, we have found in db/db mice that the AMPAR receptor subunit GluR1 and NMDAR subunit NR2A were severely attenuated in expression. However, rosiglitazone ameliorated this effect in the same animals after 2 weeks of oral treatment. To support these findings, we treated H-19 hippocampal neuronal cells with rosiglitazone (10μM) for 48 hours and found an increase in gene expression of NR2A and GluR1. To better understand the mechanism as to how NR2A and GluR1 were increased by rosiglitazone treatment, we found that both the postsynaptic auxiliary protein PSD-95 and neurotrphic factor BDNF increased in expression. PSD95 is known to be a scaffolding protein, which inserts the glutamate receptors to postsynaptic sites and enhances LTP. To determine the molecular signaling mechanism responsible for postsynaptic receptor up regulation by rosiglitazone we measured the mRNA levels of PPARγ targets and found that IRS-2 was down regulated in the hippocampus of db/db mice. Most surprisingly, this was ameliorated by rosiglitazone treatment. It is well known that IRS-2 induces an increase in the activation of AKT and GSK3β, which results in a reduction of Tau protein hyper-phosphorylation. To determine the central effect of rosiglitazone, we

measured the gene expression of direct PPARγ targets in the hippocampus. Interestingly, we found that the insulin-degrading enzyme (IDE), which is transcriptionally regulated by PPARγ, was significantly increased in our diabetic mice after treating with rosiglitazone. Furthermore, other direct PPARγ targets such as CD36, FATP, FABP, and N-Cadherin increased in expression in these same animals hippocampus. These results suggest that rosiglitazone can cross the blood brain barrier and have an effect upon the hippocampus. Consequently, we have found that PPARγ activation in the diabetic hippocampus induces the increase in mediators involved in the insulin-signaling cascade such as the insulin receptor (IR), insulin growth factor-2 (IGF-2) and insulin receptor substrate-2 (IRS-2). To better understand, if the PPARγ had direct effects in the glutamergic receptors, we utilized hippocampal H-19 cell lines, and found that rosiglitazone treatment induced an increase in syanptogenesis, dendritogenesis and axonal branching of neurons. Our findings predict that type 2 diabetes induces cognitive dysfunction via altering hippocampal synaptic transmission by down regulating postsynaptic glutamate receptors and impairing insulin signaling in the hippocampus. Moreover, PPARγ activation ameliorates diabetes induced cognitive dysfunction via improving basal synaptic transmission and synaptic plasticity. This may be due to an increase in postsynaptic glutamate receptor expression and improved insulin singaling in the hippocampus. Consequently, we believe that PPARγ may be a possible potential molecular therapeutic target in the brain for diabetes mediated cognitive dysfunction as well as for AD and aging related cognitive deficits.

INTRODUCTION

Diabetes mellitus is at epidemic proportions today affecting more than 24 million Americans. The prevalence of diabetes increases with age (National diabetes statistics, 2007, Cowie et al., 2003, Wilson et al., 1986) and is expected to reach 9% in 2025. It's a complex metabolic disorder characterized by hyperinsulinemia resulting from impaired insulin secretion or increased insulin resistance to insulin action. Long-term tissue and organ damage as well as systemic dysfunction involving the eyes, kidneys, nervous and vascular systems are the major causes of chronic hyperglycemia and metabolic dysfunctions of diabetes (Diabetes care 2009, American diabetes association 2009). Type 1 diabetes is defined by impaired insulin secretion and type 2 diabetes is defined by insulin resistance and hyperinsulinemia. Type 2 diabetes is the major class of diabetes and it is one of the major causes of cognitive impairment due to its similarities to AD pathology.

Impaired glucose homeostasis, hyperglycemia, hyperinsulinemia, insulin resistance, impaired insulin signaling and hypercholesterolemia are major causes of type 2 diabetes induced cognitive dysfunction. The hippocampus is where memory is encoded and consolidated and is the major region of the brain that is highly vulnerable for AD and type 2 diabetes mediated alterations. Inhibition of cell proliferation, neurogenesis and the induction of apoptosis were observed in the hippocampus of diabetic rodents. Reduced activity of glycolytic enzymes in the diabetic brain causes elevated superoxide levels and accumulation of free radicals leads to oxidative stress. Inflammatory nuclear factor B transcription factors up regulated by advanced glycation end products (AGEs), and S100 protein (causes brain injury) that can bind to RAGEs, were both up regulated in the hippocampus of diabetic rats. Insulin resistance, hyperinsulinism and hyperglycemia, are involved in the accumulation of AGEs. Reactive oxygen species from

the hyperglycemic state may trigger a cascade of events that leads to accelerated neuronal aging and hippocampal atrophy, which may represent the initial neuronal damage in diabetes mellitus. Neurochemical changes contribute to cognitive dysfunction and insulin resistance impairs long-term potentiation (LTP), a fundamental mechanism for memory consolidation. Neurotransmitter functions which are altered in diabetes mellitus include decreased acetylcholine production, decreased serotonin turnover, decreased dopamine activity, and increased norepinephrine; all leading to the development of cognitive dysfunction.

Aβ plaques and hyperphosphorylated tau tangles are increased in the brain of type 2 diabetes patients with age, which are the major hallmarks of AD. Although insulin resistance, impaired insulin signaling, increased AGEs and ROS as well as oxidative stress are implicated in diabetes-induced memory deficits, the specific molecular mechanism for the development of diabetes induced cognitive dysfunction remains relatively unknown.

Several diabetic animal models including DIO-Rats (diet induced obesity) and transgenic models have been studied to elucidate the mechanism for cognitive impairment and synaptic deficits. Impaired hippocampal spatial memory and impaired synaptic transmission have been found in type 1 STZ-induced diabetic models, to be associated with deficits in the glutamatergic system, which is responsible for fast excitatory synaptic transmission in the brain. Recently intracranial STZ-induced rodent models have attracted specific attention because it shows pathology associated with sporadic AD. This animal model has shown spatial memory deficits, impaired synaptic transmission and glutamatergic deficits in the hippocampus (Shonessy et al., 2011, de lamonte et al., 2006).

Type 2 diabetic rodent models are either diet challenged or genetically modified (db/db mice, ob/ob mice, Zucker fa/fa rats and Zucker diabetic fa/fa rats). In general, these animals

display obesity, insulin resistance and hyperinsulinaemia, with variable degrees of hyperglycemia. Although these models mimic certain endocrine disorders associated with type 2 diabetes reasonably well, most of them have not been sufficiently characterized.

Nuclear receptor class of ligand activated heterodimeric transcription factor peroxisome proliferators-activated receptors (PPARs) have attracted recent attention to metabolic diseases and drug therapy (Blanquart et al. 2003, Feige et al. 2006; Michalik and Wahli 2007) because of their pronounced abilities to regulate lipid metabolism and glucose homeostasis in the brain, adipose tissue and placenta (Michalik et al. 2006; Kummer and Heneka 2008). Three different PPAR isoforms have been identified namely PPARα, PPARβ/δ, and PPARγ, with similar protein structures despite differences in coding genes (Nuclear Receptors Nomenclature Committee 1999) and display distinct physiological and pharmacological functions depending on their target genes and their tissue distributions (Bishop-Bailey, 2000; Buchan and Hassall, 2000). Among them PPARγ is highly expressed in the brain especially in the hippocampus and cortex and down regulated in AD. PPARγ has been shown to have anti-inflammatory and neuroprotective properties in AD animal models and primary neuronal cells. Few studies in AD animal models have shown improved cognitive function by PPARγ agonist treatment. However, these studies fail to implicate a specific molecular mechanism by which PPARγ improves memory.

Therefore, we have investigated how type 2-diabetes impairs cognitive function by altering the postsynaptic glutamatergic system in the hippocampus. We have hypothesized that PPARγ activation in the brain ameliorates diabetes-induced cognitive dysfunction via altering postsynaptic glutamatergic system. To test this hypothesis we used 8 weeks old leptin knockout db/db male mice and treated them orally for two weeks with the PPARγ agonist rosiglitazone (10 mgs/kg body weight). We then investigated hippocampal recognition memory, spatial memory

performances as well as synaptic transmission and LTP (synaptic plasticity). Moreover, we studied the beneficial effects of PPARγ activation upon the expression levels of postsynaptic glutamatergic receptors AMPAR and NMDAR and their subunit compositions in diabetic hippocampal tissue. Additionally, we studied changes in expression of postsynaptic auxiliary proteins, neurotrophic factors, kinases, insulin signaling cascades and transcription factors. This study will help to characterize the mechanistic basis of synaptic deficits in diabetes and identify potential molecular targets for developing novel therapeutic options for diabetes-induced cognitive dysfunction. In addition, it will help to understand the molecular link between AD, diabetes and age related cognitive dysfunction.

MATERIALS AND METHODS

Chemicals

BCA protein assay reagent kit and Coomassie plus protein assay reagent kit were purchased from Pierce Biotechnology Inc. (Rockford, IL). All other chemicals were purchased from Sigma chemicals, St. Louis, MO, USA, otherwise stated.

Db/db type 2 diabetes Mice

Twenty leptin receptor knockout obese diabetic mice (8 weeks in age) were purchased from Jackson laboratory (PA). This strain is called BKS.Cg-Dock7m +/+ Leprdb/J. Control male C57/B6 mouse (Jackson Laboratories, PA) were acquired at 8 weeks of age and randomly divided into 2 groups. The one group received 10 mg/kg PPARγ agonist Rosiglitazone for 2 weeks by oral administration and other group of animals received same volume of saline. Leptin receptor knock out db/db type 2 diabetes mice were acquired with the same age group and

divided into two random groups and received oral administration of rosiglitazone and control saline. All animals were caged and maintained in auburn university animal facility and received food and water *ad libitum* and maintained in 12 hours of dark-light cycle.

Mice homozygous for the diabetes spontaneous mutation (*Lepr*^{db}/ *Lepr*^{db}) become hyperglycemic and insulin insensitive at 6-8 weeks in age. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. The severity of disease on this genetic background leads to an uncontrolled rise in blood sugar, severe depletion of the insulin-producing beta-cells of the pancreatic islets, and death by 10 months of age. Exogenous insulin fails to control blood glucose levels and gluconeogenic enzyme activity increases. Peripheral neuropathy and myocardial disease are seen in C57BLKS-*Lepr*^{db} homozygotes. Wound healing is delayed, and metabolic efficiency is increased. The course of the disease is markedly influenced by genetic background. A number of features are observed on the C57BLKS background, including an uncontrolled rise in blood sugar, severe depletion of the insulin-producing beta cells of the pancreatic islets, and death by 10 months of age. Female homozygotes exhibit decreased uterine and ovarian weights, decreased ovarian hormone production and hypercytolipidemia in follicular granulosa and endometrial epithelial tissue layers (Garris et al., 2004, Garris et al., 2004).

Although normal in body weight, blood glucose, and plasma insulin, heterozygotes (*Lepr*^{db}/+) also have increased metabolic efficiency and can survive a prolonged fast longer than controls. Experiments involving destruction of the ventromedial nucleus of the hypothalamus suggest that *Lepr*^{db} may cause a defect in the hypothalamus. Steroid sulfotransferase enzymes, aberrantly expressed in diabetic mice, interact with the *Lepr*^{db} mutation as modifiers of gender differences in obesity-induced diabetes susceptibility. Because of the sterility of *Lepr*^{db}

homozygotes, the misty (Dock7m) mutation has been incorporated into stocks for maintenance of the diabetes mutation. (Woolley, 1941, 1945; Truett et al. 1998; Sviderskaya et al. 1998.).

