

**Role Of Integrin-Linked Kinase In Synaptic Plasticity And Memory:
Diabetes/Alzheimer's Link**

by

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Abstract

Type 2 diabetes mellitus (T2DM) has recently been proven to be a risk factor for cognitive dysfunction and dementia such as Alzheimer's disease (AD). However, the exact pathophysiology of cognitive dysfunction in patients who suffer T2DM remain to be clearly defined. Our recent study using intracerebroventricular streptozocin (ic. STZ)-injected rats- a model of sporadic AD with central insulin resistance and brain insulin deficiency revealed that hippocampal synaptic impairments and deficits in long term potentiation (LTP - a cellular model of memory), correlated well with alterations in integrin linked kinase (ILK) signaling, a serine-threonine kinase that functions downstream of growth factor and β -integrin receptors to regulate cell growth and differentiation. However, a causal link between ILK, synaptic transmission and plasticity has not yet been investigated. In the current study, inhibition of ILK activity by using a potent and specific pharmacological modulator resulted in impaired synaptic transmission and plasticity in the hippocampus. Additionally, induction of chemical LTP (cLTP) in organotypic hippocampal slice cultures enhanced ILK activity. Taken together, this study demonstrated for the first time, an essential role of ILK in hippocampal synaptic transmission and plasticity required for information storage (memory) in the brain.

A Recent study reported that phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity, is upregulated in the fat tissue of T2DM patients and type 2 diabetic mouse model db/db mice. In addition, studies demonstrated that activation of the nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR) delta, modulates PTEN activity and enhance ILK expression and activity. PPAR delta agonists have been recently proven to exert neuroprotective effects in experimental models of AD. However, it remains

unclear if activation of PPAR delta will improve cognitive dysfunction caused by type II diabetes. Therefore, in view of the above findings and due to wide spread prevalence of PPAR delta in the brain, we investigated the effect of high affinity PPAR delta agonist GW0742 on hippocampal long-term potentiation and spatial memory in the type II diabetic animal model db/db/ mice. Our findings suggest that molecular targets such as PPAR delta may offer potential therapeutic targets for cognitive dysfunction in patients with T2DM.

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1. INTRODUCTION

Several lines of evidence suggest a relationship between type II diabetes mellitus (T2DM) and cognitive dysfunction such as Alzheimer's disease (AD). (Leibson et al., 1997; Janson et al., 2004; Biessels et al., 2006; Kulstad et al., 2006; Zhao et al., 2009; Park, 2011). There are now more than 250 million diabetes patients all over the world, and it is predicted that by 2030, 360 million people will have diabetes (Wild et al., 2004). Diabetes is not the only disorder that will increase in prevalence over this time period. The latest World Alzheimer report, released by Alzheimer Disease International estimated that, in 2010, more than 35 million people are living with dementia. Researchers predict that the global prevalence of AD, the most common form of dementia, and accounting for approximately 90% of all cases (Ritchie and Lovestone, 2002) will almost double every 20 years to be 65.7 million in 2030 and 115.4 million in 2050. In a clinical context, patients with type II diabetes appear to have two to three fold increased relative risk for AD (Stolk et al., 1997; Ott et al., 1999; Petot et al., 2003; Zhao et al., 2009) and more than 80% of AD patients appear to have type II diabetes (Craft et al., 1998; Messier, 2003; Janson et al., 2004; Cole and Frautschy, 2007; Zhao et al., 2009; Park, 2011). In addition, AD patients show peripheral hyperinsulinemia, insulin resistance and brain insulin deficiency (Craft et al., 1998; de la Monte et al., 2006), making AD a neuroendocrine disorder that represents a brain-specific form of diabetes, i.e. type 3 diabetes (Steen et al., 2005; Kronez, 2009). Cognitive impairments have also been reported in experimental models of type II diabetes. For example, impaired hippocampus-dependant long-term potentiation and spatial memory have been demonstrated in type II diabetic mouse strain db/db, characterized by hyperinsulinemia and hyperglycemia (Li et al., 2002; Sharma et al., 2010; Dinel et al., 2011). Similar deficits have been also reported in intracerebroventricular streptozocin-injected rats (ic.

STZ), a model of sporadic AD with central insulin resistance and brain insulin deficiency (Biessels et al., 1996; de la Monte et al., 2006; Shonesy et al., 2012). The exact pathophysiology of cognitive dysfunction in patients who suffer T2DM is not completely understood (Kodl and Sequist, 2008) and the mechanisms underlying this association remain to be clearly defined (de la Monte et al., 2006; Watson and Craft, 2006). Recently, we assessed the synaptic mechanisms underlying learning and memory deficits in ic.STZ animals. Changes in glutamatergic synaptic physiology in ic.STZ rats have been examined at cellular, molecular and neuronal circuit level. Results of these studies revealed abnormalities in expression and function of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) (AMPA) and N-Methyl-D-aspartic acid (NMDA) synaptic receptors. Most importantly, these changes in glutamate receptors expression and function in ic.STZ rats were accompanied by a down-regulation of one of the downstream effectors of integrin and insulin signaling, “Integrin-Linked kinase” (ILK) (Shonsey et al., 2012). ILK is a serine-threonine kinase that binds to integrins and couples insulin receptors to downstream signaling pathways that modulate crucial cell functions involving protein kinase B/AKT (PKB/AKT), and glycogen synthase kinase-3 β (Delcommenne et al., 1998; Dedhar et al., 1999). The results of these recent studies suggested a correlation between LTP deficits in ic. STZ animals, and alteration in integrin-linked kinase (ILK)-glycogen synthase kinase 3 beta (GSK3- β) signaling. The aim of the present study is to establish causal link between ILK, synaptic plasticity and memory. Furthermore, novel experimental techniques are used to investigate the molecular mechanisms by which ILK mediate synaptic transmission and plasticity from a single receptor level to more complex mechanisms that affect memory formation.

A recent study reported that, phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity, is upregulated in fat tissue of T2DM patients and type 2 diabetic mouse model

db/db mice (Yu et al., 2011). In addition, studies demonstrated that activation of nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR) delta, modulated PTEN activity and enhanced ILK expression and activity (Di-Poï et al., 2002). PPAR alpha, gamma and delta represent PPAR subfamily of the nuclear hormone receptor superfamily. These receptors regulate gene transcription in response to ligand binding to specific promoter regions of target genes (Schoonjans et al., 1996; Blumberg et al., 1998; Willson et al., 2000; Chawla et al., 2001). PPARs play a role in regulation of lipid and glucose metabolism and they have been successfully exploited to be clinically used in the treatment of metabolic diseases like hyperlipidemia and type-2-diabetes (Robinson and Grieve, 2009; Bilsen and Nieuwenhoven, 2010). Activation of PPARs has been demonstrated to exert additional benefits on the central nervous system. In this regard, Pedersen et al (2006) reported that treatment of Tg2576 mice with the PPAR gamma agonist rosiglitazone significantly enhanced spatial memory and decreased brain level of β -amyloid 1-42 (Pedersen et al., 2006). Clinical trials of rosiglitazone reported significantly improved cognition in AD patients (Chalimoniuk et al., 2004; Watson et al., 2005; Risner et al., 2006). In addition, activation of PPAR delta which is the predominant isotype in the brain (Braissant et al., 1998; Woods et al., 2003) has been recently proven to exert neuroprotective effects. Early treatment with PPAR delta agonists, prevented neurodegeneration and associated cognitive deficits in an experimental model of sporadic AD (de la Monte et al., 2006). Activation of PPAR delta by the high affinity synthetic drug GW0742 decreased brain inflammation and amyloid burden in transgenic animal model of AD (Kalinin et al., 2009). However, it remains unclear if activation of PPAR delta will improve cognitive dysfunction caused by type II diabetes. Therefore, In view of the above findings, we investigated the effect of the high affinity

PPAR delta agonist GW0742 on hippocampal long-term potentiation and spatial memory in the type II diabetic animal model db/db/ mice.

In summary, T2DM might contribute to cognitive deficits observed in AD. Understanding the cellular and molecular mechanisms by which these two disorders are linked, may shed light on novel diagnostic tools and therapeutic targets for AD. This study was designed to establish a causal link between ILK and synaptic plasticity and to elucidate the mechanistic basis of the role played by ILK in synaptic plasticity and memory. The results of this study highlight important mechanisms by which T2DM and AD are linked. Most importantly, the clinical relevance of the use of PPAR delta agonists as new therapeutic regimen to bring about the desired improvement of T2DM-induced cognitive functions was also investigated. Successful use of these new therapeutic regimens could, at least, in part, ameliorate cognitive deficits in patients with T2DM and AD.

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2. LITERATURE REVIEW

Memory and the hippocampal formation:

Memory is a brain process that refers to the capacity to encode, store and retrieve information. Understanding the neural mechanisms of encoding, storing, retrieving and transforming unstable memory traces into more persistent structural and biochemical changes in the nervous system is a great challenge in neuroscience. It will enable the development of novel therapeutic targets for cognitive disorders (Morris, 2006). For decades, the hippocampus has been one of the most widely studied brain regions. In part, this is because of the vital role that the hippocampus plays in initial storage of memories (Kerchner and Nicoll, 2008). The central role of hippocampus in memory formation, has been arised from the clinical observations of Scoville and Millner (1957), showing that the damage of the hippocampus in neurological patients resulted in anterograde amnesia. Another attractive feature of the hippocampus is its simple and consistent laminar pattern of neurons and neural pathways which could facilitate the rapid encoding and storage of a huge numbers of arbitrary associations (Neves et al., 2008). Three main types of excitatory neurons in the hippocampus have been identified; a) (Dentate gyrus) granule cells project through their axons (mossy fibres) to the proximal apical dendrites of CA3 pyramidal cells. These CA3 neurons project through Shaffer collaterals to ipsilateral CA1 pyramidal cells. The CA1/Shaffer collateral neurons are responsible for the main output of the hippocampus proper. In addition, the CA3 neurons, through commissural connections, project to contralateral CA3 and CA1 pyramidal cells (Morris, 2006; Neves et al., 2008) (Fig.2.1.). However, the hippocampus does not operate in isolation, but it has important functional interactions with the neocortex, resulting in the transfer of older memories overtime out of the hippocampus and into the cortex (Morris, 2006). External inputs via different pathways from the

cortex are conveyed to neurons in the hippocampus. The major inputs project via the perforant pathway input from layer II of the entorhinal cortex to the dentate gyrus. A direct input from layer II entorhinal cortex also innervates CA3 pyramidal cells. Moreover, the CA1 pyramidal neurons are innervated by a direct input from layer III cells of the entorhinal cortex (Morris, 2006; Neves et al., 2008).

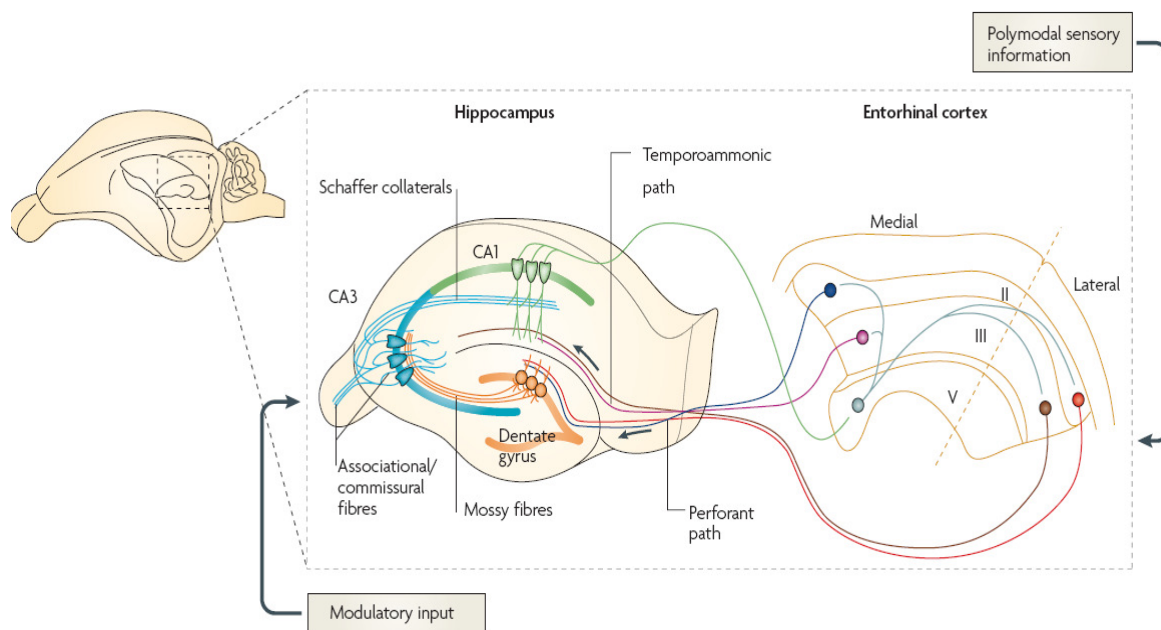


Fig.2.1. Excitatory neurotransmission in the hippocampus. Dentate gyrus projects through mossy fibres to the commissural fibers, which then connects the CA3 neurons to contralateral CA3 and CA1 pyramidal cells. **Adapted from Neves G., Cooke S.F., Bliss T.V.P. (2008).** Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nature Neurosci* 9: 65-75.

Synaptic plasticity and memory:

One of the most fundamental and fascinating feature of the mammalian central nervous system is its plasticity ie; its ability to modify neural circuit function and hence subsequent feelings, thoughts and behaviors (Citri and Malenka, 2008). The term “synaptic plasticity”, specifically refers to activity-dependant changes in the efficiency of synaptic transmission that lead to strengthening of association between neurons (Lynch, 2004; Citri and Malenka, 2008). It has

been proposed for over a century that synaptic plasticity in the brain plays a vital role in transforming transient experiences into persistent memory (Citri and Malenka, 2008). The Spanish Nobel laureate Santiago Ramon Y Cajal, over 100 years ago, hypothesized that memories are generated by an activity- dependant, long-lasting changes in the pattern of synaptic strength. In the late 1940, this hypothesis was further advanced by Donald Hebb, who proposed that, when presynaptic activity correlates with postsynaptic firing, synaptic modifications that strengthen connections will be able to generate memories (Hebb, 1949).