Behavioral Studies for Hippocampal spatial memory

Animals:

8 weeks old male db/db and C57/BL6 mice (Jackson lab, PA) weighing 20–25g (db/db) and 40-50g (control) were used for behavioral study. All experiments were carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and approved by the corresponding committee at the Auburn University IACUC.

Behavioral Tasks

Hippocampal spatial memory tasks of mice were analyzed in two behavioral tasks: first, in the Object Recognition task; and second, in the Two trial Y Maze task; in order to evaluate the effect of PPARγ activation by rosiglitazone on cognitive rescue. Mice were intensively handled (5 days by ~5 min per mouse) and weighed once a week before behavioral testing. During the entire training session and observational sessions all group of animals were caged in the same room of animal facility of Auburn University.

Object Recognition Task (ORT)

Mice were divided into four experimental groups (10 mice per group) as follows: Control (C57/BL6), db/db diabetic, db/db + rosiglitazone (10mg/kg) and control + rosiglitazone. Object recognition task was performed as described elsewhere (Rutten et al., 2005). The apparatus consisted of a Plexiglas open-field box (52×52×40cm). Half of the 40cm high wall was covered with blue paper and the other half was left transparent. Two transparent glass bottles (diameter

2.5cm, height 6.5cm) filled with sand were placed symmetrically about 5cm away from the blue wall. During the second trial, a gray color plastic cube (2.5×2.5×6cm) was placed for one of the glass bottle. A testing session comprised of two trials. The duration was 5 minutes for first (exploration) and 5 minutes for the second (retention test) trial respectively. Subsequently, after a pre-determined delay interval 4h and 24h, the mouse was put back in the apparatus for the second trial (T2). Before each trial, the surface of the box was sprayed with a diluted ethyl alcohol solution (70%) to erase any scent cues. In the present experiment, the inter trial interval of 24h and 4h were used because pilot data indicated that a 5 minutes training trial induces significant retention at a 1h and 24h, delay with normal mice. Cognitive behavior was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. Times spent exploring each object during T1 and T2 was recorded manually using a personal computer and a timer. In order to avoid the presence of olfactory cues the objects were always thoroughly cleaned using diluted ethyl alcohol before each trial.

Two Trial Y Maze Test

Two trial Y Maze was used to measure spatial recognition memory as described previously (Ma et al., 2007). The Y-maze test consisted of two trials separated by a time interval. In the first (acquisition) trial, one arm of the maze (subsequently called novel arm) was closed with a guillotine door. Mouse was placed at the distal end of one arm (starting arm), head pointing away from the center of the maze, and was allowed to visit the two accessible arms of the maze for 15 minutes. At the end of the acquisition trial, each mouse was placed in their cage for a period, called inter-trial interval (ITI) of 4hrs and 24h. During the second (Retention test)

trial mouse was given free access to the three arms and was allowed to explore the maze for 5 minutes. Behavior of mice in maze was recorded by using a video camera mounted above the experimental apparatus and tapes were analyzed off-line by a trained observer who was unaware of the treatment condition. This test is based on the interest of mice for novelty; hence, they explore preferentially unknown territories. The number of explorations of each arm was counted every minute and the percentages of visits in the novel arm with respect to the total number of visits in the three arms during each minute of the test was calculated.

Basal synaptic transmission and LTP

Hippocampal Slice Preparation

Mice from all 4 treatment groups (n=4) control C57, db/db, db/db + Rosi, Control + Rosi, age of 8 weeks after two weeks oral treatment of 10mg/kg rosiglitazone were placed in a closed chamber into which CO2 was slowly released until the animal reached an anesthetic state, after which it was quickly decapitated using a guillotine. A midline incision was made into the top of the head and the skin was pulled back to expose the skull. Following this, the skull was cut down the sagital suture with small iris scissors and was peeled back using a pair of bone rongeurs. The brain was quickly removed by placing a small spatula under the frontal lobe and elevating the brain away from the base of the skull. The brain was then briefly rinsed with a dissection buffer consisting of (in mM): NaCl 85, KCl 2.5, $MgSO_4$ 4.0, $CaCl_2$ 0.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5, saturated with 95% O_2/5% CO_2 at a temperature of 0°C. The cerebellum was dissected away and the brain was placed onto a vibratome chamber filled with ice-cold dissection buffer bubbled with 95% O_2 / 5% CO_2. The brain was held in place with cyanoacrylate glue and was placed with the frontal lobe facing up

and the basal aspect of the brain resting against a block of 10% agar. The brain was then cut into hemispheres and the two hemispheres were simultaneously cut into coronal slices of 400 μm thickness. These slices were then placed in a slice incubation chamber for 30 minutes at 30°C in artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26, dextrose 11.0 and saturated with 95% O_2 and 5%CO_2. After 30 minutes, they were transferred to ACSF with a temperature of 24°C where they remained for at least another 30 minutes before being used for electrophysiological recordings. The slice incubation chamber consisted of a round plastic vessel (7 cm in diameter and 6 cm in height) placed inside a glass crystallizing dish (10 cm in diameter and 8 cm in height). The slices were placed at the bottom of the cylinder, which was made of nylon stockings stretched and glued across the edges of the plastic vessel. The dish was filled with aCSF, and an aquarium stone was placed at the bottom of the vessel to provide the 95% O_2 and 5%CO_2.

Extracellular Field Recordings

Basal Synaptic transmission and Long-term potentiation (LTP)

A slice containing an anterior portion of the hippocampus was transferred to a submersion-recording chamber using a paintbrush. The recording chamber was continuously superfused at 2 ml per minute with aCSF saturated with 95% O_2 and 5% CO_2. A ring with silk strings stretched across was placed on top of the slice in such a way that the mesh did not touch the hippocampus. Healthy slices do not normally stick to the paintbrush and have a clearly visible stratum pyramidal and stratum granulosum. Recordings in CA3-CA1 synapses were performed by placing the stimulating and recording electrodes on surface of the slice

approximately 350-400 μm apart from each other in the stratum radiatum where the Shaffer collateral fibers are located.

All recordings were performed at 24°C. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 with a glass electrode with a resistance of 1-4 MΩ filled with aCSF. Test pulses were delivered by a constant current stimulus isolator driven by input signals from the computer. The stimulus electrode was a platinum bipolar electrode. Stimulation (50-70 μA, 0.2 ms duration) was applied to CA3 axons every 20 seconds and the recording and stimulation electrodes were slowly advanced towards the slice until the maximal amplitude of responses was attained. After ten to fifteen minutes of stable responses, the stimulus-response relation was recorded in response to stimulation pulses (0.2 ms, every 20 s) of increasing intensity by varying stimulus intensity between 0 μA to 200 μA in steps of 50 μA and measuring the slope of the fEPSPs as well as the fiber volley. Field EPSPs were recorded until a population spike (upward going inflection) appeared on the decaying phase of fEPSPs. Typically, the amplitude of supra maximal fEPSPs was greater than one mV and the amplitude of presynaptic volleys were less than one third that of the fEPSPs. Field EPSPs were elicited every twenty seconds for at least ten minutes with the stimulus intensity that elicited fEPSPs with a slope of 40% of the supramaximal. Obtaining stable responses of fEPSPs during the baseline period is of utmost importance to insure that the correct stimulus intensity is used. Following stable baseline recordings of at least ten minutes, a high frequency stimulus (HFS) was applied using the test pulse stimulus intensity. The HFS protocol for inducing LTP consisted of three trains of 100 pulses (100 Hz), with an inter-train interval of 20 seconds. LTP was measured as the percentage of the baseline fEPSP slope. During the 20 minute baseline and 1 hour following the tetanus, fEPSP peak amplitude and slope were analyzed online using WinLTP acquisition software

(Anderson and Collingridge, 2007). The data is presented as mean ± SEM. Significance was determined using a two-tailed Student's t-test. Paired-pulse facilitation (PPF) was evaluated by stimulating the synapses with twin pulses at interpulse intervals of 40, 60, 80, 150, and 200 ms. fEPSPs, were recorded at 40% of maximal response, as determined from an input–output curve for each experiment. Following stable baseline recordings of at least 20 minutes, high frequency stimulation (HFS) was applied using the test pulse stimulus intensity. The HFS protocol for inducing LTP consisted of three trains of 100 pulses (100 Hz), with an inter-train interval of 20 seconds. LTP was measured as the percentage of the baseline fEPSP slope. During the 20 minute baseline and 1 hour following the tetanus, fEPSP peak amplitude and slope were analyzed online using WinLTP acquisition software (Anderson and Collingridge, 2007). It is important to compare baseline fEPSPs to responses after induction of LTP. If the slice is healthy and recording, conditions are ideal the stimulus artifact and the amplitude of presynaptic volley should be identical prior to the application of the high frequency stimulation and at the end of the recording. It is important to select appropriate parameters for slope measurements so that linear changes in the slope of the fEPSPs would be detectable for all stimulation intensities used to estimate the stimulus-response curve. In addition to the plot of stimulus intensity versus fEPSP slope, the presynaptic fiber volley is plotted as a function of stimulus intensity and of fEPSP slope. This function provides analysis of the number of activated axons (presynaptic volleys) and resulting fEPSPs. It is important to keep recording and stimulating electrode at a consistent and sufficiently long distance, as the presynaptic fiber volley amplitude is directly proportional to this distance. The mean magnitude of fEPSPs recorded during the baseline (at least 10 min before TBS) is taken as 100 % and changes are expressed relative to this as a means of normalizing the responses amongst multiple recordings from multiple slices. The values of LTP

are calculated as the relative increase in the mean slopes of averaged fEPSPs measured at 50-60 minutes after induction of LTP. The data is presented as mean ± SEM. Significance was determined using a two-tailed Student's t-test.

Gene Expression

Hippocampus from all four treatment groups were stored at -80°C and thawed and homogenized by QIAZOL (Qiagen, Valencia, CA). Total RNA was isolated from 50-100 mg of whole hippocampal lysates using an RNeasy Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was determined by optical density absorption spectrophotometry at 260 nm. Then cDNA was synthesized according to the manufacturer's protocol utilizing 1 μg of RNA with a iScript cDNA synthesis kit (Bio Rad, Hercules, CA). Real-time PCR was performed in triplicate with 25μl of reaction mixture in MicroAmp optical 96-well reaction plates on an iCycler real-time PCR detection instrument (Bio-Rad) using 1 μl cDNA, 3.0 μl 1.25 μM (final concentration) primer mix, 9 ml RNAase free water and 12.5 μl iQ SYBR Green Supermix (Bio-Rad) per reaction. Rat gene specific primers were selected and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Primer sequences for the genes can be found in Table 5.1. The following PCR amplification cycle (40X) was used: 95° C, 180 sec; 95° C, 15 sec; 58° C, 30 sec; 72° C, 30 sec. Melting curve analysis was performed to determine specificity of products formed. Relative gene expression was determined by the comparative C_T method (Pfaffl, 2001). Gene expression was normalized to β-actin for each experimental gene tested. The data is presented as mean ± SEM. Significance was determined using a two-tailed Student's t-test.

Primers List

Table 5.1 Primer sequences of Synaptic plasticity genes.

1	GluR1	F	ATG CAA TCC ATT CCC TGC ATG AGC
		R	GTG GTT GGA GGC GGT GTT TCA TTT
2	GluR2	F	TTA CGT GAG TCC TGG CAT GGG AAT
		R	ATC GGA TGC CTC TCA CCA CTT TGA
3	NR1	F	CAA GCT GGC ACG GAC CAT GAA TTT
		R	TTG TTG CTG TTG TTT ACC CGC TCC
4	NR2A	F	ACT GGG TCA CAG AGC AAC ATG CTA
		R	AGT CCA TTC GCG AGG AGT TCA TGT
5	NR2B	F	TCT GTC CAC CAT TCC TGT TCC CAT
		R	AAA GCC TCG CTC AAA GTG AAT CGC
6	BDNF	F	CAT TGA GCT CGC TGA AGT TGG CTT
		R	AAC TTC TTT GCG GCT TAC ACC ACC
7	Synapsin	F	ATG CCT TCA GCATGG CAC GTA ATG
		R	TGT GTA GTC GAA CCATCT GGG CAA
8	IRS-2	F	AAA GTG GCCTAC AAC CCT TAC CCA
		R	TCA TCG CTC TTG CAG CTATTG GGA
9	IRS-1	F	ACT ATG CCA GCATCA GCT TCC AGA
		R	AAG ACGTGA GGT CCT GGTTGT GAA
10	CBP	F	GCC CAG CAT GCA GAT GAATCA CAA
		R	CAT TAG CCG CGC CAA CAA GAA GAA
11	C/EBP β	F	ATG CAA TCC GGA TCA AAC GTG GCT

		R	TTT AAG TGA TTA CTC AGG GCC CGG CT
12	Ins-1	F	ACCTGG AGA CCT TAATGG GCC AAA
		R	ATG ACCTGC TTG CTG ATG GTC TCT
13	IR	F	GTT CTT TCC TGC GTG CAT TTC CCA
		R	ATC AGG GTG GCC AGT GTGTCT TTA
14	IGF-1	F	AAG CAA GAG GCT AGG GAT TTG GGA
		R	ATT TGA CTG AGGTCA CAG GGT GGT
15	IGF-2	F	ACA AGG CCC ATC CCA AAT TTC CTG
		R	AGC CTG GTC ACA CAT AGA GCC AAT
16	IGFR1	F	ACC ATC GAT TCG GTG ACT TCT GCT
		R	TGA AGT TCT CCA ACT CCG AGG CAA
17	IGFR2	F	TCC TTC CAT GAT GAC AGC GAC GAA
		R	TGA CAG ACG CAT CAT CGA GCT GAA
18	IDE	F	TTC CAG GAA GAG CAT CTC AGG CAA
		R	TTC CAG GAC CTT CGT GCC CAATTA
19	PPAR-γ	F	ACA TAA AGT CCT TCC CGC TGA CCA
		R	AAA TTC GGA TGG CCA CCT CTT TGC
20	PPAR-δ	F	GCC CAA GTT CGA GTT TGC TGT CAA
		R	ATT CTA GAG CCC GCA GAA TGG TGT
21	B-actin	F	TTG CTG ACA GGA TGC AGA AGG AGA
		R	ACT CCT GCT TGC TGA TCC ACA TCT

Protein Extraction

Whole hippocampal homogenates or synaptoneurosomes were lysed in a chilled buffer containing protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4, 1% NP- 40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). The lysates were incubated for 15 minutes on ice and centrifuged for 15 minutes at 10,000 x *g*, at 4°C. The supernatants containing the protein extract were collected, and their protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Duplicate samples for individual rats were subjected to 10% SDS-PAGE and subsequently blotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were saturated with 5% non-fat dry milk (Biorad) or 1% BSA and then incubated with anti-Akt (Cell Signaling), anti-pAkt (Cell Signaling), anti-GluR1(Millipore), anti-GluR2 (Millipore), anti-NR2A (Santa Cruz), anti-NR2B (Santa Cruz), anti-BDNF (Millipore), anti-Stargazin (Cell Signaling), anti-PSD-95 (Santa cruz), CBP and IRS-2 (Epitomics), anti-GSK-3beta and anti-pGSK3beta, Anti-Tau anti-pTau (ser 9), anti-CREB), anti-pCREB and anti-β actin antibodies were all purchased from (Cell Signaling) (overnight at 4°C. The membranes were subsequently incubated at room temperature for 1 hour with the corresponding anti-rabbit or anti-mouse antibodies (Santa Cruz). The membranes were then incubated in Immobilon Western HRP Substrate (Millipore) for 5 minutes and imaged using the Molecular Imager ChemiDoc XRS system (Bio-Rad). Signals were subsequently quantified by densitometric analysis using Quantity One Analysis software (Bio-Rad). The densities of each band, which represented individual animals, were normalized to β-actin and these normalized values were averaged for control and diabetes and rosiglitazone treated groups**.** The data is presented as mean ± SEM. Significance was determined using a two-tailed Student's t-test.

Immunohistochemistry

Hippocampal sections were fixed overnight in 4% buffered paraformaldehyde followed by several washed in PBS. Sections were then blocked for 1 hour with blocking solution containing 5% goat serum in PBS, followed by overnight incubation with monoclonal rabbit antibody against BDNF, PSD-95, GluR1, NR2A and NR2B at 4^0C in PBS containing 5% goat serum. Sections were then washed three times for 10 minutes in PBS. A secondary anti-rabbit IgG antibody coupled to Alexa-Fluor-488 (Invitrogen) was applied for 1 hour at room temperature. Finally, sections were rinsed three times for 10 minutes in PBS and mounted in ProLong gold+ DAPI (Invitrogen). Images of stained sections were acquired by a Nikon inverted fluorescence microscope.

OGGT

Oral glucose tolerance test was performed to evaluate the diabetic nature of all treated 4 groups of animals by tail vein blood samples. Glucose (150 mg/kg) was given to each animal orally followed by blood glucose monitoring at specific timed intervals (30, 60, 90, 120, 150, 180 minutes) from tail vein blood using Glucose test kit (One touch). Each group was analyzed on the basis of time taken for them to regulate their glucose level to normal.

H19 Hippocampal Neuronal Culture

H19-IGF-1R hippocampal neuronal cell lines were grown at 34^0 C and 10% fetal bovine serum. Cells were differentiated incubation at 39°C with serum supplemental with insulin or IGF-2 in physiological concentration. These neuronal cells were treated with rosiglitazone in 10μM concentration.

PPARγ agonist Rosiglitazone Treatment

All mice were grouped into 4 groups (n=10) as control, db/db, control + Rosi and db/db + Rosi. Negative control groups were treated by saline buffer. The db/db + rosi and control + rosi animal groups received 10mg/kg of rosiglitazone via oral gavages every day and control groups received same volume saline buffer solution in the same time for 2 weeks. Body temperature and body weight of all the animals were measured every day. After 2 weeks of treatment, animals performed behavioral tasks and euthanized. Hippocampus and cortex tissues were dissected and stored at -80°C.

Statistical Analysis

Experiments were performed with the investigator blinded to the treatment the animals received. Data were expressed as mean ± SEM. Electrophysiological results were normalized to the basal values and analyzed by ANOVA with Tukey's post-hoc analysis. The level of significance was set for $p<0.05$. All statistical calculations were performed on a computer using a commercially available statistical package.

RESULTS

Behavioral Analysis

We investigated the effect of PPARγ activation by rosiglitazone treatment 10mg/kg for two weeks on hippocampal spatial memory and object recognition memory in type 2 diabetes induced cognitive impairment by using a battery of behavioral tests. There is a lack of reports on memory enhancing ability of rosiglitazone in type 2 diabetic animal models (both transgenic and DIO models).

PPARγ activation improves object recognition memory in the diabetic mouse.

To investigate the effect of rosiglitazone on object recognition memory, mice were allowed to explore two identical objects followed by a 24 hour delay after which memory retention was tested with a novel object. Post-hoc analysis showed that the level of exploration as measured by object discrimination index (DI) was significantly decreased in type 2 diabetes db/db mice group compared to control group (Fig.5.1.A, $p<0.05$) and DI increased significantly (Fig.5.1.A, $p<0.05$) in rosiglitazone treated db/db group to control group DI and there was no significant difference in DI between rosiglitazone treated group and non-treated control group. In between several repetitions of trials, enough time had been given (48 hours) to all mice groups to lose the recognition memory to discriminate between the novel object and familiar object and the DI was of chance level.

PPARγ activation improves spatial memory in the diabetic mouse.

Thereafter we investigated the effect of rosiglitazone on hippocampal spatial memory by first restricting them to explore in two arms of Y maze for 15 minutes followed by a delay of 4 hours of memory retention, which was administered by allowing the mice to explore in all arms for 5 minutes. The effect of rosiglitazone on the number of entry in novel arm, entry frequency (%) in novel arm, and time spent in the novel arm, the arm opened only during second trial, in Y maze is presented in Fig.5.1.B (i), (ii) and (iii). The spatial memory was significantly reduced in diabetic db/db mice ($p<0.05$) when compared to their control group. Furthermore rosiglitazone treatment (10mg/kg for two weeks) significantly improved ($p<0.05$) the diabetes induced cognitive impairment.

PPARγ activation improves Long-term potentiation (LTP) and Post-tetanic potentiation (PTP).

To investigate how PPARγ activation in the brain can affect different forms of plasticity, we analyzed the magnitude of PTP and LTP. A robust theta burst tetanization paradigm consisting of five trains with an inter-train interval of 20 s was used to induce LTP. PTP is another form of short-term plasticity affected by diabetes type 2 and rosiglitazone treatment. PTP was significantly decreased in db/db mice ($p<0.01$) and increased to normal level by rosiglitazone treatment ($p<0.01$) (Fig.5.2.A&C). 1-7 minutes after the theta burst high frequency stimulation, slope of fEPSP were 156.52±4.62 percent in db/db mice treated with rosiglitazone, 121±3.56 percent in db/db without treatment, control group showed 163.26% ± 4.34 percent and control treated with rosiglitazone showed 149.52±4.23 percent. Percent of fEPSP slope in rosiglitazone treated control group and non-treated control group did not show significant changes. (Fig 5.2.A & C). These results showed that type 2 diabetes impairs short-term plasticity which is rescued by PPARγ activation.

Interestingly, LTP was significantly reduced ($p<0.001$) in type 2 diabetes db/db mice compared to their control group. This effect was ameliorated by PPARγ agonist rosiglitazone (Fig.5.2.A&B); sixty minutes after the induction of LTP the slope of fEPSP were 138±3.3 percent in db/db mice treated with rosiglitazone and 102±4.7 percent in db/db without treatment. Percent of fEPSP baseline in rosiglitazone treated control group and non-treated control group did not show significant changes (Fig.5.2.A&B, control 137.72±4.21, control treated with rosiglitazone 138.04±3.87).

PPAR expression and activation

We investigated the change of gene expression levels of PPARγ and PPARδ receptors in the hippocampus of type 2 diabetes db/db mice and control group. PPARγ gene expression was significantly reduced in diabetes type 2 db/db mice compared to their control group (Fig.5.3.A, $p<0.001$). Moreover PPARγ agonist rosiglitazone oral administration for two weeks significantly increased ($p<0.05$) (Fig.5.3.B) PPARγ receptor gene expression in the db/db mice to levels found in control mice. Furthermore, there was no significant difference in PPARγ gene expression between rosiglitazone treated control group and non-treated control group.