Long-term potentiation and memory:

In 1973, Bliss and Lomo, reported that, in the hippocampus, repetitive activation of excitatory synapses caused strengthening of synaptic connections that could last for hours or even days. This phenomena has termed “Long-term potentiation” (LTP), and it is widely believed that this particular form of synaptic plasticity provides an important key to understanding the molecular and cellular mechanisms by which memories are generated (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008). The LTP observed in the CA1 region of the hippocampus is the most extensively studied form of synaptic plasticity (Lynch, 2004; Citri and Malenka, 2008; Neves et al., 2008). The three well described properties of LTP “cooperativity, associativity and input specificity”, make it an attractive cellular mechanism for storage of informations and memory. “Cooperativity”, indicates that coincident activation of a critical number of synapses induces LTP. “Associativity”, means that when a weak input is activated in association with a strong input, it could be potentiated. “Input specificity”, is the capacity to induce LTP only at synapses stimulated at afferent synapses and not on adjacent inactive synapses located on the same postsynaptic cell (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008). Other evidence has consolidated the hypothesis that LTP may be a biological substrate for information storage and

memory. Like memories, which are classified into short-term and long-term memories, LTP consists of an early phase which lasts 2-3 hours (E-LTP) and long-lasting phase (L-LTP), which lasts for several hours (Lynch, 2004).

***N*-methyl-D-aspartate (NMDA) receptors NMDARs -dependant LTP:**

The two major types of glutamate receptors are NMDARs and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPA). These receptors are often co-localized at the postsynaptic density. Activation of AMPARs provides most of the inward current that contributes to the excitatory synaptic responses. This is due to the permeability of the AMPAR channels to monovalent cations (Na^+ and K^+). In contrast to AMPARs, NMDARs have little contribution to the postsynaptic response during basal synaptic activity. This is because of the block of NMDAR channels by extracellular magnesium at negative membrane potentials. However, upon depolarization of the cell and the subsequent dissociation of magnesium from its binding site on the NMDAR channels, monovalent cations (Na^+ and K^+) and divalent cations (Ca^{+2}) enter the cell. As NMDARs require both presynaptic release of glutamate and postsynaptic depolarization in order to elicit post synaptic response, these receptors are often referred as a 'coincidence receptor' (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008).

Properties of NMDARs:

NMDARs are heteromeric assemblies of NR1 (GluN1, with 8 different splice variants), NR2 (GluN2A, GluN2B, GluN2C and GluN2D) and NR3 (GluN3A and GluN3B) subunits (Cull-Candy and Lesskiewicz, 2004; Rebola et al., 2010). NMDARs subunits consist of a long extracellular N-terminal domain, three membrane-spanning domains, a re-entrant loop that form the pore region, and an intracellular C-terminal domain of variable length (Lau and Zukin, 2007; Rebola et al., 2010). NR2A and NR2B subunits have been extensively studied as they are widely

expressed in the brain and they play fundamental roles in synaptic plasticity (Yashiro and Philpot, 2008). NR2A and NR2B NMDARs subunits are present as either di-heteromers (NR1/NR2A or NR1/NR2B) or tri-heteromers (NR1/NR2A/NR2B). NR2A and NR2B-containing NMDARs exhibit different channel kinetics, protein binding partners and synaptic localization (Yashiro and Philpot, 2008). An important determinant of the functional properties of NMDARs, is the N-terminal domain of NR2 subunits (Law and Zukin, 2007; Rebola et al., 2010). The NR2A subunit confers faster deactivation and greater channel open probability than does the NR2B subunits (Law and Zukin, 2007; Yashiro and Philpot, 2008). However, the low peak current of NR2B subunits carries two fold more charge than NR2A subunits for a single synaptic event. This occurs because; the deactivation of NR2B subunits is slow enough to compensate for the low open probability of NR2B subunits. In addition, NR2B subunits were found to carry more calcium per unit current than does the NR2A subunit (Yashiro and Philpot, 2008). The C-terminal domain of NR2A and NR2B subunits contain PDZ-binding motifs, through which they can interact with a family of synaptic scaffolding proteins known as membrane-associated guanylate kinase (MAGUK). The interaction with these scaffolding proteins physically links NMDARs to intracellular signaling pathways and controls their synaptic localization (Law and Zukin, 2007; Yashiro and Philpot, 2008; Rebola et al., 2010). Postsynaptic density protein-95 (PSD-95; also known as PSD-protein of 95 kDA), and synapse-associated protein-102 (SAP-102) are members of the large MAGUK family that are essential for anchoring NMDARs in the postsynaptic density. In addition, these scaffolding proteins play an important role in the intracellular trafficking and synaptic delivery of NMDARs. PSD-95 promotes clustering and surface expression of NMDARs. Moreover, PSD-95 was found to affect NMDAR channels kinetics, it reduces the desensitization of NMDARs responses and enhances

NMDA channel opening (Lin et al., 2004; Lin et al., 2006; Law and Zukin, 2007). The differential association of NMDARs subunits with MAGUK family members is controversial. It was assumed that NR2A preferentially binds to PSD-95, while NR2B preferentially binds to SAP-102. However, a recent immunoprecipitation study suggests that PSD-95 and SAP-102 interact at comparable level with NR2A and NR2B subunits (Yashiro and Philpot, 2008).

In addition to its role in the NMDARs/MAGUK proteins interaction, the C-terminal domain of NR2A and NR2B subunits plays an important role in NMDARs internalization. NR2A and NR2B subunits contain distinct internalization motifs in their distal C-termini, (LL) in NR2A and (YEKL) in NR2B subunits. These internalization motifs interact with the clathrin and adaptor protein (AP2) and initiate clathrin-dependant endocytosis at somewhat different rates. The internalization of NR2B occurs more slowly than that of NR2A. Following NMDARs endocytosis, NR2B subunits are preferentially targeted for recycling, whereas, NR2A receptors are preferentially targeted for degradation. Studies have shown that NMDARs move laterally between synaptic and extrasynaptic domains. This lateral mobility of NMDARs is likely to be fundamental step in NMDARs internalization. NR2B was found to exhibit faster lateral mobility ($\approx 500 \times 10^{-4} \mu\text{m}^2 \text{sec}^{-1}$) than NR2A ($\approx 2 \times 10^{-4} \mu\text{m}^2 \text{sec}^{-1}$) (Law and Zukin, 2007). Therefore, it has been proposed that NR2B-containing NMDARs are more likely to occupy extrasynaptic sites, while NR2A-containing NMDARs are preferentially targeted to the central portion of the synapse. However, the assumption that the subunit composition of NMDARs differ between synaptic and extrasynaptic sites is controversial (Yashiro and Philpot, 2008).

Induction of *N*-methyl-D-aspartate (NMDA) receptors NMDARs -dependant LTP:

The critical event leading to the induction of NMDA-dependant LTP in the CA1 region of the hippocampus is the increase in postsynaptic calcium concentration to reach some critical

threshold value, as a consequence of strong postsynaptic depolarization and NMDARs activation. This can be also triggered experimentally, by applying theta-burst stimulation or high-frequency tetanic stimulation protocols (Lynch, 2004; Citri and Malenka, 2008). Several signal transduction proteins have been suggested to contribute to the translation of the calcium signal that is required to induce LTP into long-lasting rise in synaptic strength (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008). For defining a role of a protein as a mediator of LTP induction, some basic criteria had been suggested; for example, if the induction of LTP can be blocked by inhibiting the activation of such protein or if a potentiation of synaptic transmission will occur as a result of activation of this protein (Citri and Malenka, 2008). Among the early findings was that calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII), one of the most abundant proteins in neurons, is an essential component of the molecular machinery for LTP. It has been shown that, after induction of LTP, persistent activation of CaMKII occurs as a result of autophosphorylation at Thr-286 (Lynch, 2004; Citri and Malenka, 2008). Several studies further, indicated the implication of many other kinases in the triggering of LTP. Activation of the cyclic adenosine monophosphate-dependent protein kinases (PKA) are enhanced after LTP induction (Lisman 1989; Mackinston et al., 1999). In addition, the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway has also been shown to be essential for LTP, as inhibition of this kinase resulted in suppression of LTP in CA1 (Impey et al., 1998). Moreover, PI3-kinase has been implicated in the early expression of LTP and triggering LTP was found to be associated with the PI3-kinase activation (Sanna et al., 2002). Protein kinase C and specifically the atypical PKC isozyme PKM ξ , has been implicated in long-term maintenance of LTP (Serrano et al., 2005).

Expression of LTP:

It has been acknowledged for decades the importance of AMPARs in fast excitatory synaptic transmission and that, an activity-dependant increase in the number of AMPARs within the postsynaptic density could significantly contribute to the expression of LTP at hippocampal CA1 synapses (Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir 2002; Brecht and Nicoll 2003; Derkach et al., 2007). The proposal of the “silent synapse” hypothesis further strengthened the importance of AMPARs incorporation into the postsynaptic plasma membrane as a primary requirement for the expression of LTP (Malenka and Nicoll., 1997; Lynch, 2004; Citri and Malenka, 2008). Silent synapses are those synapses which only contain NMDARs, they lack or they may contain few AMPARs. These synapses does not exhibit detectable post synaptic responses to synaptically released glutamate at normal resting membrane potentials, however, during LTP, these synapses are unsilenced via the insertion of AMPARs into the postsynaptic density (Liao et al., 1995; Brecht and Nicoll, 2003; Malinow and Malenka, 2002, Song and Huganir, 2002; Citri and Malenka., 2008).

Properties of AMPARs:

In the CNS, most AMPARs are tetramers composed of four subunits: GluR1-4 (or GluRA-D) (Braithwaite et al., 2000; Jiang et al., 2006; Victor et al., 2007; Citri and Malenka, 2008). At mature hippocampal CA3-CA1 excitatory synapses, AMPARs are heteromers that consist predominantly of GluR1/GluR2 AMPARS subunits. Each AMPARs subunit consists of an extracellular N-terminus, 3 transmembrane-spanning domains (TM1, TM3 and TM4), one re-entrant transmembrane domain (TM2) and an intracellular C-terminus. The N-terminal domain and the loop between TM3 and TM4 are extracellular and they form the ligand-binding core of the receptor. The C-terminal domain is intracellular, and it represents the interaction site for the

proteins that regulate AMPARs trafficking and signaling (Braithwaite et al., 2000; Jiang et al., 2006) (Fig.2.2.).

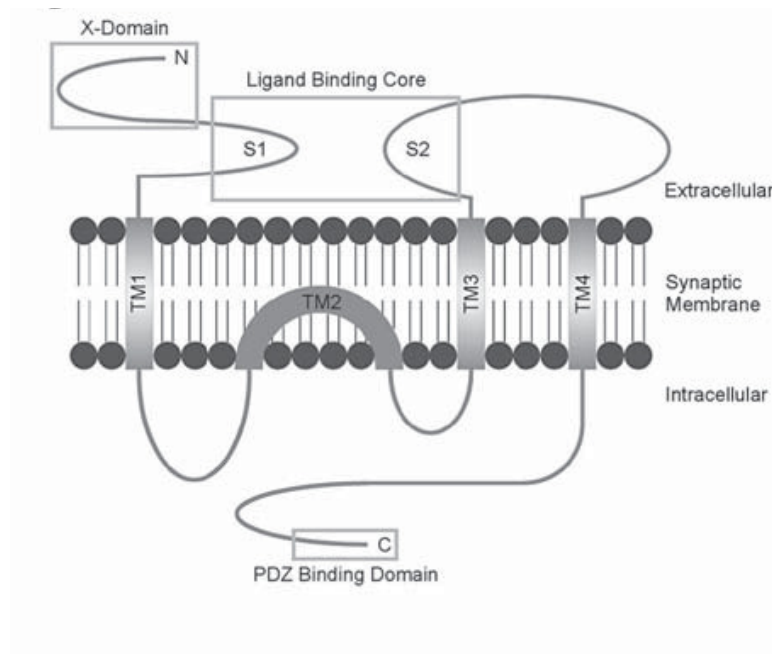


Fig.2.2. Structure of AMPAR. Each AMPAR subunit consists of four hydrophobic domains (TM1-4), an extracellular N-terminal domain and an intracellular C-terminus. **Adapted from Jiang J., Suppiramaniam V., Wooten M. (2006).** Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. *Neurosignals* 15: 266-282.

The subunit composition of AMPARS has a profound consequence on their functional properties and trafficking. GluR2 AMPARs subunits undergo RNA editing such that, the arginine (R) codon replaces the glutamine (Q) codon for residue 607 (Fig.2.3.). This editing profoundly controls calcium permeability and other channel properties of GluR2-containing AMPARs including, channel conductance, kinetics and the affinity of receptor for glutamate. GluR2-containing AMPARs exhibit a lower calcium permeability, open probability, channel conductance and rectification, whereas GluR2-lacking AMPARs have high channel conductance, calcium permeability and probability of opening (Braithwaite et al., 2000; Jiang et al., 2006; Derkach et al., 2007). In addition, an arginine codon can be altered to a glycine codon in GluR2

and GluR3, resulting in the production of ‘flip and flop’ isoforms of AMPARs which have differing properties of desensitization and resensitization (Braithwaite et al., 2000; Jiang et al., 2006). In addition, it has been suggested that the synaptic delivery of AMPARs is also governed by a subunit specific rules (Derkach et al., 2007; Citri and Malenka, 2008). The incorporation of GluR1-containing AMPARs into synapses under basal conditions is found to be slow. However, following NMDARs activation, the insertion of GluR1-containing AMPARs subunits into the post synaptic density is strongly stimulated. GluR2/3 heteromeric AMPARs are inserted constitutively on a rapid time scale. Therefore, the GluR1-containing AMPARs seem to be crucially implicated in the activity-dependant insertion of AMPARs into the postsynaptic density during LTP (Braithwaite et al., 2007; Citri and Malenka, 2008).