Post synaptic Glutamate receptors and auxiliary proteins expression

Glutamate receptors AMPAR and NMDAR expression in the postsynaptic surface and their subunit composition play a crucial role in fast excitatory synaptic transmission in the mammalian brain thus leading to synaptic plasticity and cognitive function. Moreover postsynaptic auxiliary proteins PSD-95 and stargazin play key roles in AMPAR and NMDAR trafficking and ion channel kinetics. Therefore, we investigated mRNA and protein expression levels of the receptor subunits such as AMPAR- GluR1 and GluR2 and NMDAR- NR2A and NR2B and post synaptic auxiliary proteins PSD-95 and stargazin. Protein ($p<0.01$) and gene ($p<0.001$) expression of GluR1 decreased in db/db mice compared to control mice (Fig.5.4.A.i & B.i). PPARγ activation by rosiglitazone oral treatment increased GluR1 gene ($p<0.001$) (Fig.5.4.A.i) and protein ($p<0.01$) (Fig.5.4.B.i) expression to control levels. NR2A gene and protein levels were found to be down regulated in db/db mice when compared to control groups. This was ameliorated by two weeks rosiglitazone treatment (Fig.5.4.A.ii & B.ii). However, these changes were not apparent in control groups treated with rosiglitazone. Furthermore, there was

no significant change in expressions levels of GluR2 and NR2B in all groups. To further explore the protective effects of PPAR upon improving postsynaptic receptor expression, we found that rosiglitazone treatment in H19 hippocampal neuronal cells demonstrated increased ($p<0.001$) gene expression of GluR1 and NR2A when compared to non-treated counterpart after differentiation (Fig.5.8.C).

The postsynaptic auxiliary proteins PSD-95 and stargazin, which are involved in rafficking of the glutamate receptors to the postsynaptic sites of the synapse and they were found to be reduced in expression ($p<0.01$) in the db/db mice. These findings were ameliorated by rosiglitazone treatment ($p<0.01$) (Fig.5.4.D.i). Interestingly immunohistochemical analysis also showed attenuated expression levels of PSD-95 in the CA1-CA3 region of Schaffer-collateral in the hippocampus and increased to their control counterpart by rosiglitazone treatment (Fig.5.4.B.iii). This data supports our postsynaptic receptor differential expression pattern in diabetes and their rescue by PPARγ activation in the brain.

Expression of CBP/CREB/BDNF

CREB is a master regulatory switch of synaptic plasticity in the hippocampus and cortex. CREB binding protein CBP plays a major role in activating CREB by phosphorylation and activating BDNF expression a major neurotrophic factor in the brain. Therefore, we investigated the expression levels in the hippocampus from db/db mice. Our gene and protein expression studies showed significant attenuation of CBP ($p<0.001$), CREB ($p<0.01$) and BDNF ($p<0.01$) in db/db mice. These cognitive impairments observed in diabetes, were ameliorated by rosiglitazone treatment (Fig.5.5.A and B). However, rosiglitazone offered no changes in the control groups. Interestingly rosiglitazone treatment significantly increased CBP protein

expression in the control group (Fig.5.5.B). Furthermore, immunohistochemical analysis of BDNF expression found reduced levels in db/db hippocampal Schaffer-collateral CA1-CA3 region and improved to control level with rosiglitazone (Fig.5.5.C).

Expression of Insulin signaling Molecules

Insulin signaling plays a crucial role in synaptic plasticity and cognitive function. However, the specific signaling mechanism involving insulin-mediated effects upon synaptic plasticity has not been established. Therefore, we explored how improvement in central insulin signaling affects the hippocampus. We found that molecules associated with the insulin-signaling cascade such as IR, IRS-2, IGF-2 and IDE gene expressions were significantly down regulated in db/db mouse hippocampus. Rosiglitazone treatment after 15 days, improved this insulin-signaling pathway in the hippocampus (Fig.5.6.A.i & B & Fig.5.6.A.ii). However, rosiglitazone did not improve the insulin receptor expression levels. Furthermore, rosiglitazone treatment increased both IRS-2 and IDE gene expression level. Interestingly IRS-2 promoter sequence analysis revealed that the PPRE; PPARγ binding element, is located in the IRS-2 promoter. Thus suggesting that IRS2 may be a potential target in which PPARγ may improve the insulin signaling in the diabetic hippocampus. Recent studies showed IDE also has a PPRE in the promoter region. This explains direct binding of PPARγ to IRS-2 and IDE promoter and results in higher expression of IDE and IRS-2 by direct PPARγ activation.

This data strongly support that insulin resistance and insulin signaling impairment in the hippocampus of the type 2 diabetes and also rosiglitazone treatment improves central insulin sensitivity by enhancing insulin signaling cascade in the CNS.

AKT/GSK3β signaling and Tau hyperphosphorylation

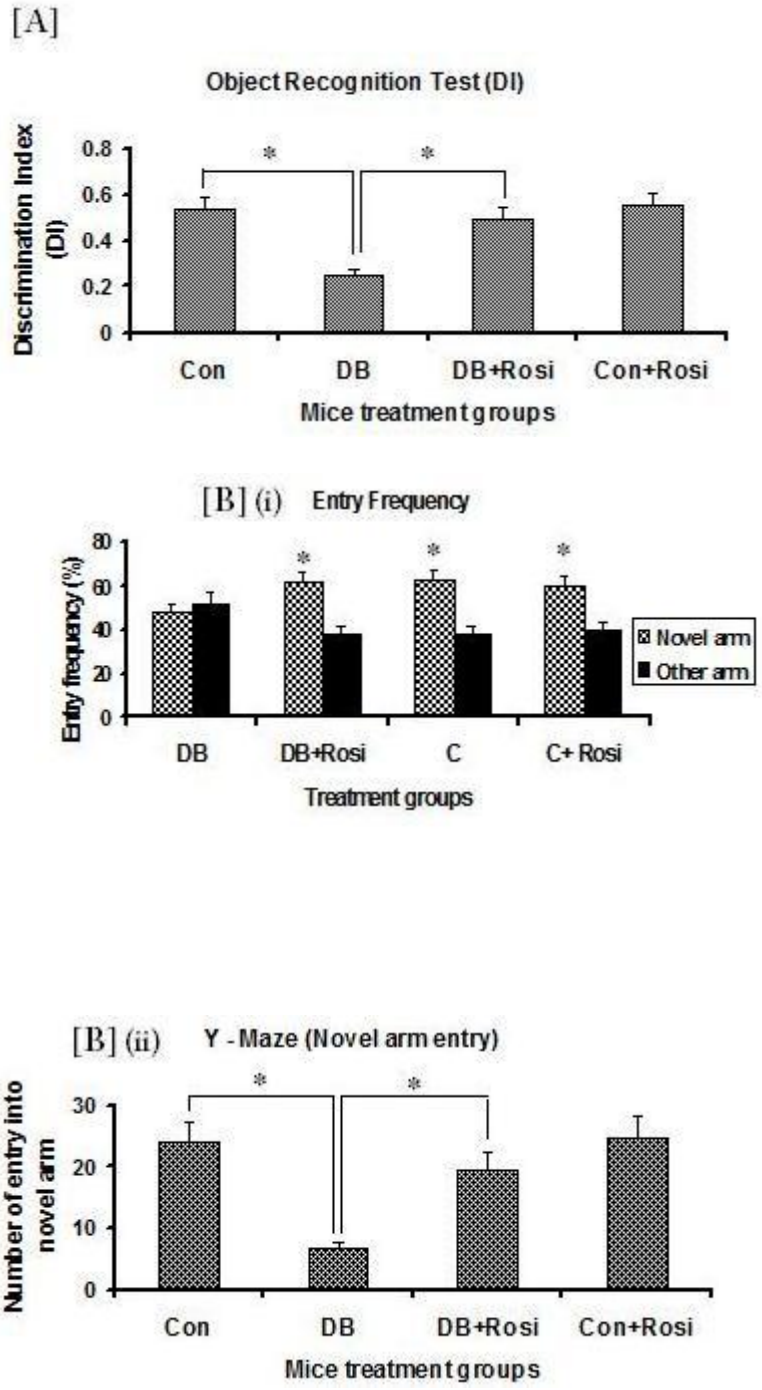
AKT/GSK3β signaling cascade plays a significant role in the insulin signaling and synaptic plasticity in the brain. We found that db/db mice showed reduced phosphorylation of AKT ($p<0.01$) and increased phosphorylation of GSK3β (Fig.5.7.A&B). Interestingly Tau hyperphosphorylation was found to be increased in db/db mice and was attenuated by rosiglitazone treatment to control levels found in control mice (Fig.5.7.C). This data offers that impaired AKT/GSK3β signaling and increased Tau hyperphosphorylation is associated with type 2 diabetes. Rosiglitazone treatment improves the AKT/GSK3β signaling and reduces Tau hyperphosphorylation.

Dendritogenesis and Arborization of H19 neuronal cells

To further explore, our findings involving the protective mechanism offered by PPARγ against diabetes induced cognitive impairment in the diabetic animal model, we applied our findings to the H19 hippocampal neuronal cell line. Rosiglitazone (10μM) treatment showed dendritogenesis and arborization of H19 hippocampal neuronal cells compared to non-treated control counterpart (Fig.5.8.A & B). This hippocampal cell line is useful in studying the effects of the insulin-signaling pathway upon synaptogenesis and neurogenesis because stimulation of the IGF promoter is required for differentiation of the neuron. Consequently, we have found that neuronal differentiation is enhanced by rosiglitazone.

FIGURES AND FIGURE LEGENDS

Fig.5.1



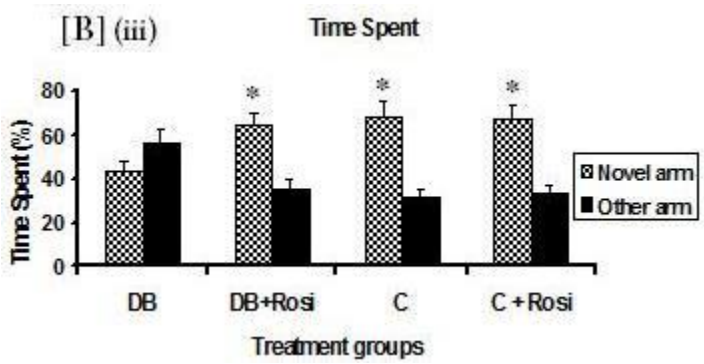


Fig.5.1: PPAR gamma activation improves object recognition memory and spatial memory in type 2 diabetes: Memory impairments is observed in db/db diabetic mice which is ameliorated by the PPARγ agonist rosiglitazone for two weeks (10mg/kg). These results are found in panel **A**; object memory test and Panels B1-3where spatial learning memory was tested. Panel **B(i)** Novel arm entry, number of entries to novel arm [(**ii**)], time spent in the novel arm [**B** (**iii**)]. Discrimination index (DI) was calculated as a ratio of additional time mouse spent with novel object to the total time spent on exploring objects. Db/db mice displayed impaired preference for the novel arm after 4 hours training interval. However, rosiglitazone enhanced the preference for the Novel arm significantly in db/db mice. Furthermore, these mice after receiving rosiglitazone improved in the spatial orientation test. The number of entries in each arm during the second trial was analyzed against the number of entries into the novel arm. Males, n =10 for each treatment group control, diabetes, diabetes + Rosi and control + Rosi. Statistical significance is evaluated by student's t-test unpaired two tailed, * $p<0.05$, n =10. Values are mean ± SEM.

Fig.5.2

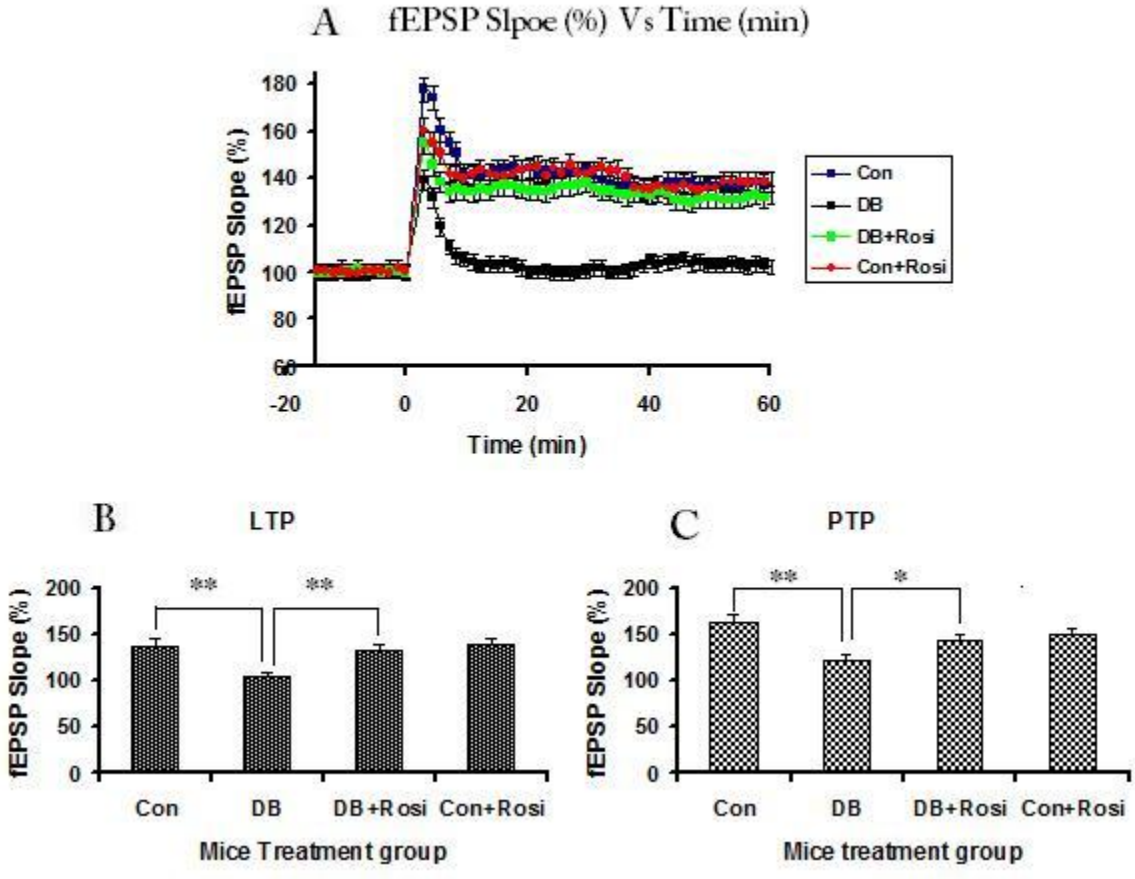


Fig.5.2: PPARγ activation enhances LTP and PTP in the diabetic hippocampus. Long-term potentiation (LTP) was induced with a tetanic stimulus at 100 Hz and an intensity that evoked 50% of a maximal fEPSP (**A**). The fEPSPs slopes were significantly diminished ($p<0.01$) in the db/db mouse and was ameliorated in mice treated with rosiglitazone ($p<0.01$). Rosiglitazone did not induced changes in control mice. Representative traces are shown for all four groups at baseline (Trace 1) and 60 minutes (Trace 2) after tetanic stimulation. LTP (B) and PTP (C) in db/db, rosiglitazone treated db/db, rosiglitazone treated control and untreated control mice measured at 55–60 minutes (LTP) and 1-7 minutes (PTP) respectively. At 1–7 minutes, db/db mice showed attenuated PTP which was improved in db/db mice treated with rosiglitazone. There was no

difference in PTP between control and rosiglitazone treated control groups. (*n*=6 slices; 5 mice) compared to control mice ($p<0.01$). At 55–60 minutes LTP was significantly reduced in db/db mice (*n*=6 slices; 5 mice) when compared to control mice ($p<0.01$) and LTP was found to be proportional to control levels (P<0.01) in db/db mice treated with rosiglitazone (n=6 slices, 5 mice). Statistical significance was evaluated by student's two tailed t-test unpaired, ** p<0.01, N=5. Values are mean ± SEM.

Fig.5.3

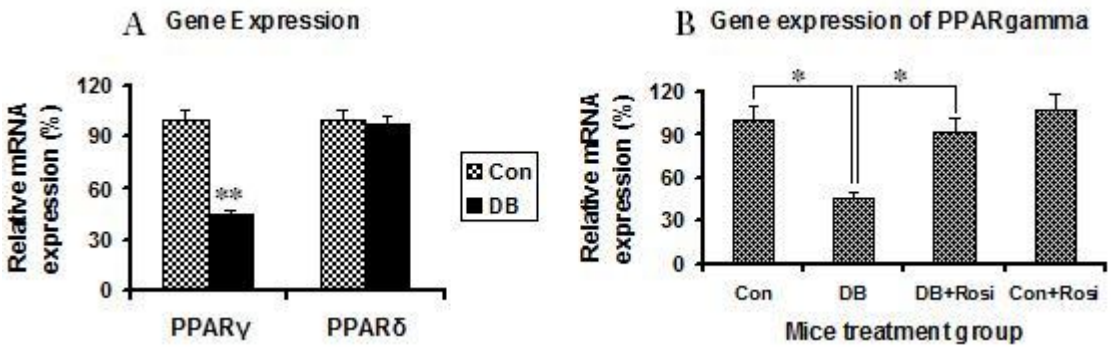
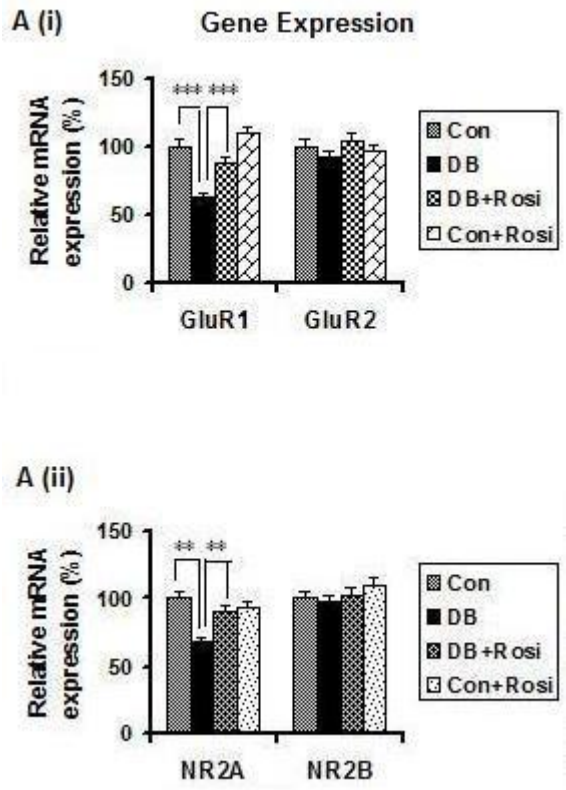


Fig.5.3: PPARγ receptor levels are altered in diabetic hippocampus (**A**) PPARγ receptor mRNA levels were significantly decreased in the hippocampus in db/db mice (p<0.01) however there was no significant difference in the expression of PPARδ. (B) Rosiglitazone improves the relative mRNA expression of PPARγ in the diabetic hippocampus (p<0.05) however, rosiglitazone had no effect upon PPARγ mRNA expression levels in the control group (**B**). All gene expression values are normalized to housekeeping gene β-actin. Statistical significance is evaluated by Student's two tailed t-test unpaired, ** P<0.01, * P<0.05, N=5. Values are mean ± SEM.

Fig.5.4


A (i)
Gene Expression
Relative mRNA expression (%)
150
100
50
0
*** ***
GluR1
GluR2
Con
DB
DB+Rosi
Con+Rosi
A (ii)
Relative mRNA expression (%)
150
100
50
0
** **
NR2A
NR2B
Con
DB
DB+Rosi
Con+Rosi

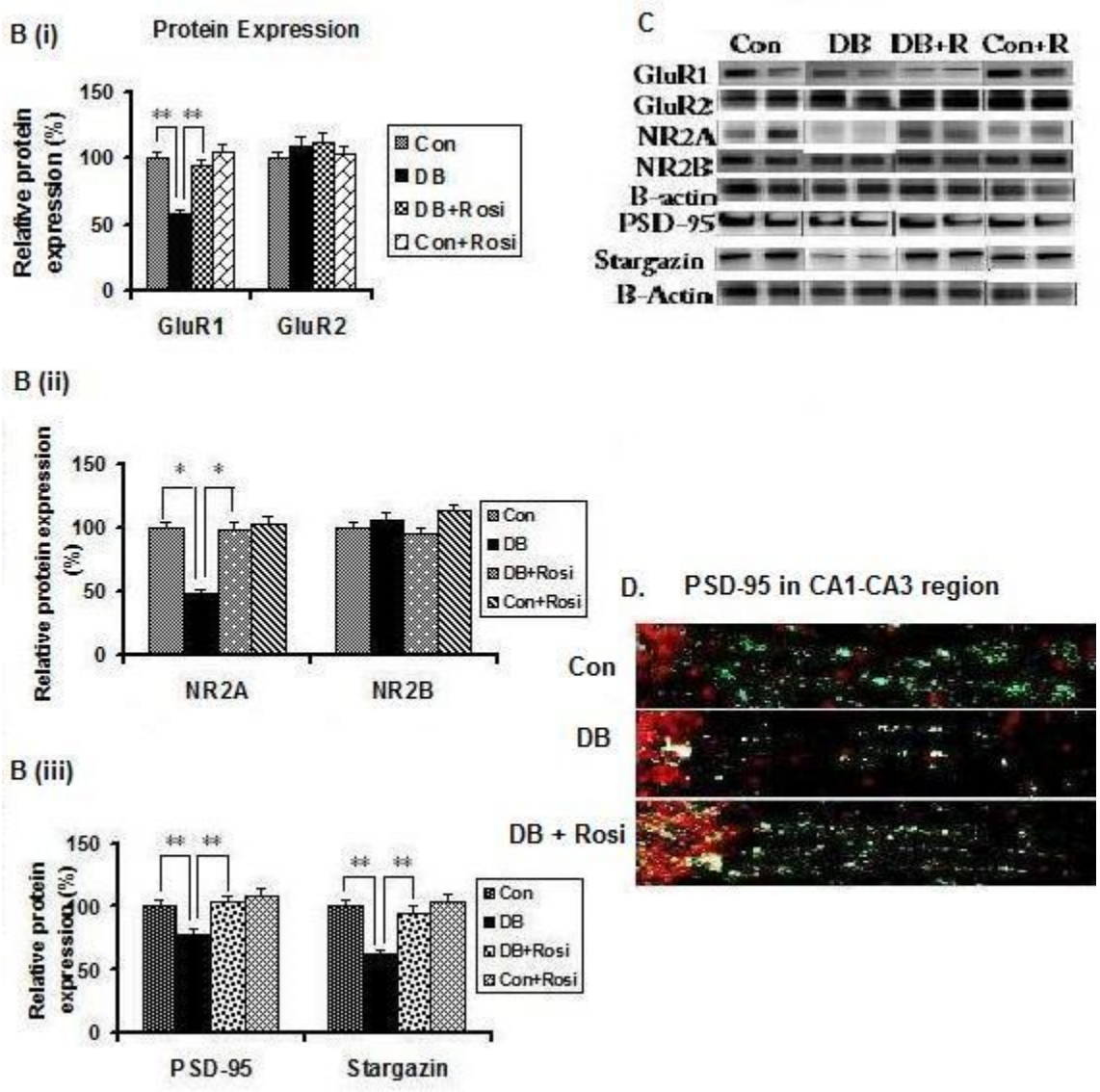


Fig.5.4: PPARγ activation improves the expression levels of postsynaptic glutamate receptors AMPAR, NMDAR, and postsynaptic auxiliary proteins PSD-95 and Stargazin. Relative mRNA expression and protein expression levels were attenuated in db/db mice as shown in figures [**A**] (**i**) & (**ii**). In figures [**B**] (**i**) the AMPAR receptor subunit GluR1 ($p<0.001$, $p<0.01$) and (**ii**) NMDAR subunit NR2A decreased significantly ($p<0.01$,$p<0.05$) in db/db mice hippocampus and increased significantly in db/db mice hippocampus after rosiglitazone treatment. However, there was no significant difference in AMPAR subunit GluR2 and NR2B