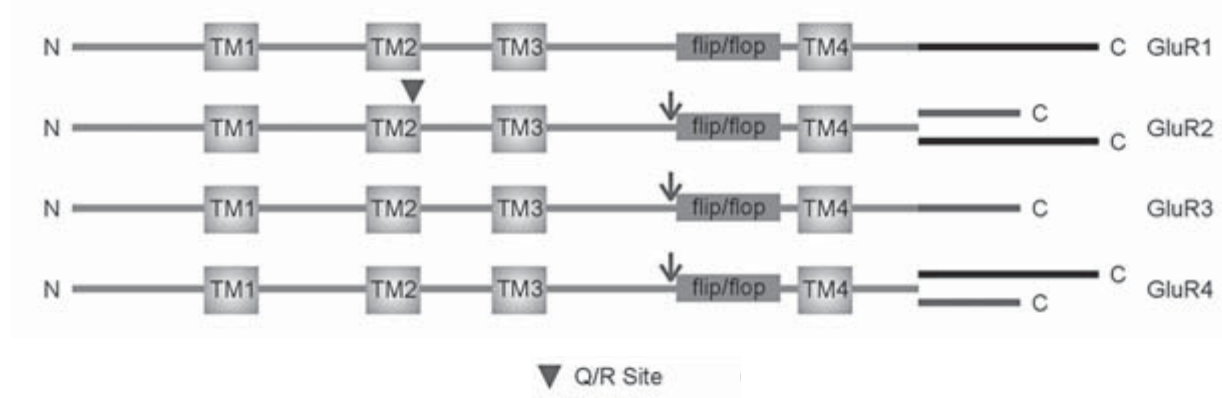


Fig.2.3. Posttranscriptional modifications of AMPARs. The Q/R RNA editing site is present within the TM2 domain of GluR2 AMPAR subunit. **Adapted from Jiang J., Suppiramaniam V., Wooten M. (2006).** Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. *Neurosignals* 15: 266-282.

Under basal conditions of synaptic transmission, AMPARs are constitutively recycled between the postsynaptic membrane and the cytosol. They are either degraded, or reinserted at synapses. However, during LTP, AMPARs are more actively recycled to enhance exocytosis through an endosomal pathway. Recent findings indicate that AMPARs don't appear to be exchanged directly between synapses and the cytosol. Instead, AMPARs are exocytosed at extrasynaptic

sites from which, AMPARs laterally diffuse in the plasma membrane and incorporated within the post synaptic density (Fig.2.4.) (Derkach et al., 2007; Citri and Malenka, 2008). This extrasynaptic trafficking of AMPARs is not NMDARs dependant; however, it is mediated by protein kinase A (PKA)-induced phosphorylation of ser 845 in the GluR1 AMPARs subunit (Wang et al., 2005; Jiang et al., 2006; Derkach et al., 2007; Citri and Malenka et al., 2008). The subsequent incorporation of GluR1-containing AMPARs into the synaptic sites during NMDARS-dependant LTP requires NMDARs/calcium dependant mechanisms, which involves phosphorylation of ser 818 in GluR1 AMPARs subunits by protein kinase C (PKC). Therefore, trafficking of AMPARs to synapses probably occurs in two steps: The first one, is mediated by (PKA)-induced phosphorylation of ser 845 in the GluR1 AMPARs subunit, it occurs under basal synaptic transmission and it mobilizes AMPARs to extrasynaptic sites. The second step promotes lateral diffusion of GluR1-containing AMPARs into the post synaptic membrane to induce synaptic potentiation. This last step is NMDARs/Calcium-dependant and it requires phosphorylation of ser 818 in GluR1 subunits by PKC (Derkach et al., 2007; Jiang et al., 2006 and Citri and Malenka, 2008). During LTP, GluR1-containing AMPARs undergo a regulated phosphorylation on the PKC/CaMKII site (serine 831), resulting in an increase in their single channel conductance. LTP also appears to involve a phosphorylation driven increase in the single channel open probability of GluR1-containing AMPARs on the PKA-sensitive site (serine 845) (Boehm and Malinow, 2005; Wang et al., 2005).

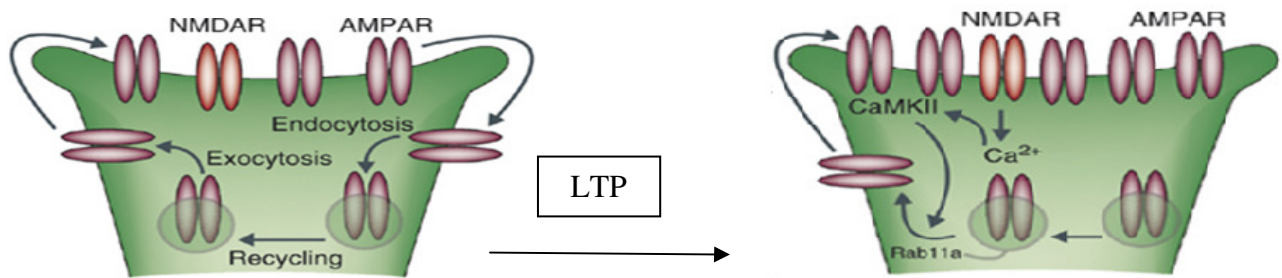


Fig.2.4. AMPARs trafficking during LTP. AMPARs are recycled between synapses and the cytosol under basal conditions, where they are either reinserted at postsynaptic membrane or degraded. During induction of LTP, AMPARs, are recycled through an endosomal pathway more actively and stabilized at the synapse. **Adapted from Citri A. and Malenka R.C. (2008).** Synaptic plasticity: Multiple forms, functions, and mechanisms. *Neuropsychopharm* 33: 18-41.

Within synapses, AMPARs are much less mobile due to anchoring to the so-called “slot proteins”. These proteins function to physically interact with AMPARs and limit their lateral mobility. MAGUKs are attractive candidates for these slot proteins. PSD-95 appears to be particularly important for the surface expression of GluR1-containing AMPARs. Surprisingly, PSD-95 does not appear to directly bind to AMPARs, but rather, does so through a family of AMPARs auxillary subunits termed ‘Transmembrane AMPARs regulatory proteins (TARP) (Jiang et al., 2006; Derkach et al., 2007; Citri and Malenka, 2008). The first identified transmembrane interactor of AMPARs is termed ‘Stargazin’ (Jiang et al., 2006). Stargazin, the prototypical TARP, binds through its intracellular C-terminus to PSD-95, to mediate the synaptic localization of GluR1-containing AMPARs (Jiang et al., 2006; Nicoll et al., 2006; Citri and Malenka, 2008). Stargazin also has been found to mediate the extrasynaptic delivery of GluR1-containing AMPARs (Chen et al., 2000; Nicoll et al., 2006). In addition, stargazing influences the biophysical properties of GluR1-containing AMPARs channel by enhancing the channels conductance and slowing glutamate-induced GluR1-containing AMPARs deactivation and desensitization (Nicoll et al., 2006; Jiang et al., 2008). A novel auxillary AMPARs subunit has been recently identified and termed ‘Cornichons’. Cornichon proteins are found to be

coassembled with AMPARs. They increase the surface expression of AMPARs and modify their channel gating by slowing desensitization and deactivation kinetics (Shwenh et al., 2009).

Maintaining LTP:

LTP resembles memory in that, it is unstable initially and then progressively consolidates (stabilizes) with time (over 30 min) (Lynch, 2004; Lynch et al., 2007). The incorporation of AMPARs into the post synaptic density has been suggested to be accompanied by cytoskeletal reorganization in the synapses and dendritic spines (Lynch, 2004, Lynch et al., 2007; Citri and Malenka, 2008). The stabilization of these newly morphological organizations represents the mechanism underlying the initial phase of LTP consolidation (Lynch et al., 2007). Following repetitive afferent stimulation, an influx of calcium through the NMDARs, activates kinases and the protease calpain (Perlmutter et al., 1988; Kennedy, 2000), resulting in, the breakdown of spectrin and other proteins that cross-link and stabilize actin filaments (e.g. actinin and activity-regulated cytoskeleton-associated protein (Arc)). The latter effect will lead to disassembly of actin cytoskeleton (Dosemeci and Reese, 1995). The Post synaptic density and the spine, in turn change towards rounder configuration which allows the insertion of additional AMPARs into the post synaptic density, and mediates better access to proteins that increases current flow through AMPARs. At the same time, increased synaptic signaling from activated adhesion receptors, particularly integrin receptors, causes actin polymerization. The newly reorganized cytoskeleton is stabilized by cross-linking and anchoring of the newly assembled actin filaments to adhesion receptors (Fig.2.5.) (Lynch et al., 2007).

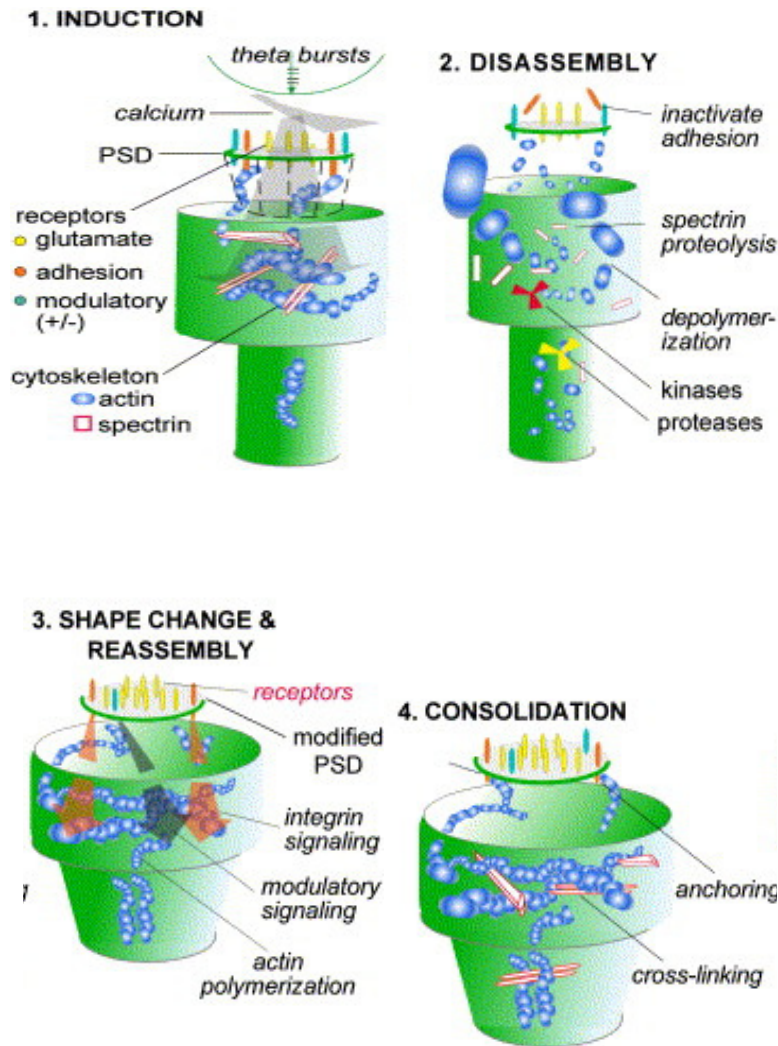


Fig.2.5. Cytoskeleton reorganization and modulation of spine morphology during LTP consolidation. Calcium influx during induction of LTP mediates disassembly of actin filaments. Actin depolymerization leads to cytoskeleton collapse and the post synaptic density (PSD) will be changed into a rounder shape, so that it can accept additional AMPARs. **Adapted from Lynch G., Rex C.S. and Gall C.M. (2007).** LTP consolidation: substrates, explanatory power, and functional significance. *Neuropharmacol* 52: 12-23.

Stabilizing the newly reorganized cytoskeleton does not seem to be the end of the process; it represents the mechanism of the early phase of LTP consolidation (Lynch et al., 2007). The mechanisms that allow LTP to persist for hours, days or even longer (Late phase of LTP), is dependant upon new protein synthesis which supplies the synapse with the proteins that are crucial for maintaining synaptic strength (Lynch, 2004; Reymann and Frey, 2007 & Citri and

Malenka, 2008). The newly synthesized proteins may include cytoskeletal proteins such as Arc, receptors as GluR1-containing AMPARs or signaling proteins as CaMKII and other receptor trafficking regulators (Lynch, 2004; Soulé et al., 2006; Carvalho et al., 2008). However, how does synaptic plasticity that leads to LTP regulate gene expression? Several lines of evidence have implicated endogenous brain derived neurotrophic factor (BDNF) in the regulation of gene expression and local mRNA translation required for synaptic consolidation. BDNF is an activity dependent modulator of neuronal structure and function in the adult brain. Sustained release of BDNF, and activation of its postsynaptic receptor tyrosine kinase 'Tropomyosin receptor kinase B' (TrkB) has been observed following bursts of excitatory synaptic activity (Lynch, 2004; Soulé et al., 2006; Lynch et al., 2007; Carvalho et al., 2008). Activation of TrkB increases the translation in spines and transcription of Arc mRNA in cell bodies. Sustained translation of Arc leads to phosphorylation of a protein 'Cofilin'. Phosphorylation of cofilin on ser 3 impairs its activity, resulting in actin polymerization and subsequent actin-dependent enlargement of synapses and LTP consolidation (Soulé et al., 2006; Rex et al., 2009). Moreover, activation of TrkB stimulates the transcription of GluR1-containing AMPARs and thus increases their protein expression (Carvalho et al., 2008). Several studies have indicated a role for mammalian target of rapamycin (mTOR)-PI3K dependent pathway as a signaling mechanism that operates downstream of TrkB- in BDNF-induced regulation of dendritic protein synthesis (Lynch, 2004; Soulé et al., 2006; Carvalho et al., 2008). In addition, to the delayed BDNF signaling that is coupled to stimulation of transcription and protein synthesis, BDNF was found to promote the synaptic trafficking of GluR1-containing AMPARs. This effect is associated with phosphorylation at ser 831 and most probably phosphorylation at ser 818 of GluR1-containing AMPARs (Carvalho et al., 2008).

Chemical LTP (cLTP):

In addition to the electrical protocols used for induction of LTP, LTP can be also triggered by application of chemicals that directly induce biochemical LTP mechanisms. For example, application of BDNF could induce LTP in hippocampal slices. This effect is mediated by the release of intracellular calcium and new protein synthesis. However, because of the difficulty in the delivery of BDNF into slices, it is difficult to reproduce this method. Bath application of agents that raise cAMP level such as the adenylate cyclase activator (Forskolin) and the phosphodiesterase inhibitor (Rolipram), is a frequently used method for inducing LTP (Otmakov et al., 2004). These agents directly activate components of the mTOR pathway including PI3K and AKT/protein kinase B (PKB) required for protein synthesis in late LTP (Gobert et al., 2008). cLTP induced by cAMP elevation was shown to be NMDARs dependant (Otmakov et al., 2004). In acute slices, induction of cAMP-dependant LTP requires low frequency stimulation of presynaptic fibres. In contrast, cAMP-dependant LTP induction in slice culture, although, it is still NMDARs dependant, but it does not require electrical stimulation. This is due to the fact that, in acute slices the majority of CA3-CA1 connections are severed during slice preparation, so that presynaptic CA3 cells will not be able to burst (Otmakov et al., 2004), a condition that is required for cLTP induction (Mackinson et al., 1999). However, in cultured slices, cut axons are replaced by collaterals from CA3 cells, so that CA3 activity can potentially activate all CA1-CA3 synapses. Therefore, in slice culture, application of Forskolin/Rolipram is a useful protocol for a detailed examination of the morphological changes and the biochemical pathways implicated in NMDARs-dependant LTP (Otmakov et al., 2004; Gobert et al., 2008).

Short-term synaptic plasticity:

Several short-lasting forms of synaptic plasticity (lasting from milliseconds to several minutes), have been observed at excitatory synapses in the mammalian brain. These forms of short-term synaptic plasticity are induced by short bursts of activity, resulting in a transient increase in presynaptic calcium. The latter effect induces a change in the probability of neurotransmitter release (Citri and Malenka, 2008).

Paired pulse facilitation:

This form of plasticity occurs as a result of variation in the probability of neurotransmitter release (P). Delivering two stimuli within a short interval, the response to the second stimulus can be either increased or decreased relative to the first stimulus (Wu and Saggau 1994; Zuker and Regehr, 2002). This phenomena can be simply explained by the fact that, an additional neurotransmitter release during the second stimulation caused by the residual calcium left over from the invasion of the first action potential (Citri and Malenka, 2008).

Integrin-Linked Kinase:

ILK was first discussed in 1996 as serine threonine kinase that is directly anchored to the cytoplasmic domain of $\beta 1$ integrin subunit (Hannigan et al., 1996; Dedhar et al., 1999). ILK acts as a kinase and as an adaptor protein (Dagnino, 2011), to mediate the signal transduction of biochemical pathways that control crucial process such as cell survival, differentiation and gene expression (McDonald et al., 2008). The multifunctional capacity of ILK is attributed to its tripartite structure that comprises N-terminal domain, a central pleckestrin homology (PH)-like domain and a C-terminal domain (Brakebusch and Fässler, 2003). The three domains of ILK serve distinct functions. The N-terminal domain of ILK consists of four ankyrin repeats that mediate the interaction of ILK with PINCH (Particularly interesting new cysteine-histidine

protein), a LIM domain-adaptor protein which is required for the localization of ILK to focal adhesion sites (Zhang et al., 2002). In addition, PINCH can also bind to receptor tyrosine kinase through an adaptor Nck2, thereby linking ILK to growth factor signaling (Wu and Dedhar, 2001). The PH-like domain, in the central region of ILK binds to phosphatidyl inositol-3,4,5-triphosphate (Ptd Ins(3,4,5)P₃) in response to cell adhesion or insulin and mediate PI3K-dependant ILK activation (Delecommene et al., 1998; Hannigan et al., 2005). The activity of ILK also be negatively regulated by ILK-associated protein serine/threonine phosphatase of the PP2 family (ILKAP), or phosphatase and tensin homolog (PTEN) (Wu and Dedhar, 2001). The C-terminal kinase domain of ILK exerts control over several downstream effectors that mediate fundamental cellular functions (Delecommene et al., 1998; Guo et al., 2007; Mc Donald et al., 2008). In particular, ILK as a kinase, once activated directly phosphorylates, PKB/AKT on serine 473. ILK also phosphorylates glycogen-synthase kinase-3 β (GSK-3 β) on serine 9 and inhibits its activity (Dedhar, 2000). In addition to its catalytic activity, the C-terminal domain also mediates the binding of ILK to integrins and the actin-binding adaptor proteins, paxillin (Nikolopoulos and Turner, 2001; Nikolopoulos and Turner, 2002), α , β and γ parvins and their recruitments to the focal adhesions, and thus linking ILK to the actin cytoskeleton (Hannigan et al., 2005; Hannigan et al., 2007; Legate et al., 2006). Inhibition of ILK activity was found to be sufficient to impair localization of these proteins to focal adhesions, resulting in disruption of actin cytoskeleton reorganization (Qian et al., 2005). Through its C-terminal domain, ILK also binds to Src protein, resulting in phosphorylation of cofilin at serine 3, an actin severing protein and actin cytoskeleton remodeling (Kim et al., 2008) (Fig.2.6).

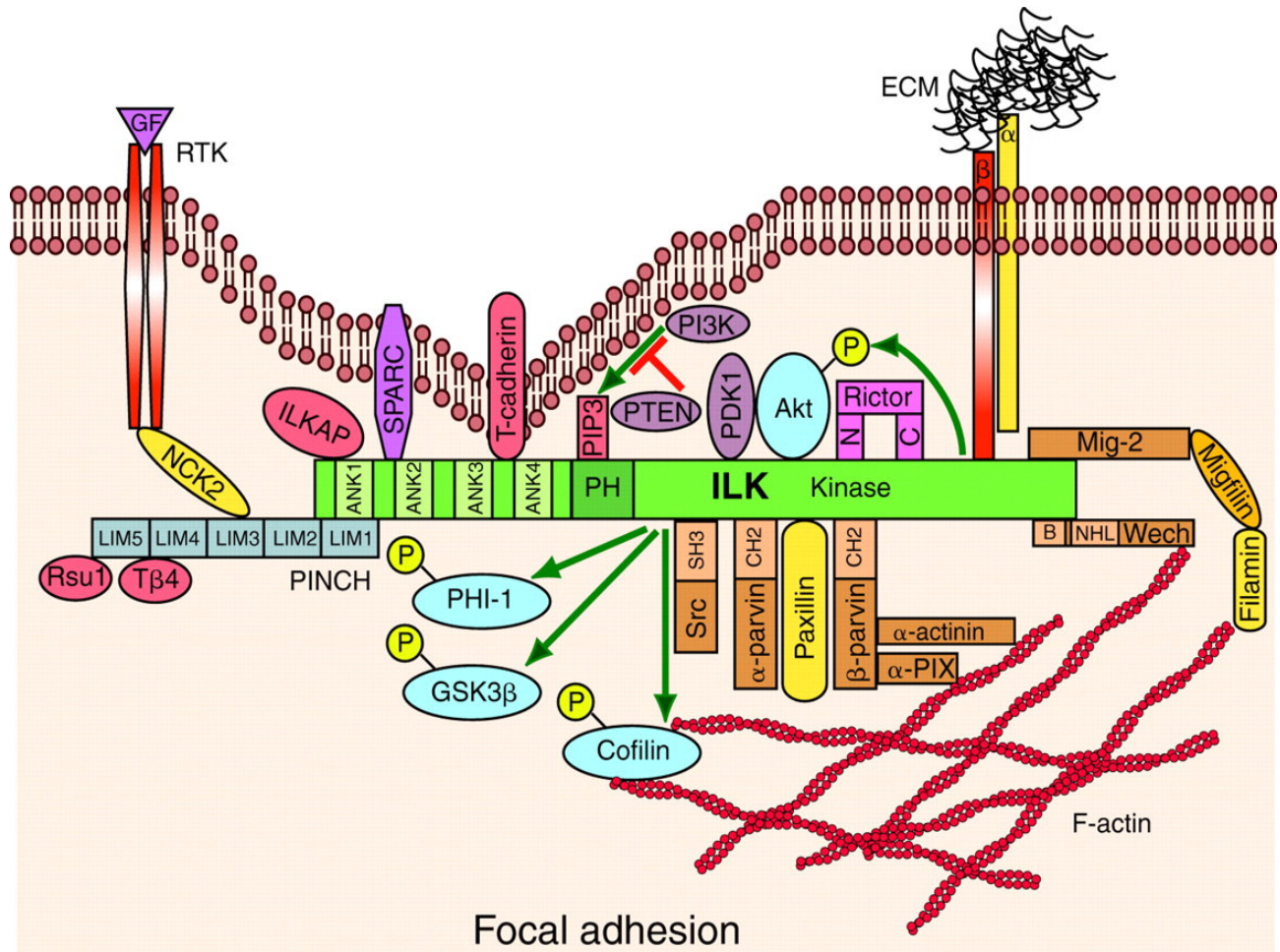


Fig.2.6. ILK structure and interactions. ILK consists of 3 domains: N-terminal domain, a central pleckstrin homology (PH)-like domain and a C-terminal domain. Activation of PI3K leads to binding of PIP3 to the central PH domain and ILK activation. Activated ILK has been shown to phosphorylate two downstream effectors of PI3K; PKB (or AKT on serine 473) and GSK-3β on serine 9, resulting in gene expression and cell survival. Following its activation, ILK is recruited together with other actin binding proteins (Paxillin, parvins and Src) to focal adhesions where it can interact with these proteins by its C-terminal domain resulting in cofilin phosphorylation and actin polymerization, thus ILK is coupled to the actin cytoskeleton. **Adapted from McDonald: McDonald P.C., Fielding A.B., Dedhar S. (2008).** Integrin-linked kinase--essential roles in physiology and cancer biology. *J Cell Sci* 121(Pt 19):3121-3132.

In the CNS, ILK is found to be enriched in neurons, and it plays fundamental role in CNS development and neuronal function (Mc Donald et al., 2008). Reports suggested that ILK is the

dominant kinase for GSK-3 β inactivation in the hippocampus. ILK/AKT/GSK-3 β signaling pathway is found to be essential in the development and functions of neurons. For example, it has been shown that downregulation of ILK expression impairs dendrite formation through inhibition of GSK-3 β phosphorylation (Naska et al., 2006). In addition, ILK-induced GSK-3 β inactivation was found to be essential for axon formation in hippocampal neurons (Oinuma et al., 2007). Moreover, ILK-induced AKT activation is required to mediate hippocampal neuronal survival signaling (Gary et al., 2003). ILK also, through activation of AKT can participate in axon remyelination (Pereira et al., 2009).). The role of ILK in synaptic physiology was limited to its ability to regulate dendrite formation in developing hippocampal neurons (Guo et al., 2007& Naska et al., 2006). A study, recently reported that ILK signaling is required for maintaining the expression of brain-derived neurotrophic factor (BDNF), an essential mediator for LTP (Guo et al., 2008). Another recent report stated that ILK mediates neural plasticity induced by cocaine-sensitization, by interacting with PSD-95, GluR1 and phospho-GluR1 serine 845 and increasing their protein levels in the nucleus accumbens core (Chen et al., 2010). We recently reported that ILK expression is decreased in the hippocampus of an experimental rodent model of sporadic Alzheimer's disease and central insulin resistance. In addition, deficits in LTP reported in these animals correlated well with alterations in ILK signaling (Shonesy et al., 2012).

Synaptic deficits and cognitive dysfunction in Alzheimer's disease:

Alzheimer's disease (AD), the most common age-related neurodegenerative disorder, is characterized by early decline in learning and memory that progresses to global deterioration in higher cognitive function (Hsieh et al., 2006; Watson and Craft, 2004). Early Alzheimer's disease is specifically disease of the medial temporal lobe, especially the hippocampus. Therefore, the majority of early AD patients have selective hippocampal-dependant memory

deficits (Rowan et al., 2004), that are likely to be the result of impairments of synaptic functions (Ting et al., 2007). The majority of AD cases have a sporadic AD (de la Monte et al., 2009). A familial form of AD caused by mutation of some genes such as presenilin and APP is identified in very small proportion of AD patients (Rowan et al., 2004). AD is characterized pathologically by severe atrophy in the hippocampus and cortex, and also the presence of the two characteristics pathological hallmarks; senile plaques and the neurofibrillary tangles. The amyloid beta ($A\beta$) peptides are the major constituent of senile plaques; the neurofibrillary tangles are composed mainly of hyperphosphorylated microtubule-associated protein “tau” (Parameshwaran et al., 2008; Erol, 2009; Götz et al., 2009; Huang and Jiang, 2009). Animal studies demonstrated that $A\beta$ peptides and hyperphosphorylated tau cause synaptic dysfunction and neuronal loss (Cole and Frautschy, 2010). According to the amyloid beta hypothesis, $A\beta$ peptides are considered the primary causative agent for AD (Parameshwaran et al., 2008). In addition, several in vivo and in vitro studies showed that $A\beta$ peptides can inhibit LTP in the hippocampal perforant path (Chen et al., 2000; Wang et al., 2002; Rowan et al., 2004). $A\beta$ peptides attenuate AMPARs and NMDARs surface expression and function, resulting in impairment of glutamatergic transmission. The latter effect could contribute to the synaptic deficits and cognitive decline observed in AD patients (Parameshwaran et al., 2008).