mRNA and protein expression level among all treatment groups. [**B**] (**iii**) Relative protein expression of post synaptic auxiliary proteins PSD-95 and stargazin decreased significantly ($p<0.01$) in db/db mice hippocampus which was significantly improved by rosiglitazone treatment in db/db mice. (D) Immunofluorescence microscopy showed decreased expression of PSD-95 protein in db/db mice hippocampal Schaffer-collateral CA1-CA3 region compared to control group and PSD-95 protein increased in expression in the db/db mice hippocampus after oral treatment of rosiglitazone for two weeks (10mg/kg). All gene expression values and protein band density values are normalized to housekeeping gene and protein β-actin. Statistical significance is evaluated by student's two tailed t-test unpaired, *$p<0.05$, **$p<0.01$, ***$p<0.001$, n=5. Values are mean ± SEM.

Fig.5.5

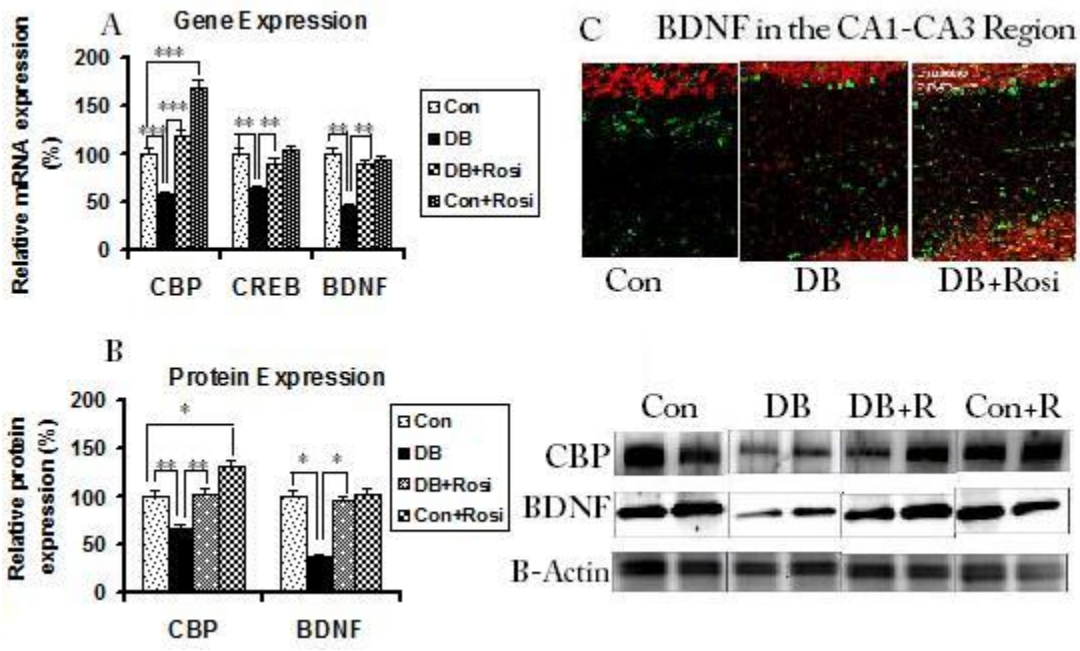


Fig.5.5: PPARγ activation induces change in CBP, CREB and BDNF expression in the diabetic hippocampus. (**A**) Relative mRNA expression of CBP CREB and BDNF is significantly reduced in in db/db mice hippocampus when compared to control animals. These

changes were ameliorated by rosiglitazone. Further more rosiglitazone also increased CBP mRNA expression in the control groups. (**B**) Relative protein expression of CBP and BDNF were significantly reduced in db/db mice hippocampus compared to control group. Rosiglitazone improved reduced expression levels of CB found in the diabetic hippocampus. (**C**) Immunohistochemical confocal microscopy showed decreased expression of BDNF protein in db/db mice hippocampal Schaffer-collateral CA1-CA3 region compared to control group and BDNF protein increased in expression in the db/db mouse hippocampus after oral treatment of rosiglitazone for two weeks (10mg/kg). All gene expression values and protein band density values are normalized to housekeeping gene and protein β-actin. Statistical significance is evaluated by student's two tailed t-test unpaired, *p<0.05, **p<0.01, ***p<0.001, N=5. Values are mean ± SEM.

Fig.5.6

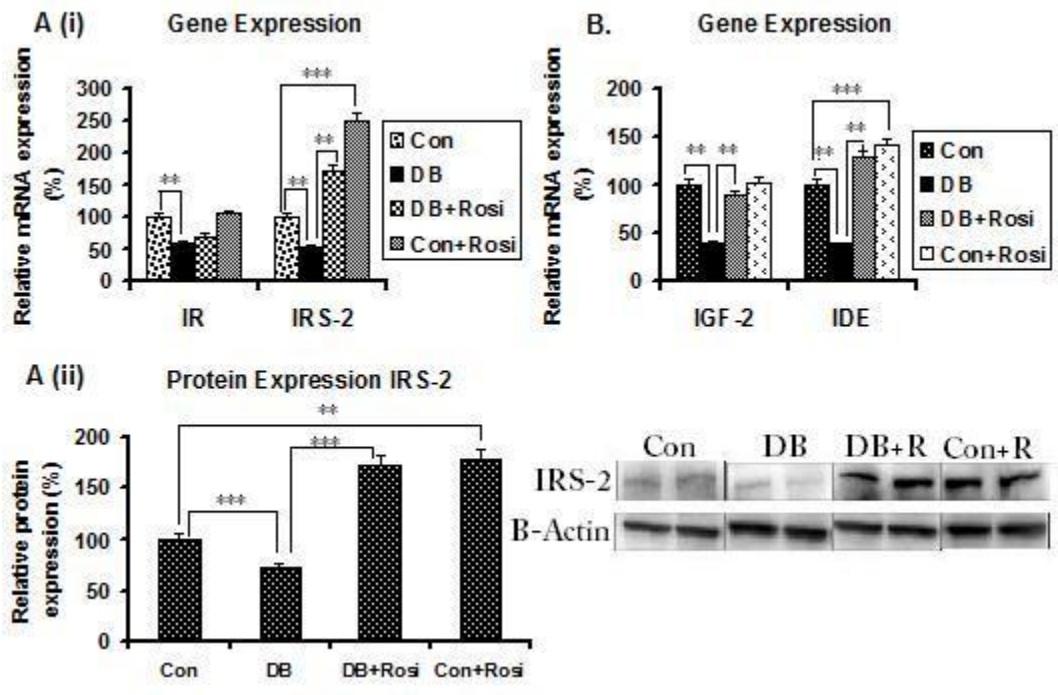


Fig.5.6: PPARγ activation improves the expression of Insulin signaling cascade in the diabeteic hippocampus. [**A**](**i**) Relative mRNA expression of IR and IRS-2 decreased significantly in db/db mice hippocampus, however rosiglitazone (10mg/kg for two weeks) only improved IRS-2 mRNA expression levels in the hippocampus from db/db mice. [A] (ii) Relative protein expression of IRS-2 was significantly reduced in the diabetic hippocampus and was a significantly improved after rosiglitazone treatment. (**B**) Relative mRNA expression of IGF-2 and IDE decreased significantly in db/db mice hippocampus was increased to near control levels after rosiglitazone for two weeks (10mg/kg). IDE, a direct PPARγ target, was found to increase after rosiglitazone treatment in both diabetic and control mice. (**B**). All gene expression values and protein band density values were normalized to housekeeping gene and protein β-actin. Statistical significance is evaluated by student's two tailed t-test unpaired, significant level **p<0.01, ***p<0.001, n=5.Values are mean ± SEM.

Fig.5.7

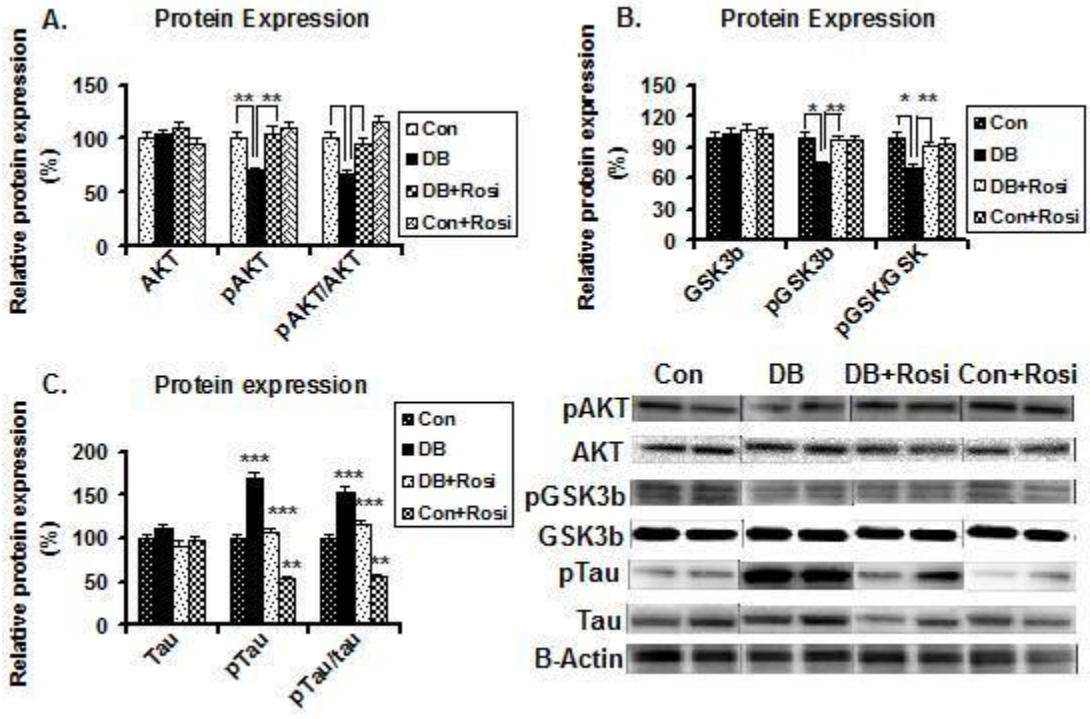


Fig.5.7: PPAR gamma activation improves AKT signaling in the diabetic hippocampus. [A] Rosiglitazone improved the reduced expression levels of p-AKT in the diabetic hippocampus. [**B**] GSK3β a direct effector of AKT, was also found to be attenuated in the diabetic hippocampus and was rescued to normal levels after rosiglitazone treatment (10mg/kg for 2 weeks). [**C**] Tau hyperphosphorylation was found to increase in the diabetic hippocampus and was attenuated by rosiglitazone treatment. Surprisingly Tau hyperphosphorylation also decreased in rosiglitazone treated control groups when compared to non-treated control group. All gene expression values and protein band density values are normalized to housekeeping gene and protein β-actin. Statistical significance is evaluated by student's two tailed t-test unpaired, *$p<0.05$, **$p<0.01$, ***$p<0.001$, N=5. Values are mean ± SEM.