Diabetes and cognitive dysfunction:

Type 2 diabetes mellitus (T2DM) has recently been proven to be risk factor for cognitive dysfunction and dementia such as Alzheimer’s disease (Leibson et al., 1997; Janson et al., 2004; Biessels et al., 2006; Kulstad et al., 2006; Zhao et al., 2009; Park, 2011). In a clinical context, it has been reported that patients who suffer from T2DM develop cognitive deficits and have 2 to 3 fold higher relative risk for AD (Stolk et al., 1997; Ott et al., 1999; Petot et al., 2003; Zhao et al.,

2009). In addition, clinical studies demonstrated that more than 80% of patients with AD are type 2 diabetic patients (Craft et al., 1998; Messier, 2003, Janson et al., 2004; Cole and Frautschy, 2007; Zhao et al., 2009; Park, 2011). The T2DM/AD association was further strengthened by experimental studies that showed deficits in hippocampal-dependant LTP and spatial memory in db/db mice, a T2DM animal model (Li et al., 2002; Sharma et al., 2010; Dinel et al., 2011). Similar cognitive impairments have been reported in ic-STZ rodents, a sporadic AD model with brain insulin resistance (Biessels et al., 1996; de la Monte et al., 2006; Shonsey et al., 2012).

Molecular mechanisms of T2DM-induced cognitive deficits:

The exact pathophysiology of cognitive dysfunction relevant to T2DM is not yet clearly understood, but it is likely that insulin resistance and hyperinsulinism play fundamental role (Kodl and Seaquist, 2008). Peripheral hyperinsulinemia and insulin resistance have been noted in patients with cognitive disorders such as AD (Andreasen et al., 2001; Pedersen and Flynn, 2004). In addition, reduced cerebrospinal fluid (CSF) insulin level has been reported in AD patients (Craft et al., 1998; de la Monte and Wands, 2006). In view of these findings, AD has been recently considered as brain type diabetes and has termed “type 3 diabetes” (Steen et al., 2005). Previous studies reported that peripheral hyperinsulinemia and insulin resistance are the leading cause for the low CSF level of insulin in AD patients (Lee et al., 1999; Kaiyala et al., 2000). Little or no insulin is produced in the CNS, and the majority of brain insulin is of pancreatic origin (Woods, 2003). In T2DM, insulin resistance and peripheral hyperinsulinemia, disrupts the hypothalamic pituitary axis activity, resulting in elevation of serum cortisol level, which impairs carrier-mediated insulin transport across the blood brain barrier (BBB), leading to central hypoinsulinemia (Tojo et al., 1996; Lee et al., 1999; Kaiyala et al., 2000). Insulin receptors are

distributed throughout the CNS with the highest occurrence in the hippocampus and cortex (Bondy and Cheng, 2004). Insulin-receptor-mediated signal transduction activates several downstream effectors in a cascade-like manner. Activated insulin receptors initiates signaling through PI3K resulting in activation of PKB/AKT (Alessi and Cohen, 1998; Vanhaesebroeck and Alessi, 2000). PKB in turn, phosphorylates the N-terminal region of GSK-3 leading to inhibition of GSK-3 activity (Cross et al., 1995). Activated GSK-3 increases the production of amyloid-beta and is critically involved in tau phosphorylation and subsequent formation of neurofibrillary tangles. (Smith et al., 1995; Hoshi et al., 1996; Ko et al., 1999; Grimes and Jope, 2001; Turenne and Price, 2001; Kaytor and Orr, 2002; Mazanetz and Fisher, 2007; Muyliaert et al., 2008). Therefore, disruption in insulin receptor signaling in the brain, will result in inhibition of GSK-3 inactivation and subsequent increase in brain level of amyloid-beta and hyperphosphorylated tau, which are the major constituents of senile plaques and the neurofibrillary tangles, the two characteristic pathological hallmarks of Alzheimer's disease (Gasparini and Xu, 2003; Parameshwaran et al., 2008; Erol, 2009; Götz et al., 2009; Huang and Jiang, 2009). Moreover, central insulin resistance, impairs beta amyloid clearance by decreasing the expression of lipoprotein receptor related protein (LRP), which is an important mediator for the transport of beta amyloid across BBB, or its reuptake into microglia (Misra et al., 1999), and insulin degrading-enzyme (IDE), which is involved in the local enzymatic degradation of beta amyloid (Zhao et al., 2004). Indeed, central insulin resistance would lead to accumulation of beta amyloid in the brain via increasing the production and impairing the clearance processes (Zhao and Townsend, 2009). Beta amyloid could also hinder insulin receptor activation by competing with insulin for insulin receptor binding, and treatment with high dose of insulin overcomes beta amyloid-induced LTP deficits (Townsend et al., 2007; Lee et al., 2009). Therefore, amyloid beta

accumulation and central insulin resistance reinforce each other to induce cognitive dysfunction (Zhao and Townsend, 2009) (Fig.2.7.).

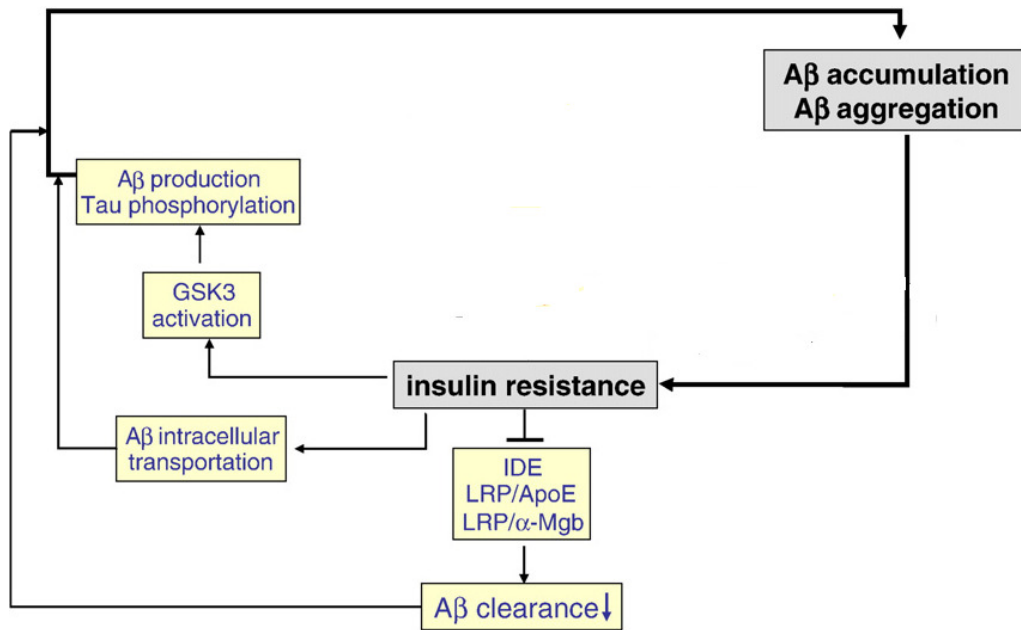


Fig.2.7. Insulin resistance and beta amyloid accumulation. In the brain, increased beta amyloid leads to central insulin resistance by competition on insulin receptor binding. Neuronal Insulin resistance could also lead to accumulation of beta amyloid by increasing beta amyloid production and inhibiting clearance processes. **Adapted from: Zhao W.Q., Townsend M. (2009).** Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. *Biochim Biophys Acta* 1792(5):482-496.

Animal Models of Diabetes with cognitive dysfunction:

The most widely used animal model to investigate the relation-ship between DM and AD was STZ –injection murine model. However, this animal model exhibits T1DM which is less common in AD patients than T2DM. In addition, STZ cannot cross the BBB due to the absence of its transporter GLUT2, so this model only reveals the effects of peripheral hypoinsulinemia on the brain (Szkudelski, 2001; Park, 2011). On the otherhand, ic. STZ animals, although they are not diabetic, develop central insulin resistance and central hypoinsulinemia, or the so called

“type 3 diabetes” similar to those reported in the postmortem brains of AD patients. Moreover, significant beta amyloid and tau-related pathology were observed in these animals (Lester-Coll et al., 2006; de la Monte et al., 2006; Shonsey et al., 2012). The bio-breeding zucker diabetic rat/wor rats and db/db mice T2DM animal models are widely used now to assess the T2DM/AD link. These animal models demonstrated neurodegeneration and synaptic loss, and extensive tau phosphorylation (Planel et al., 2007; Jolivald et al., 2008; Kim et al., 2009). Db/db mice are leptin receptor knockout animals, they serve as a good animal model of T2DM at early ages, and they exhibit hyperinsulinemia and hyperglycemia. Impaired hippocampus-dependant long-term potentiation and spatial memory has been demonstrated in the type II diabetic mouse strain db/db (Li et al., 2002; Sharma et al., 2010; Dinel et al., 2011).

Peroxisome proliferator-activated receptors (PPARs):

Peroxisome proliferator-activated receptor (PPAR) alpha, gamma and delta represent the PPAR subfamily of the nuclear hormone receptor superfamily. These receptors regulate gene transcription in response to ligand binding to specific promoter regions of target genes (Schoonjans et al., 1996; Blumberg et al., 1998; Willson et al., 2000; Chawla et al., 2001). PPARs consist of four functional domains, known as A/B, C, D and E/F that comprise five or six structural regions. A ligand-independent activation function-1 (AF-1) within the aminoterminal A/B domain modulates receptor activity upon phosphorylation in an isotype-dependant manner. Two zinc finger-like structures are present in the C domain that is responsible for binding of PPAR to a peroxisome proliferators response element (PPRE) in the promoter regions of target genes. The C-terminal ligand binding domain or E/F domain is responsible for specificity of ligands and promotes PPAR activation upon binding to PPRE. A docking site for co-factors is found within the D domain. Like other nuclear hormone receptors, PPARs form heterodimers

with the 9-cis retinoic acid receptors (retinoid X receptor, RXR) upon activation by endogenous or synthetic ligands. The resulting complex then binds to the PPRE which is specific for PPAR/RXR heterodimer and consists of two direct repeats spaced by one nucleotide (5'-AGGTCA n AGGTCA-3'). Either ligands of PPAR or RXR can induce gene transcription independently; however, simultaneous activation of PPAR and RXR leads to synergistic stimulation of gene transcription (Robinson and Grieve, 2009) (Fig.2.8).

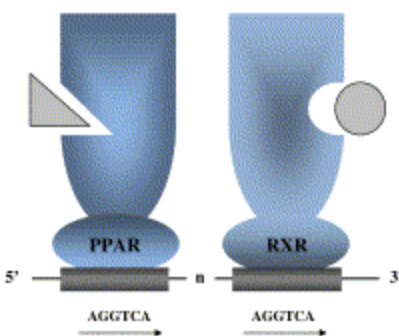


Fig.2.9. Mechanism of action of PPARs. PPARs binds to RXR and form heterodimer which can then bind to PPRE in the promoter region of target gene, resulting in synergistic induction of gene transcription. **Adapted from: Robinson E., Grieve D.J. (2009).** Significance of peroxisome proliferator-activated receptors in the cardiovascular system in health and disease. *Pharmacol Ther* 122(3):246-263.

PPARs play a role in regulation of lipid and glucose metabolism and they have been successfully exploited to be clinically used in the treatment of metabolic diseases like hyperlipidemia and type-2-diabetes (Robinson and Grieve, 2009; Bilsen and Nieuwenhoven, 2010). More recently, activation of PPARs has been demonstrated to exert additional benefits on the central nervous system. In this regard, Pedersen et al., reported that treatment of Tg2576 mice with the PPAR gamma agonist rosiglitazone significantly enhanced spatial working and reference memory and also decreased brain level of β -amyloid 1-42 (Pedersen et al., 2006). Clinical trials of rosiglitazone in AD patients reported that rosiglitazone therapy significantly improved cognition

in AD patients (Chalimoniuk et al., 2004; Watson et al., 2005; Risner et al., 2006). In addition, activation of PPAR delta which is the predominant isotype in the brain (Braissant et al., 1998; Woods et al., 2003) has been recently proven to exert neuroprotective effects. Early treatment with PPAR delta agonists, prevented neurodegeneration and associated cognitive deficits in an experimental model of sporadic AD (de la Monte et al., 2006). Activation of PPAR delta by the high affinity synthetic drug GW0742 decreased brain inflammation and amyloid burden in transgenic animal model of AD (Kalinin et al., 2009).

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3. Integrin linked kinase is required for excitatory synaptic transmission and plasticity in the hippocampus

ABSTRACT

The role of integrin linked kinase (ILK) in hippocampal synaptic plasticity has not been elucidated yet. In the current study, we demonstrated that selective inhibition of ILK activity results in deficits in hippocampal synaptic transmission and plasticity. Our recent study revealed that deficits in long term potentiation (LTP) in an animal model of sporadic Alzheimer's disease, correlated well with disruption in ILK/glycogen synthase kinase 3-beta (GSK3- β) signaling. In order to establish a causal link between ILK and hippocampal synaptic plasticity, we performed extracellular electrophysiological recordings and biochemical assays in rodent hippocampal slices. Treatment of acute hippocampal slices with an ILK activity inhibitor KP-27389 (50 μ m), resulted in significant and irreversible impairment in basal synaptic transmission ($p < 0.01$) and LTP ($p < 0.001$). In addition, the mean amplitudes of AMPA and NMDAR mediated sEPSCs recorded from KP-27389-treated slices were significantly ($p < 0.01$ and $p < 0.001$ respectively) reduced. The deficits in LTP and basal synaptic transmission were accompanied by alterations in the function and expression of synaptic AMPA and NMDAR. Furthermore, co-immunoprecipitation studies in the rat hippocampus revealed that, ILK complexes with GluR1, NR1, NR2A subunits and their associated proteins, postsynaptic density protein-95 (PSD-95) and stargazin; this interaction was disrupted by inhibition of ILK activity. Interestingly, increased ILK activity ($p < 0.05$) was observed in organotypic hippocampal slice cultures after induction of chemical LTP with forskolin. Taken together, our data suggest that ILK activity is required for hippocampal synaptic transmission and plasticity by maintaining the trafficking and function of glutamate receptors.

INTRODUCTION

Characterization of novel proteins that affect hippocampal synaptic plasticity will shed light into molecular mechanisms of memory processing. Our recent study using an experimental rodent model of sporadic Alzheimer's disease revealed that hippocampal synaptic impairments and deficits in Long term potentiation (LTP) correlated well with alterations in the integrin linked kinase (ILK) signaling (Shonesy et al., 2012). ILK is a serine-threonine kinase that functions downstream of growth factor and β -integrin receptors to regulate cell growth and differentiation (Dedhar et al., 1999). The kinase activity of ILK is regulated in a PI3K-dependent manner (Delecommene et al., 1998). Following its activation, ILK phosphorylates PKB/AKT on serine-433 residue. ILK also phosphorylates GSK-3 β on serine-9 residue and inhibits its activity (Delecommene et al., 1998; Dedhar, 2000). Inactivation of GSK-3 β has been reported to be crucial for LTP induction (Hooper et al., 2007; Peineau et al., 2007; Zhu et al., 2007; Jo et al., 2011). Reports suggested that ILK is the dominant kinase for GSK-3- β inactivation in the hippocampus (Naska et al., 2006). The current understanding of the role of ILK in synaptic physiology is limited to its ability to regulate dendrite formation in developing hippocampal neurons (Naska et al., 2006). A study, recently reported that ILK signaling is required for maintaining the expression of brain-derived neurotrophic factor (BDNF), an essential mediator for LTP (Guo et al., 2008). Another recent report stated that ILK mediates neural plasticity induced by cocaine-sensitization in the nucleus accumbens core (Chen et al., 2010). However, the role of ILK in hippocampal synaptic transmission and plasticity has not yet been investigated.

In the current study, we investigated the synaptic transmission and plasticity in the hippocampus after inhibition of ILK activity by using a potent and specific pharmacological approach. Additionally, we assessed ILK activity in the hippocampus after induction of chemical LTP (cLTP) in organotypic slice culture. In this study, we demonstrated for the first time, the existence of a causal link between ILK and hippocampal synaptic plasticity.

We further explored the role of ILK in mediating hippocampal synaptic plasticity at a single receptor level to the more complex mechanisms that affect the plasticity in the hippocampus. The AMPA and NMDARs play complementary roles in long-term potentiation (LTP) in the hippocampus. Modifications of the kinetics and/or the number of functional AMPA and NMDARs at the membrane of post synaptic density will have extensive implications on the induction and expression of LTP respectively (Huang and Stevens, 1998; Kleshchevnikov, 1998; Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir 2002; Brecht and Nicoll, 2003; Derkach et al., 2007). Stargazin and post synaptic density-95 (PSD-95) are members of MAGUKs (for membrane-associated guanylate kinase), a family of proteins that play an important role in anchoring AMPA and NMDARs to the membrane of postsynaptic densities (Wheal et al., 1999, Chen et al., 2000; Nicoll et al., 2006). In addition, reports showed that stargazin and PSD-95 affect AMPA and NMDARs biophysical properties respectively (Lin et al., 2004; Lin et al., 2006; Nicoll et al., 2006; Law and Zukin, 2007). Our findings suggest that ILK by interacting with AMPA, NMDARs and their associated proteins can affect their function and expression at the postsynaptic density and thereby mediates hippocampal synaptic plasticity.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA), were housed in a temperature controlled room, with free access to food and water *ad libitum* water. Animal care and all experimental procedures were approved by the Institutional Animal Care Committee of Auburn University.

Acute hippocampal slice preparations:

Acute hippocampal slices were prepared using standard experimental procedure, previously described by (Zeng et al., 1995) and (Zeng and Tietz, 1999). Male Sprague-Dawley rats (7-8 weeks of age) were decapitated under CO₂ anesthesia. After rapid dissection, the brains were immediately placed in ice-cold oxygenated (95% O₂, 5% CO₂) dissection buffer consisting of (in mM): NaCl 85, KCl 2.5, MgSO₄ 4.0, CaCl₂ 0.5, NaH₂PO₄ 1.25, NaHCO₃ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5). Coronal hippocampal slices of 350 μm thickness were obtained using a vibratome Series 1000 tissue sectioning system (Technical products international Inc., St. Louis, MO,USA). These slices were incubated for 2 hours at 30°C in holding chamber of artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 119, KCl 2.5, MgSO₄ 1.3, CaCl₂ 2.5, NaH₂PO₄ 1.0, NaHCO₃ 26, dextrose 11.0, and continuously bubbled with 95% O₂/5% CO₂.

Treatment with the ILK inhibitor (KP-27389):

Acute hippocampal slices were treated with 10 and 50 μM KP-27389 (US patent) (unless otherwise specified), an inhibitor of ILK kinase activity, for 2 hours. An equivalent amount of vehicle control (DMSO) was added to all control samples.

ILK kinase activity assay

ILK kinase activity was determined as previously published (Eke et al., 2009). Briefly, slices were homogenized in ice-cold cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with complete protease inhibitor cocktail. The extracts were centrifuged for 15 minutes at 10,000 x g, at 4 °C to remove cellular debris, and the protein content of the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL). Anti-ILK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with gentle roking at 4 °C overnight with protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). 500 µg of total protein were incubated with immobilized anti-ILK antibody cross-linked to the A/G plus agarose beads with gentle roking at 4°C for 4 hours. After ILK was selectively immunoprecipitated from the cell lysates, the immunoprecipitated products were washed twice in lysis buffer and twice in kinase assay buffer (Cell Signaling, Danvers, MA), containing (25 mM Tris, pH 7.5, 10 mM MgCl₂, 5 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, and 2 mM dithiothreitol). The samples were then resuspended according to the manufacturer's protocol (Cell Signaling, Danvers, MA) in 50 µl of kinase assay buffer containing 200 µM ATP and 1µg of GSK-3β fusion protein. After 30 min incubation at 30°C, the kinase reaction was terminated by the addition of Laemmli SDS sample buffer (Bio-Rad, Hercules, CA). ILK kinase activity was measured subsequent to SDS-PAGE and Western blotting, by detection of phosphorylated GSK fusion protein by using anti-GSK3α/β S21/9 antibodies.

Western Blot Analysis.

For western blotting, hippocampal cell lysates were resolved by 10% SDS-PAGE and blotted to PVDF membranes (immobilon- P; Millipore, Bedford, MA). Membranes were blocked with 5%

non-fat dry milk in PBS containing 0.01% Tween 20 for 1 h and then incubated with anti-GSK3 α/β S21/9 antibodies (1:1000; Cell Signaling Technology), anti-ILK (1:1000; Cell Signaling Technology) and anti- β -actin (1:1000; Cell Signaling Technology) overnight at 4°C. Membranes were then probed with corresponding anti-rabbit or anti-mouse horseradish peroxidase conjugated second antibodies for 1 h (1:5000; Cell Signaling Technology, 1:5000; Cell Signaling Technology) and developed using the enhanced chemiluminescence (SuperSignal West Femto ECL reagent (Pierce Biochem)). All immunoreactive bands were scanned with a desktop scanner and were subsequently quantified by densitometric analyses using Quantity 1 analysis software (Bio-Rad, Hercules, CA, USA). The densities of each band which represented individual animals were normalized to β -actin and then compared with control levels for both control and treated groups. The data represent the mean \pm SEM. Significance was determined using, one way ANOVA, post tukey's test and a two-tailed Student's *t*-test.

Extracellular field recordings:

For electrophysiological recordings a single slice was then transferred to a submerged-type recording chamber and held between two nylon nets. The submersion chamber was continuously perfused with oxygenated ACSF (30°C) at a flow rate of 2–3 ml/min. A platinum bipolar electrode was placed in the stratum radiatum to deliver test pulses (0.033 Hz) to the schaffer-collateral commissural pathway of the hippocampal CA1 region. Glass microelectrodes (1–4 M Ω) were pulled from 1.5-mm-outer diameter glass tubing with the use of a micropipette puller (Narishigie scientific instruments Lab, Tokyo) and filled with (ACSF). The glass electrodes were used for recording field excitatory postsynaptic potentials (fEPSPs) from the CA1. Recordings of fEPSPs were made in the middle of the stratum radiatum with an Axoclamp 2B (Axon Instruments, Foster City, CA) (Shonsey et al., 2012). To examine the effects of kinase inhibitor

on basal synaptic transmission and LTP, the slices were pretreated with KP-27389 (50 μ m) or control vehicle for 2 hours and experimental levels of LTP were measured on slices prepared from the same hippocampus. Input/output curves were recorded by varying stimulus intensity between 0 and 200 μ A in steps of 50 μ A and measuring the slope of the fEPSPs as well as the amplitude of fiber volley. Paired-pulse facilitation (PPF) curves were generated by stimulating the schaeffer collateral bundle with twin pulses of equal magnitude at inter-pulse intervals (IPI) of 25, 50, 75, 100, 150 and 200 ms. The percentage increase in initial slope of the second response relative to that of the first response in each pair determines the degree of facilitation. Test intensities were set to obtain fEPSP slopes of 50% of those at which population spikes were detectable as determined from input/output curves for each slice. Following stable baseline recordings of at least 20 minutes, theta burst stimulation consisted of one episode of the following: 10 bursts of stimuli, each of four pulses at 100 Hz; interburst interval, 200 msec was applied to the schaeffer-collaterals and fEPSPs were measured in *stratum radiatum*. LTP was measured as the percentage of the baseline fEPSP slope. During the 20 minutes baseline and 1 hour following the tetanus, fEPSP peak amplitude and slope were analyzed online using Win-LTP acquisition software (Anderson and Collingridge, 2007). The data are presented as means \pm SEM. For plasticity experiments, significance was determined using 2-way repeated measures ANOVA, and a two-tailed unpaired *t*-test was used to determine significance for all basal measures.

Organotypic slice cultures

Organotypic hippocampal slices were prepared and maintained similarly to the protocol described in (De Simoni and Yu, 2006). Briefly, transverse brain slices (400 μ M) were prepared from P5–7 Sprague–Dawley rat pups using a vibratome Series 1000 tissue sectioning system

(Technical products international Inc., St. Louis, MO, USA). The brain was removed and dissected in sterile, ice cold Hanks balance salt solution (Invitrogen Corporation, Carlsbad, CA) containing: 137.9 mM NaCl, 5.33 mM KCl, 0.41 mM MgSO₄, 0.49 mM MgCl₂, 1.26 mM CaCl₂, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 0.34 mM Na₂HPO₄ and 10 mM glucose and gassed with 95% O₂/5% CO₂. These slices were plated on millicell culture plate inserts (Millipore, Billerica, MA, USA) and allowed to recover in a culture media containing 25% Earle's balanced salt, 50% MEM and 25% heat-inactivated horse serum (Invitrogen Corporation, Carlsbad, CA), and was supplemented with 1 mM glutamine (Invitrogen Corporation, Carlsbad, CA) and 36 mM glucose. To prevent microbial growth penicillin, streptomycin, and fungizone (Invitrogen Corporation, Carlsbad, CA) were also added at a proportion of 1:100 and the culture media was filtered sterile with 0.22 µm pore filters. The slices were then kept at 37°C in a humidified atmosphere (95% O₂, 5% CO₂) for 7-10 days, and the media was changed every other day.

Chemical LTP (cLTP) protocol:

After 10 days of culture, the slices were isolated from the inserts by cutting the insert membrane around the slice. The slices were allowed to recover for 1 h in slice holding chambers containing ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1 mM NaH₂PO₄, 26 mM NaHCO₃ and 11 mM dextrose; pH 7.4; 95% O₂/5% CO₂ osmolarity 310 mOsmol). After recovery of the slices, chemical LTP was induced for our molecular experiments as described previously (Otmakkhov et al., 2004). In brief, slices were transferred to 0Mg²⁺ ACSF containing NMDA (Sigma, St. Louis, MO), (200 nM final) for 10 min, followed by incubation in 0Mg²⁺ ACSF containing cAMP elevating reagents [forskolin (Tocris, Ellisville, MO) , (50 µM final) + rolipram (Tocris, Ellisville, MO), (0.1 µM final)] for 15 min and then transferred to normal ACSF.

ILK kinase activity assay

After cLTP induction, slices were snap frozen on dry ice, and ILK kinase activity was determined as described above.

Synaptosome preparation

Isolation of synaptosomes was performed as previously described (Johnson et al., 1997) with some modifications. Briefly, male Sprague-Dawley rats (7-8 weeks of age) were decapitated under CO₂ anesthesia. After rapid dissection, the brains were immediately placed in ice-cold, oxygenated (95% O₂, 5% CO₂) artificial cerebral spinal fluid (ACSF). Coronal hippocampal slices of 350 μ m thickness were obtained using a vibratome Series 1000 tissue sectioning system (Technical products international Inc., St. Louis, MO,USA). Rat hippocampal sections were dissected from whole brain coronal sections. Hippocampal sections were then homogenized in ice-cold modified Krebs-Henseleit buffer (mKRBS) containing: 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO₄, 2.5 mM CaCl₂, 1.18 mM KH₂PO₄, 24.9 mM NaHCO₃, 10 mM dextrose, 10 mM adenosine deaminase, pH 7.4 (by bubbling with 95% O₂/5% CO₂) using a potter homogenizer (10 strokes). To minimize proteolysis, protease inhibitors (0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin, 5 mM Benzamide) were included in the homogenization buffer. Homogenates were diluted with 500 μ l of additional ice cold mKRBS buffer. This mixture was loaded into a 1 ml syringe attached to a 13 mm diameter Millipore syringe filter holder and forced through a 100 μ m pore cell strainer (BD Falcon, Bedford, MA) pre-wetted with 150 μ l of mKRBS. This diluted filtrate was collected in a 1.5 ml eppendorf tube. Another 1 cc tuberculin syringe was used to load the nylon pre-filtered mixture, and the filtrate was forced through a pre-wetted 5 μ m pore low protein binding filter (Millex-SV; Millipore Corp., Bedford, MA). To minimize proteolysis, homogenates were kept at ice-cold temperatures

at all times. The filtered homogenate was then microcentrifuged at 1000×g for 15 min at 4°C. The supernatant was removed and the pellet rich in synaptosomes was resuspended in 20 µl mKRBS and stored at -80°C.