Fig.5.8

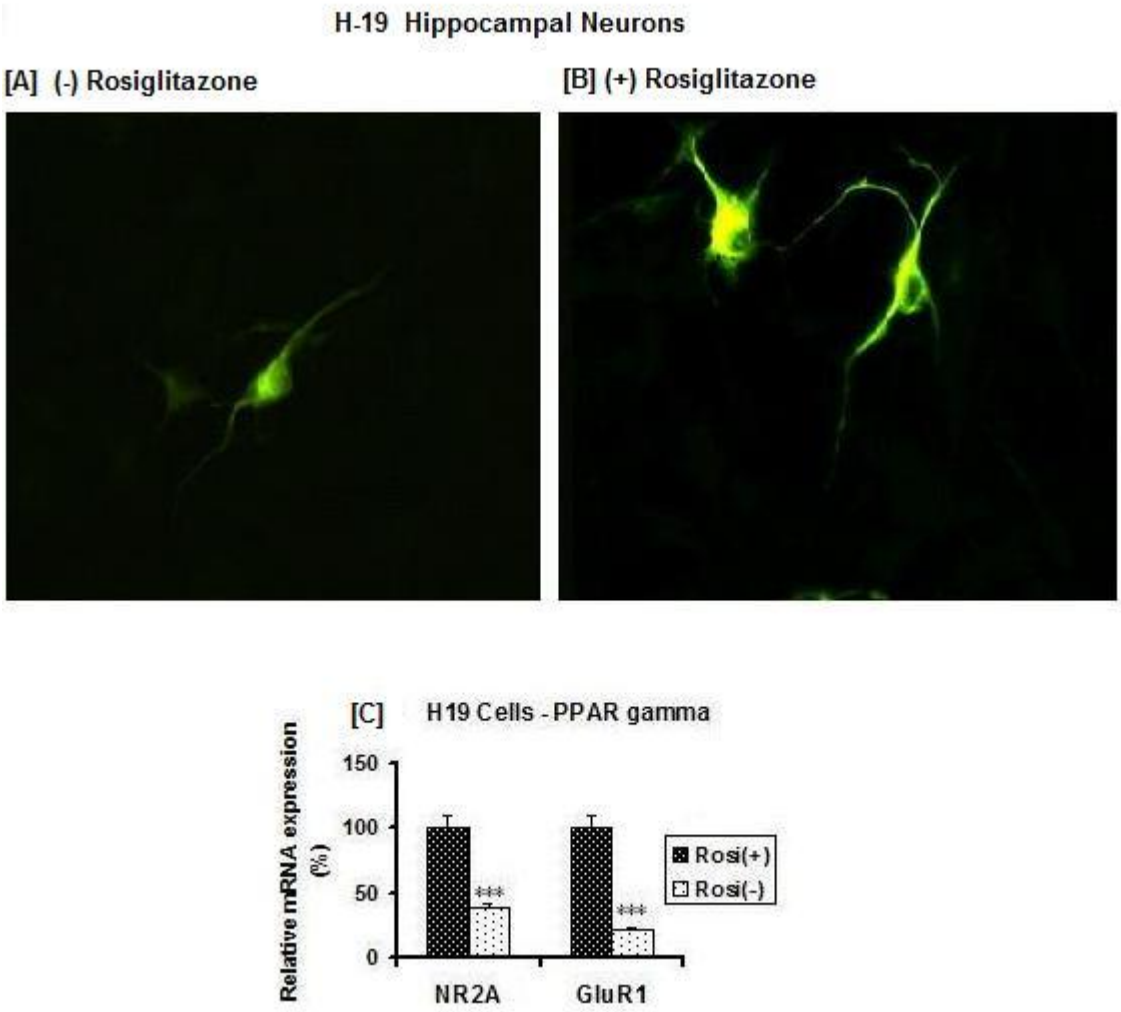


Fig.5.8: PPARγ induces dendritogenesis, spine formation, and dendritic arborization and mRNA expression of glutamate receptor subunits in neuronal cell cutures. (A) Differentiated H19 neuronal cell dendritogenesis and dendrite arborization is visualized by fluorescence microscopy with and without Rosiglitazone treatment. (**B**) Rosiglitazone induces and increases dendritogenesis and dendrite arborization in H19 neuronal cells as visualized by fluorescence microscopy. (C) Relative mRNA expression level of AMPAR subunit GluR1 and NMDAR subunit NR2A increased significantly) in differentiated H19 hippocampal pyramidal neuronal cells treated with rosi (+Rosi) compared to non-treated (-Rosi) H19 cells. Dendrite

arborization and spine formation was found to increase after rosiglitazone treatment when compared to control H-19 neurons. Postsynaptic glutamate receptor genes increased in expression after rosiglitazone treatment in differentiated H19 neuronal cells. All gene expression values are normalized to housekeeping gene β-actin. Statistical significance is evaluated by student's two tailed t-test unpaired, *$p<0.05$, **$p<0.01$, ***$p<0.001$, N=5. Values are mean ± SEM.

DISCUSSION

Our studies clearly demonstrated that hippocampal spatial memory and recognition memory are impaired in type 2 diabetes and can be ameliorated by two weeks oral treatment of PPARγ agonist rosiglitazone. This *in vivo* oral administration possitively modulates the hippocampal function by enhancing basal synaptic transmission and synaptic plasticity. The cognitive function is mainly determined by hippocampal synaptic plasticity (Milner et al., 1998). There are no sufficient reports on the characterization of type 2 diabetes-induced cognitive dysfunction and synaptic deficits. Most of the studies were reported on STZ-induced type 1 diabetic animal models with related cognitive dysfunction. Recently intra-cerebroventricular (icv) STZ-induced central insulin resistance model of rodents attracted specific attention due to its resemblances to sporadic AD pathology and cognitive dysfunction (De lamonte et al., 2006; Shonesy et al., 2011). Understanding the molecular mechanisms of type 2 diabetes induced cognitive deficits may lead to the development of potential molecular targets for pharmacological therapy.

Presently PPARγ agonist rosiglitazone is a drug that is found to improve peripheral insulin sensitivty. However, its effects upon the brain have not been elucidated. Few studies have

found the role of PPARγ in neurprotection in strokes by having anti-inflammatory properties. Interestingly, PPARγ activation has been shown to enhance cognitive function in the AD model. Specific molecular mechanisms of PPARγ mediated enhancement of synaptic plasticity is not yet elucidated. Our preliminary data showed that PPARγ gene expression was down regulated in diabetes type 2 db/db mice hippocampus and there was no change in PPARδ expression. Interestingly, daily rosiglitazone oral treatment for two weeks enhanced PPARγ gene expression in the hippocampus. This finding supports the role of PPARγ in diabetes mediated cognitive decline. Therefore, we studied db/db type 2 diabetes mice model and how their congnitive function and synaptic transmission are altered and how it can be ameliorated by PPARγ activation by administering PPARγ agonist rosiglitazone.

Daily administration of rosiglitazone for 15 days (10 mg/kg) was capable of producing enhanced hippocampal function and synaptic plasticity in our studies. PPARγ is expressed in the hippocampus and cortex of mice and rats. Therefore, a specific PPARγ agonist can act on these brain regions to enhance cognitve function. Recent studies showed the evidence for PPARγ agonist rosiglitazone to cross the blood brain barrier. Therefore, oral administration can provide sufficient rosiglitazone to the hippocampus of the brain. To support this, our preliminary data showed that PPARγ direct gene targets in the hippocampus increased in expression after rosiglitazone treatment such as IDE, IRS-2, SREBP and also expression of PPARγ receptor. Synaptic plasticity is a critical component of the neural mechanisms underlying learning and memory. Long-term potentiation, an activity-dependent synaptic plasticity, plays a significant role in the forms of memory mediated by the hippocampus. Hippocampus is the major brain structure that plays a crucial role in learning and memory and widely used as a model to study synaptic plasticity. To support PPARγ mediated amelioration of hippocampal spatial memory

and recognition memory, we investigated extracellular field recordings of basal synaptic transmission and LTP in the hippocampal Schaffer-collateral CA3 and CA1 pyramidal cells. We found that there was a significant alteration in the short-term plasticity and presynaptic influence in these type 2 db/db mice due to impaired post-tetanic potentiation. Paired pulse facilitation (PPF) in consistent with post-tetanic potentiation (PTP) propose a significant contribution of presynaptic mechanism in addition to the postsynaptic mechanism in the enhanced synaptic plasticity by PPARγ activation in our study. Normal Schaffer-collateral LTP has been linked to postsynaptic mechanisms (Zamanillo et al., 1999). However, the possibility of involvement of presynaptic mechanism should be investigated further (Lisman, 2003, Sanes and Lichtman, 1999; Zakharenko et al., 2001). Enhanced LTP and PTP in our study suggest that the memory enhancing mechanism of rosiglitazone treatment might be chiefly at the postsynaptic and presynpatic glutamatergic fibers. LTP is the cellular and molecular model to explain synaptic plasticity and long-term memory and glutamatergic system, plays a crucial role in fast excitatory synaptic transmission in the mammalian brain and it is responsible for hippocampal long-term synaptic plasticity. Moreover, long-term potentiation (LTP) is impaired in diabetes type 2 and ameliorated to normal level by rosiglitazone treatment. To explain this behavioral and electrophysiological finding we investigated postsynaptic glutamate receptor expression in the hippocampus. Present evidence supports that LTP could occur due to an increase in single ion channel conductance or an increase in the number of AMPARs expressed at synapses (Benke et al., 1999; Luthi et al., 2004). LTP expression also involves additional AMPARs insertion into the postsynaptic membrane and stimulation of their biosynthesis. Phosphorylation of AMPARs is one means by which the biophysical properties such as ion channel kinetics of these channels are modified and ion channel trafficking also altered. Also consistent with electrophysiological

findings behavioral tests revealed that administration of rosiglitazone enhances the memory retention. The delay between first trial and retention test was pre set to 4 hours in Y maze and ORT respectively since we expected the drug treatments to improve memory performance. This delay was sufficient to produce a loss of memory retention in mice of this age group. As expected the control mice did not discriminate between the objects above chance level and in Y maze they had equal alterations in all 3 arms.

We investigated the role of postsynaptic glutamate receptor expression and the alterations in their subunit composition in diabetes and rosiglitazone treatment. The subunit composition of AMPARs has a dramatic effect on synaptic plasticity. Whereas, GluR2 containing receptors have low calcium permeability and have outwardly rectifying properties, GluR1 containing AMPARs have high calcium permeability and have an inwardly rectifying IV relationship (Hollmann et al., 1991; Burnashev et al., 1992).

Interestingly, mRNA and protein expression of glutamate receptor AMPAR subunit GluR1 and NMDAR subunit NR2A were downregulated in the type 2 diabetes hippocampus and ameliorated to its normal control level after two weeks treatment of rosiglitazone. GluR1 plays a key role in the trafficking of AMPAR receptors to the postsynaptic membrane. It is believed that mRNA transcripts are trafficked to the dendritic compartment where they are locally translated. Based on this we speculated that insulin signaling might play some role in regulating or maintaining basal mRNA trafficking of GluR1 transcripts. It is mediated by BDNF mediated phosphorylation of GluR1 and GSK3β signaling. Recent immunoprecipitation studies showed that GluR1 is co-localized with GSK3β and when phosphorylation of GSK3β is decreased, more GluR1 will be incorporated into postsynaptic sites. This was supported by auxiliary regulated proteins PSD-95 and stargazin in the postsynaptic sites. Their expression level reduced in

diabetes and rescued by rosiglitazone treatment. Furthermore, the reduction in the transmembrane AMPA receptor regulatory protein stargazin suggests possible changes in AMPARs trafficking as well as single channel properties as this protein is known to play an essential role in both (Priel et al., 2005) (Bats et al., 2007).

Postsynaptic auxiliary molecule PSD-95 supports AMPAR receptor trafficking to postsynaptic sites and AMPAR ion channel kinetics and facilitate phosphorylation of GluR1. Therefore, PSD-95 and stargazin support more incorporation of AMPAR into the postsynaptic sites and stargazin enhances ion channel conductance and kinetics of AMPAR in the postsynaptic sites thus leading to enhanced LTP in rosiglitazone treated group. NMDAR subunit NR2A gene and protein are down regulated in diabetes and ameliorated to control level by rosiglitazone treatment. Earlier studies showed that NR2A knockout mice showed memory impairment. This finding is correlated with our studies and showed the role of NR2A in the synaptic plasticity. There is no alteration in NR2B gene and protein expression level. Therefore, decreased NR2A/NR2B ratio in db/db mice supports deficits in LTP. Decreased NR2A also causes calcium excitotoxicity due to impaired calcium homeostasis in diabetes and is ameliorated by rosiglitazone treatment. The changes in glutamate receptor expression that occur in db/db mice are in contrast to the previously reported up-regulation of these proteins in spontaneous type-1 diabetic animals (Valastro et al., 2002). This interesting difference suggests that although insulin deficiency and insulin resistance both result in memory impairment, it is likely that the two are mediated by different underlying mechanisms. Interestingly, H19 hippocampal neuronal cells showed increased gene expression of GluR1 and NR2A after rosiglitazone administration in the cell culture. This finding supports rosi mediated increased expression of these receptors in the hippocampus of db/db mice.

Transcription co-factor CBP, a CREB binding protein and synaptic plasticity master regulator CREB play a key role in synaptic plasticity and LTP. They play a crucial role in neurotrophic factor BDNF regulation also. Recent studies showed that hippocampal gene delivery of CBP (CBP overexpression vector construct) increased CREB phosphorylation and elevated BDNF level and rescued cognitive function in the AD mice model (Caccamo et al., 2010). Therefore, we studied CBP/CREB/BDNF expression in the hippocampus of db/db mice and the effect of rosiglitazone treatment. Downregulation of CBP/CREB/BDNF gene expression and CBP/BDNF protein expression support the LTP deficit in db/db mice and the rosiglitazone treatment rescued CBP/CREB/BDNF expression at gene and protein level. Therefore increased CBP expression and increased CREB expression and CREB phosphorylation enhances synaptic plasticity and LTP. Our immunohistochemical studies showed diminished expression level of BDNF in the CA1-CA3 pyramidal cells in the hippocampus and they increased to normal level after rosiglitazone treatment. Therefore, increased level of BDNF in the CA1-CA3 region of the hippocampus enhances synaptogenesis, dendritogenesis and neurogenesis and suppresses neurodegeneration.

Insulin signaling plays a key role in synaptic plasticity and LTP; however, its mechanism is not well understood. Aging and AD studies showed that insulin signaling is impaired which leads to cognitive impairment and deficit in synaptic plasticity, indicating that insulin-signaling pathways are impaired in the AD brain (Frolich et al., 1999; Rivera et al., 2005; Cole and Frautschy, 2007). Epidemiological evidence that type-2 diabetes is a major risk factor for AD. This suggests an involvement of insulin signaling in synaptic physiology. Previous studies have reported decreased IR expression and IR desensitization in AD brains (Hoyer, 2004; Steen et al., 2005); moreover, since AD is also associated with decreased glutamate receptor levels (Bi and

Sze, 2002), our results add to the emerging evidence that brain insulin resistance may be a key feature of AD pathology.

Recent studies suggested the correlation between glutamatergic system and insulin signaling in AD related synaptic deficit. Therefore, we studied the expression of insulin signaling molecules and their signaling pathways in the hippocampus in db/db mice and the effect of rosiglitazone treatment. We found that db/db mice showed downregulation of IR, IRS-2, IGF-2 and IDE genes and ameliorated by rosi treatment. Protein level of IRS-2 also followed the same expression pattern and compatible with its gene expression pattern. Interestingly IRS-2 and IDE mRNA expression level increased when control group was treated with rosiglitazone. Interestingly, we found that IRS-2 promoter has a PPARγ binding element (PPRE) AGGTCA. Previous studies found that IDE also has PPRE in its promoter. Therefore, their higher gene expression level after rosi treatment can be explained by PPARγ directly binding to their promoter and increasing their expression. Rosiglitazone treatment improved mRNA expression level of IRS-2, IDE and IGF-2 but IR level was not altered. IGF-2 enhances neuronal differentiation and neurogenesis and improves synaptic plasticity. Increased expression of IDE enhances degradation of Aβ in the brain and resists AD pathology and Aβ mediated glutamatergic disruption thus enhances LTP and synaptic plasticity. IR protein level can be altered by increased trafficking. IRS-2 phosphorylation (serine) is required for insulin signaling (Rother et al., 1998). Therefore, increased IRS-2 expression by rosiglitazone treatment supports that phosphorylation of IRS-2 increases and enhances insulin signaling thus leading to increased LTP and synaptic plasticity.

IRS-2 plays a crucial role in AKT/GSK3β signaling and Tau hyperphosphorylation. In the hippocampus Akt is the primary kinase involved in the phosphorylation of glycogen synthase

kinase (GSK3β). This phosphorylation decreases GSK activity, which was recently demonstrated to be necessary for the insertion of GluR1 containing receptors during the induction of LTP. GSK3β involved in the regulation of hippocampal synaptic plasticity. These findings report that GSK3β activity is inhibited during the induction of LTP in the hippocampus. Based on this, it is not surprising that LTP is impaired in a transgenic mouse with GSK3β overexpression, which displays enhanced GSK3β activity. Moreover, these LTP deficits can be attenuated by treatment with various GSK3β inhibitors such as lithium. It has also recently been revealed that GSK3β directly complexes with AMPA receptors, the primary receptor responsible for LTP, and regulates the surface expression of these according to its phosphorylation state (Hooper et al., 2007; Peineau et al., 2007; Zhu et al., 2007). Therefore, since reduced IR expression and binding affinity are known to be present in the brains of diabetes type 2, we investigated the expression and activity of GSK3β and Akt. Our studies investigated these signaling pathways of AKT/GSK3β and found that IRS-2/AKT/GSK3β signaling is diminished in db/db and enhanced to its normal control level by rosiglitazone treatment. The db/db mice showed decreased phosphorylation of AKT and GSK3β thus decreased AKT activity and increased GSK3β activity leading to increased Tau hyperphosphorylation. This effect was reversed by rosiglitazone treatment and Tau hyperphosphorylation was reduced in db/db treated with rosiglitazone. Increased AKT phosphorylation also increased the expression of BDNF. This supports our study of elevated BDNF expression by rosiglitazone treatment. Interestingly *in vitro* studies of H19 hippocampal cells showed that rosiglitazone treatment increased dendritogenesis, spine formation and neuronal arborization. This is supported by elevated levels of BDNF and IGF-2 and improved insulin signaling after rosiglitazone treatment.

Based on our studies we suggest the crucial role of postsynaptic glutamatergic system in the hippocampal synaptic plasticity and cognitive function. In the same time, we can not exclude other signaling pathways, and our data in fact suggests that multiple factors may converge via IRS-2/AKT/GSK3β/BDNF/Tau and CBP/CREB/BDNF to modulate glutamatergic transmission in the hippocampus. We can therefore, suggest that these modulatory pathways may require a functional insulin signaling system without which severe deficits in synaptic plasticity would occur. Since diabetes is a complex disorder, its pathophysiology can be explained by more than one mechanism. Therefore, future studies are required to investigate the deficits in synaptic plasticity and cognitive impairment in different type 2 diabetes animal models. Also studying the effect of PPARγ activation in the hippocampus of diabetes type 2 mice models will help to identify molecular targets for developing therapies in the future for diabetes and AD related dementia. In addition, it will also help establish the molecular link between AD and diabetes type 2 mediated cognitive dysfunction. Moreover, PPARγ can also be a tool to elucidate the synaptic plasticity impairing mechanisms in the hippocampus in the type 2 diabetes.

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6. SUMMARY AND CONCLUSIONS

The current study investigated the mechanisms by which alterations in the glutamatergic system leads to cognitive dysfunction in models of aging. We utilized animal models of aging, AD and diabetes to study the molecular mechanisms of cognitive dysfunction.

Our results from the long-lived Ames dwarf mice suggests improvement of synaptic plasticity in the hippocampus was due to reduced inflammation, increased acetylcholine synthesis and increased energy production in the hippocampus. Furthermore, we found an increase in expression of the postsynaptic glutamate receptor and postsynaptic auxiliary protein, PSD-95 leading to enhanced synaptic plasticity. The PSD-95 protein has been shown to play a key role in trafficking of postsynaptic AMPAR receptors to the postsynaptic surfaces resulting in the enhanced synaptic plasticity.

Aβ is one of the major hallmarks of AD. Aβ (1-42) is a potential Aβ species causes neurotoxic effect and severe neurodegeneration. The mechanism by which exogenous Aβ (1-42) induces cognitive impairment is not well understood. Therefore, our study was aimed to elucidate the mechanism by which Aβ causes early cognitive decline prior to severe neurodegeneration. Our results indicate postsynaptic glutamatergic dysfunction leads to altered behavioral deficits observed in an Aβ infused model. Interestingly, cognitive deficits were observed in this model prior to oxidative stress and inflammation. This decline in memory deficits may be due to the decrease in postsynaptic glutamatergic dysfunction.

The type 2 diabetes is one of the major causes of dementia and several reports have shown glutamatergic dysfunction in animal models of diabetes. Our study suggests that PPARγ improves diabetes induced cognitive deficits by modulating glutamatergic neurotransmission by enhancing the expression and function of postsynaptic glutamate receptors. Most surprisingly,

we found an increase in BDNF, a neurotropic factor that is directly involved in the regulation of postsynaptic receptor expression and function. Lastly, we found that PPARγ enhances IRS2-AKT signaling and several mediators involved in the insulin-signaling pathway as well as in protecting against Tau-hyperphosphorylation. Consequently, a future direction for the applicability of the current findings would further validate the significance of brain specific PPARγ in protecting against diabetes induced cognitive impairment. This can be accomplished by developing a diabetic animal model with brain specific PPARγ knock-out and subsequent administration of peripheral PPARγ agonist.

Our overall findings support mechanistic basis of synaptic deficits in aging, AD and diabetes due to postsynaptic impairment of glutamatergic system including glutamate receptor expression, localization, trafficking and function. The hope of the current research will offer insights into potential therapeutic molecular targets for ameliorating aging, AD, and diabetes induced cognitive dysfunction.