Reconstitution of synaptosomal AMPA and NMDARs in lipid bilayers

Recording single channel AMPA and NMDA receptor currents from synaptosomes was performed as described previously (Vaithianathan et al., 2005). Pipettes with a resistance of 100 MΩ were pulled by the P-2000 laser puller (Sutter Instrument Company, Novato, CA). An artificial intracellular solution contained: 110 mM KCl, 4.0 mM NaCl, 2.0 mM NaHCO₃, 0.1 mM CaCl₂, 1.0 mM MgCl₂, 2.0 mM 3-N-morpholino propane sulfonic acid, pH 7.4 was loaded in the glass pipette. Bath solutions contained artificial extracellular solutions consisting of: 125.0 mM NaCl, 5.0 mM KCl, 1.25 mM NaH₂PO₄, 5.0 mM Tris-HCl, pH 7.4. The phospholipids were used to form an artificial lipid bilayer. 1,2-diphytanoil-*sn*-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) were dissolved in hexane (Aldrich Chemical Co., Milwaukee, WI) to achieve a concentration of 1 mg/ml. Using a tip-dip method, the artificial bilayer was formed by successive transfer of two monolayers onto the tip of the patch pipettes (Coronado and Latorre, 1983). After a stable membrane was formed, ~ 3–5 µl suspension of synaptosomes was added into the extracellular solutions. Gentle stirring was applied to facilitate the incorporation of AMPA and NMDARs into artificial lipid bilayers.

Single channel recording of synaptic AMPA and NMDARs

AMPA receptor single channel currents were elicited and isolated by application of 290 nM of AMPA, in the presence of [100 µM APV, 1 µM (2S, 4R)-4-methylglutamic acid (SYM 2081, Sigma-Aldrich), 100 µM picrotoxin (Sigma-Aldrich), 2 mM tetraethylammonium chloride (Sigma-Aldrich), and 1 µM tetrodotoxin (Tocris)] which block NMDA, kainate, γ-aminobutyric

acid, and glycine receptors, and potassium and sodium channels respectively. Single synaptosomal NMDA receptor channel currents were evoked by application of 3 μ M NMDA (Sigma-Aldrich) and 1 μ M glycine (Sigma-Aldrich) in the presence of AMPA, kainate, γ -aminobutyric acid, and glycine receptors, and potassium and sodium channels blockers [1 μ M 4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM 2206, Tocris), 1 μ M (2S, 4R)-4-methylglutamic acid (SYM 2081, Sigma-Aldrich), 100 μ M picrotoxin (Sigma-Aldrich), 2 mM tetraethylammonium chloride (Sigma-Aldrich), and 1 μ M tetrodotoxin (Tocris)]. Single channel AMPA or NMDA currents were amplified (Axopatch 200 B, Molecular Devices, Foster city, CA), low pass filtered at 2 kHz, sampled at 20 kHz, digitized, recorded using a PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY), and stored in VHS tape. Data were analyzed offline using pCLAMP 9.0 (Molecular Devices, Sunnyvale, CA).

Whole cell patch clamp recordings:

After the 2 hours incubation period, slices were transferred to a recording chamber perfused with ACSF solution saturated with 95% O₂/5% CO₂ at a rate of 2 ml/min Individual hippocampal slices were visualized by Nomarski differential interference contrast optics with Olympus BX51WI (Olympus,USA) equipped with a water immersion lens. Recordings from CA1 pyramidal neurons were made with patch pipettes (5–7 M Ω), pulled from borosilicate glass capillaries (nonfilamented, 1.5 mm OD, World Precision Instruments, Sarasota, FL) on a Sutter P-2000 Puller (Sutter Instruments, Novato, CA) and filled with a solution containing (in mM): 122.5 C-gluconate, 10 HEPES, 1.0 EGTA, 20 KCl, 2.0 MgCl₂, 2.0 Na₂·ATP, 2 QX-314, 0.25 Na₃·GTP·3H₂O, pH 7.3 (adjusted with KOH), 280–290 mOsm. To conserve G protein mediated responses GTP was added in the pipette solution, ATP was included to supply energy for other

intracellular phosphorylation reactions and to prevent rundown of calcium channels and Cs^+ was added to eliminate K^+ currents. By applying negative pressure to the pipette during approach to the cell, tight seals ($\geq 2 \text{ G}\Omega$ before breaking into whole cell mode) were achieved. Using the whole cell voltage-clamp technique, AMPA receptor mediated quantal events (sEPSCs) were isolated from CA1 pyramidal neurons in the presence of $50 \mu\text{M}$ aminophosphonopentanoic acid (APV) and $30 \mu\text{M}$ bicuculline methiodide (BMI). Axopatch 200-B amplifier (Axon Instruments, Inc., Foster City, CA) was used to voltage-clamp ($V_h = -70 \text{ mV}$) neurons. To isolate NMDA receptor mediated quantal events (sEPSCs) from CA1 pyramidal neurons $4 \mu\text{M}$ 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and $30 \mu\text{M}$ bicuculline methiodide (BMI) were added to the ACSF. Current output was low-pass filtered (2 kHz) and sampled at 10 kHz . The signal was continuously monitored on-line (Clampfit 9 Software, Axon Instruments), and digitized by using (Digidata, 1200, Axon Instruments). Baseline sEPSC activity was recorded in each neuron for at least 10 min. Using the Mini-Analysis program (Synaptosoft, Decatur, Ga), recorded events $\geq 4 \text{ pA}$, with a faster rise than decay were detected and analyzed. sEPSCs amplitude and decay kinetics were measured using a single exponential function. Data were compared using a student *t*-test. Results are presented as mean \pm SEM.

Coimmunoprecipitation studies

Male Sprague-Dawley rats (7-8 weeks of age) rats were euthanized and the hippocampi were dissected and immediately frozen on dry ice. Tissues were homogenized as described previously (Shonsey et al., 2012) in ice-cold cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with complete protease inhibitor cocktail. The extracts were centrifuged for 15 minutes at $10,000 \times g$, at 4°C to remove cellular debris, and the protein content of the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL). ILK was immunoprecipitated

using anti-ILK antibody or mouse-IgG (Invitrogen) (negative control), preconjugated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described above. To examine interactions between ILK and p-GluR1 Ser⁸⁴⁵, NR1A, NR2A, stargazin and PSD-95 in the hippocampus homogenates, western blots were then performed using the following antibodies; anti-GluR1 Ser⁸⁴⁵(1:1000; Millipore), anti-NR1 (1:1000; Cell Signaling Technology), anti-NR2A (Cell Signaling Technology 1:1000), anti-stargazin (1:1000; Millipore) and anti- PSD-95 (Cell Signaling Technology 1:1000). The blots were stripped and re probed with anti-ILK antibodies (Cell Signaling Technology 1:1000). To further examine these interactions, p-GluR1 Ser⁸⁴⁵, NR1A, NR2A, stargazin and PSD-95 proteins were immunoprecipitated using anti-p-GluR1 Ser⁸⁴⁵, anti-NR1A, anti-NR2A anti-stargazin and anti-PSD-95 antibodies or mouse-IgG (Invitrogen) (negative control), preconjugated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described above. Western blots were then performed using ILK antibody. The blots were stripped and re probed with anti- p-GluR1 Ser⁸⁴⁵, anti-NR1A, anti-NR2A anti-stargazin and anti-PSD-95 antibodies.

RESULTS

3.1) Kp-27389 inhibits ILK kinase activity in acute hippocampal slices:

In order to assure optimal inhibition of ILK activity, a kinase assay was performed on immunoprecipitates from total cell lysates of acute hippocampal slices treated with 10, 50 and 100 micromoles of the drug, for 2 hrs. ILK can phosphorylate and inhibit GSK-3 β at serine-9 (Delecommenne et al., 1998; Dedhar, 2000). Therefore, we examined the effect of KP-27389 treatment on protein levels of phosphorylated GSK-3 β at serine-9 using western blot analysis. A dose-dependent reduction in ILK kinase activity was found in hippocampal slices exposed to 10 μ m and 50 μ m KP-27389 as compared to an equivalent volume of the vehicle (Fig. 3.1.). No further inhibition of ILK kinase activity was detected when the dose was increased to 100 μ m, indicating that the maximal inhibitory effect of the drug was achieved at a concentration of 50 μ m (results not shown).

3.2) Impaired basal synaptic transmission and LTP in Kp-27389-treated acute hippocampal slices:

3.2.1. LTP:

We next examined synaptic plasticity in control and Kp-27389-treated acute slices, by inducing long-term potentiation with theta burst stimulation (TBS). TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ; with interburst interval 200 msec. TBS are found to be the most effective and the most physiologically relevant as it induces the normal discharge characteristic of hippocampal neurons (Albensi et al., 2007). Two-way repeated-measures ANOVA showed that LTP was significantly impaired in six of six slices, continuously incubated in 50 μ m Kp-27389 (Fig.3.2.1. A, ; $p < 0.001$; $n = 6$ slices from 6 animals) with an average of $109.5\% \pm 1.187\%$, compared with control slices in which LTP was induced in six of six slices with an

average of $140.6\% \pm 3.288\%$. LTP inhibition by Kp-27389 was irreversible, as fEPSPs slope was not recovered to a potentiated level after Kp-27389 washed out, 5 minutes after TBS delivery (Fig. 3.2.1. B; $104.2\% \pm 0.8946\%$ $p < 0.001$; $n = 6$ slices from 6 animals).). Analysis of the within TBS facilitation was performed by normalizing the amplitudes of sEPSPs with the amplitude of the first fEPSP. A two-way repeated measures ANOVA revealed a significant reduction of potentiation in Kp-27389 –treated slices compared to control slices (Fig. 2C; $p < 0.001$; $n = 6$). We then normalized the first pulse of fEPSPs from 2nd, 3rd, 4th and 5th TBS to that of the 1st TBS to assess whether subsequent TBS resulted in facilitation in Kp-27389 –treated slices. Our results revealed that there was significant facilitation with subsequent TBS, in the control, whereas such facilitation was largely impaired in the KP-27389–treated slices (Fig. 2D; $p < 0.001$; $n = 6$), suggesting that excitatory postsynaptic potentials (EPSPs) mediated by the activation of NMDARs is diminished in KP-27389–treated slices.

3.2.2. Input/output curves:

To test the strength of synaptic transmission in drug-treated hippocampal slices, we constructed input/output curves for fEPSPs. A significant reduction in fEPSP slope has been detected in Kp-27389-treated ($50 \mu\text{m}$) slices across a range of stimulus intensities (Fig. 3.2.2.A; $p < 0.01$; $n = 6$ slices from 6 animals). There were no significant differences in the presynaptic fiber volley amplitude between the control and Kp-27389-treated slices at any given stimulus intensity (Fig.3.2.2.B), indicating that the conversion of the presynaptic stimulus into axonal depolarization was not affected by Kp-27389-treatment.

3.2.3. Paired pulse facilitation (PPF):

To assess whether the decrease in fEPSP slope in Kp-27389-treated slices was due to altered probability of glutamate release, we measured the level of PPF across a range of interstimulus

interval. PPF is a presynaptic short-lasting form of synaptic plasticity where delivery of two stimuli within short-term interval, results in augmentation in the synaptic response to the second stimulus relative to the first one. This phenomenon is likely mediated by an additional neurotransmitter release during the second stimulation caused by the residual calcium left over after the first action potential (Wu and Saggau, 1994 & Zuker and Regehr, 2002). A significant enhancement of the second response relative to the first one was obtained in control and Kp-27389-treated slices. The fEPSP slope₂/slope₁ ratio observed in Kp-27389-treated slices, did not differ significantly from the control slices at any of the interstimulus interval (ISI) examined (Fig. 3.2.2C; $p < 0.05$; $n = 6$ slices from 6 animals), indicating that Kp-27389-treatment does not alter the presynaptic release.

3.3. ILK activity is increased during cLTP:

Next we verified if ILK activity is modified during synaptic plasticity. For this, we induced cLTP in organotypic hippocampal slice cultures. Bath application of agents that raise cAMP levels such as the adenylate cyclase activator (Forskolin) and the phosphodiesterase inhibitor (Rolipram) is frequently used for inducing cLTP. We ascertained that LTP is induced by our cLTP protocol with an average of $190.5 \% \pm 2.65 \%$, (Fig. 3.3.A; $p < 0.001$; $n = 6$). We then assessed the ILK activity in cLTP induced slices by performing ILK kinase assay in which we quantified the amount of P-GSK-3 β at serine-9. ILK activity estimated by P-GSK3- β Ser21/9, is significantly increased by cLTP ($45.6\% \pm 4.532\%$ relative to control, Fig. 3.3.B; $p < 0.001$; $n = 5$).

3.4. Effects of Kp-27389 treatment on AMPA and NMDAR mediated sEPSCs in acute hippocampal slices:

3.4.1. AMPAR-sEPSCs are impaired in Kp-27389 treated acute hippocampal slices:

In the hippocampus, most of the excitatory neurotransmission is mediated by AMPARs. In order to test whether alterations in AMPAR-mediated currents contribute to the reduction in basal fEPSPs and LTP deficits observed in the present study, we performed whole cell patch clamp recordings in CA1 pyramidal neurons of the hippocampus. We recorded sEPSCs that occur without inhibition of the Na⁺ channels. The NMDAR activity was blocked by APV (50 μm). Our results indicate that the mean current amplitude of AMPAR-sEPSCs was significantly reduced from (42.65 ± 4.367 pA) in control to (26.33 ± 3.712 pA) in KP-27389-treated slices (Fig.3.4.1.B and D; $p < 0.01$; $n = 6$). The frequency of sEPSCs (interevent interval) was not significantly altered (0.4283 ± 0.1861 in control slices and 0.4028 ± 0.1173 in KP-27389-treated slices), (Fig.3.4.1. C and E), indicating that the presynaptic glutamate release is not affected by Kp-27389 treatment. We also measured the decay times of AMPA receptor-mediated sEPSC in control and KP-27389-treated slices. Decay times were significantly reduced in KP-27389-treated slices ($\tau_1 = 9.84 \pm 0.78$ ms, $\tau_2 = 12.1 \pm 1.1$ ms in control slices and $\tau_1 = 5.17 \pm 0.43$ ms, $\tau_2 = 5.6 \pm 0.9$ ms in KP-27389-treated slices) (Fig. 3.4.1.F; $p < 0.05$; $n = 6$). In the glutamatergic neurons, a decrease in the decay times could be due to changes in the kinetics of channel closure or increased receptor desensitization.

3.4.2. Impaired NMDAR-mediated sEPSCs in KP-27389-treated hippocampal-slices:

Since activation of NMDARs by the TBS is required for the induction of LTP, alteration in NMDARs-mediated current could also contribute to the LTP deficits demonstrated in the present study. Therefore, we investigated the effect of KP-27389 treatment on the functional properties

of NMDAR-sEPSCs. Our results indicate that the average amplitudes of NMDARs-sEPSCs is significantly reduced in KP-27389 treated slices (22.54 ± 1.12 pA in control slices and 9.77 ± 0.8 pA KP-27389-treated slices) (Fig. 3.4.2. B and D; $p < 0.001$; $n=6$). No change in the frequency of NMDARs-sEPSCs has been detected in KP-treated slices; 0.5527 ± 0.08183 in control slices and 0.4837 ± 0.06598 in KP-27389 treated slices (Fig. 3.4.2.C and E). Decay times were significantly decreased in KP-27389-treated slices ($\tau_1=54.86 \pm 4.21$ ms, $\tau_2= 128.4 \pm 4.95$ ms in control slices and $\tau_1= 36.61 \pm 4.6$ ms, $\tau_2= 100.8 \pm 7.863$ ms in KP-27389-treated slices) (Fig.3.4.2.F; $p < 0.05$; $n=6$). As with the case of AMPARs, these results suggest that the biophysical properties and/or the synaptic expression of postsynaptic NMDARs are altered in KP-27389-treated slices.

3.5.1. Effects of Kp-27389 treatment on single channel properties of synaptosomal AMPA and NMDARs:

These experiments were designed to determine whether the decrease in amplitude of AMPAR- and NMDAR-sEPSCs in KP-27389-treated slices is due to modified single channel properties of synaptic AMPA and NMDARs respectively. Isolated synaptosomes from control and KP-27389-treated acute hippocampal slices were used to study single channel properties of synaptic AMPA and NMDARs. The isolated synaptosomes were reconstituted into artificial lipid bilayers and subsequently activated by agonists.

3.5.2. Effects of Kp-27389 treatment on single channel properties of synaptosomal AMPA receptors:

To isolate the activity of AMPARs, we supplied the ECF with blockers for sodium channels, potassium channels, NMDARs, Kainate receptors and GABAA and Glycine receptors. Application of 290 nM AMPA at a membrane potential (+200 mV), elicited synaptic AMPARs

activity that was completely blocked with CNQX (1 μ m). The channel activity is evident by upward transition of the current, representing the open state. Our experiments revealed an alteration in the single channel properties of synaptic AMPARs. The probability of channel opening (P_o) is significantly reduced in synaptosomes isolated from KP-27389-treated slices ($8.02 \pm 1.3\%$) compared to control synaptosomes ($30.15 \pm 3.4\%$) (Fig.3.5.1 G; $p<0.001$; $n=10$).

Addition of 290 nM AMPA to control synaptosomes, resulted in major conductance level of 27 Ps with the occasional presence of 14 Ps (Fig.3.5.1.A). However, synaptosomes isolated from KP-27389-treated slices displayed only the smaller conductance level (Fig.3.5.2.B). All-points current amplitude histograms for the steady state probability of observing the two current levels were fitted using Gaussian functions. We also analyzed the dwell time of channel open and close states. The histograms of channel open and close dwell times were fitted with 2 exponentials by Marquart least square methods (Fig.3.5.1.C, D, E and F). The open dwell time data showed shorter open times with the inhibitor (Fig. 3.5.1H; control: $\tau_1 0.69 \pm 0.17$ ms; $\tau_2 8.2 \pm 0.65$ ms, KP-27389: $\tau_1 0.3 \pm 0.12$ ms; $\tau_2 2.8 \pm 0.6$ ms; $p<0.001$; $n=10$). There was no change in shorter close time component (τ_1) in the KP-27389-treated synaptosomes. However, the close time component (τ_2) was significantly prolonged to nearly 3-fold that of control in KP-27389-treated synaptosomes (Fig.3.5.1.I control: $\tau_2 17.4 \pm 0.6$ ms; KP-27389, $\tau_2 71.9 \pm 6.2$ ms; $p<0.001$; $n=10$).

3.5.2. Effects of Kp-27389 treatment on single channel properties of synaptosomal NMDA

Rs:

Single channel currents mediated by NMDARs were pharmacologically isolated by adding blockers for major receptors and channels to the pseudo extracellular solution. NMDARs currents were activated by 3 μ m NMDA and voltage-clamped at +100 mV. The probability of

opening, conductance and mean open times of NMDA receptors were significantly decreased in KP-27389-treated synaptosomes. The mean single channel open probability of NMDARs for control synaptosomes was $31 \pm 3.2\%$ (n=10; Fig.3.5.2.G). KP-27389-treatment resulted in a significant reduction in P_o ($4.5 \pm 1.4\%$, Fig.3.5.2. G; $p < 0.001$; n=10). The conductance of NMDA channels was drastically reduced from 40 Ps in control to 13 Ps in KP-27389-treated synaptosomes. The mean time constants for open periods were significantly decreased by KP-27389-treatment (Fig. 3.5.2.H; control: $\tau_1 0.65 \pm 0.59$ ms; $\tau_2 15.604 \pm 0.11$ ms, KP-27389: $\tau_1 0.31 \pm 0.05$ ms $p < 0.001$; $\tau_2 8.71 \pm 1.23$ ms $p < 0.05$; n=10).

We also estimated the mean time constants for closed periods for control and treated synaptosomes. The mean values of the closed times were significantly lengthened to approximately 2-fold that of control in KP-27389 treated synaptosomes (Fig Fig. 3.5.2.I; control: $\tau_1 1.4 \pm 0.17\%$ ms; $\tau_2 25 \pm 0.19$ ms, KP-27389: $\tau_1 2.2 \pm 0.2$ ms; $\tau_2 52.2 \pm 0.1$ ms; $p < 0.01$; n=10).

3.6. Effects of Kp-27389 treatment on synaptosomal expression of AMPA and NMDARs and associated proteins:

Alteration in the expression of synaptic glutamate receptor subunits could also contribute to the impairment in synaptic transmission and plasticity. Therefore, we performed western-blot experiments to quantify the protein expression levels of the most abundantly expressed AMPARs subunit GluR1 AMPARs subunit, NR1, NR2A and NR2B NMDARS subunits. We found that the protein level of GluR1, NR1, NR2A and NR2B were decreased in the KP-27389-treated synaptosomal fraction (Fig.3.6.A, B, C and D, 100.0 ± 3.208 , 100.0 ± 4.807 and 100.0 ± 7.677 for controls and 46.11 ± 1.604 , 16.89 ± 2.415 , 52.39 ± 6.191 and 58.13 ± 2.407 for KP-27389-treated synaptosomes; $p < 0.001$ $p < 0.001$; $p < 0.01$ and $p < 0.01$; respectively, n=10). We also quantified the protein level of certain synaptic proteins that regulate AMPA and NMDARs

kinetics and postsynaptic expression. Stargazin is AMPARs auxiliary subunit that regulates AMPARs expression at synapses and enhances their conductance (Chen et al., 2000; Nicoll et al., 2006 & Jiang et al., 2008). PSD-95 is a synaptic scaffolding protein that promotes the surface expression of AMPA and NMDARs. Moreover, PSD-95 was found to affect NMDARs channel properties, it reduces the desensitization of NMDARs responses and enhances NMDAR channel opening (Lin et al., 2004; Lin et al., 2006 and Law and Zukin et al., 2007). There was a significant reduction in the protein level of stargazin and PSD-95 in the KP-27389-treated synaptosomal fraction (Fig 7.1. E and F; 100.0 ± 3.052 and 100.0 ± 4.118 for controls and 71.27 ± 2.981 and 39.81 ± 1.807 for KP-27389-treated synaptosomes; $p < 0.01$ and $p < 0.001$ respectively, $n=10$).

3.7.Effect of Kp-27389 treatment on the expression of AMPA, NMDAR subunits and their associated proteins in whole hippocampus lysate:

Alteration in the synaptic expression of GluR1, NR1, NR2A and NR2B, stargazin and PSD-95 caused by KP-27389 treatment could be due to disruption of protein synthesis. In order to address this possibility, we performed western blot experiments to measure the protein level of GluR1, NR1, NR2A, NR2B, stargazin and PSD-95 in the whole hippocampal homogenates. There were no changes in the levels of GluR1, NR1, NR2A, NR2B, stargazin proteins in the KP-27389-treated whole hippocampus lysate (Fig 3.7, A, B, C D, E $n=8$). There was a slight but a non significant decrease in PSD-95 level in the KP-27389-treated whole hippocampus lysate (Fig.3.7.F, 100 ± 4.118 for controls and 74.33 ± 25.83 for KP-27389-treated hippocampal homogenates; $p < 0.05$, $n=8$).

3.8. ILK interacts with AMPA and NMDAR subunits and their scaffolding proteins:

Since, Kp-27389 treatment impaired the surface expression of GluR1, NR1, NR2A, PSD-95 and stargazing proteins without affecting their protein synthesis, we hypothesized that ILK may physically interact with these proteins and mediate their cellular trafficking. To determine whether ILK complex with AMPA and NMDARs subunits and their associated proteins, we conducted co-immunoprecipitation experiments using homogenates from the hippocampus. ILK antibody was sufficient to immunoprecipitate p-GluR1ser845, NR1, NR2A, PSD-95 and stargazing proteins (Fig.3.8.A, B, C, D and E, n=6). To further examine these interactions, co-IP studies were performed by using p-GluR1ser845, NR1, NR2A, NR2B, PSD-95 antibodies. These antibodies were sufficient to immunoprecipitate ILK protein (Fig 3.8.G, H, I, J and K, n=6).

3.9. Effects of Kp-27389 treatment on ILK interaction with AMPA and NMDAR subunits and their scaffolding proteins:

In order to determine whether inhibition of ILK activity could impair the interaction of ILK with GluR1, NR1, NR2A, PSD-95 and stargazin and thereby impair their membrane trafficking, we performed co-IP experiments in control and KP-27389-treated acute hippocampal slices by using ILK antibody. We found a significant decrease in the co-immunoprecipitated protein levels of p-GluR1, NR1, NR2A, PSD-95 and stargazin proteins from KP-27389-treated slices compared to control (Fig 3.9.A, B, C, D and E, 100.0 ± 8.217 , 100.0 ± 8.893 , 100.0 ± 11.62 , 100.0 ± 8.462 and 100.0 ± 16.21 for controls and 66.13 ± 6.521 , 28.61 ± 2.961 , 54.44 ± 4.558 , 66.13 ± 6.521 and 40.08 ± 11.40 for KP-27389-treated hippocampal homogenates; ($p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.05$ respectively, n=8).

FIGURES AND FIGURE LEGENDS

Fig.3.1.

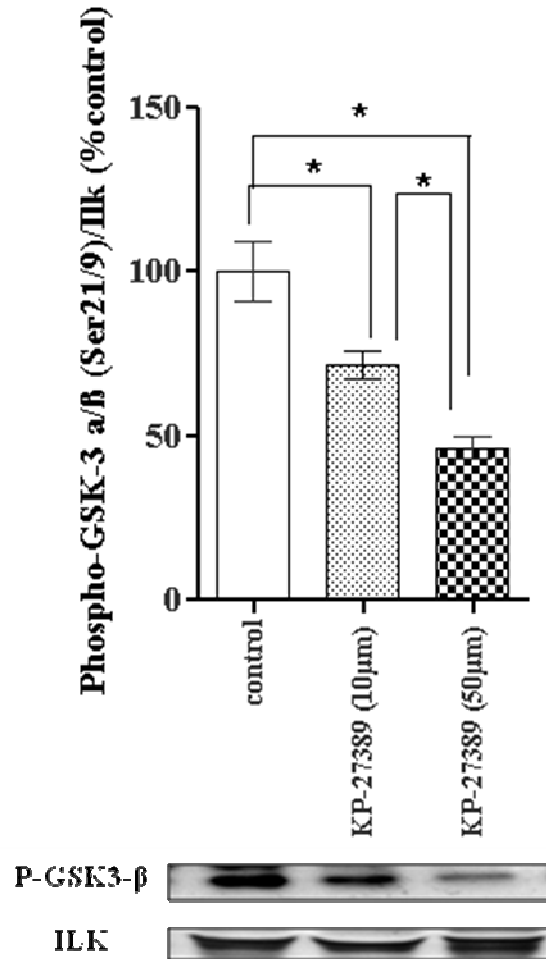


Fig.3.1. Kp-27389 modifies ILK kinase activity in a dose-dependent manner. Acute hippocampal slices were treated with 10 and 50 µm Kp-27389 (DMSO served as control) and lysed after 2 hrs. ILK was immunoprecipitated from extracts of control and Kp-27389-treated acute slices and kinase assay was performed by incubating the immunoprecipitated material with GSK fusion protein, in the presence of ATP and kinase buffer. ILK kinase activity is indicated by phosphorylation of GSK fusion protein with anti-GSK3α/β S21/9 antibodies. Percentage changes were estimated from densitometry and normalized to ILK and DMSO controls.

Fig.3.2.1

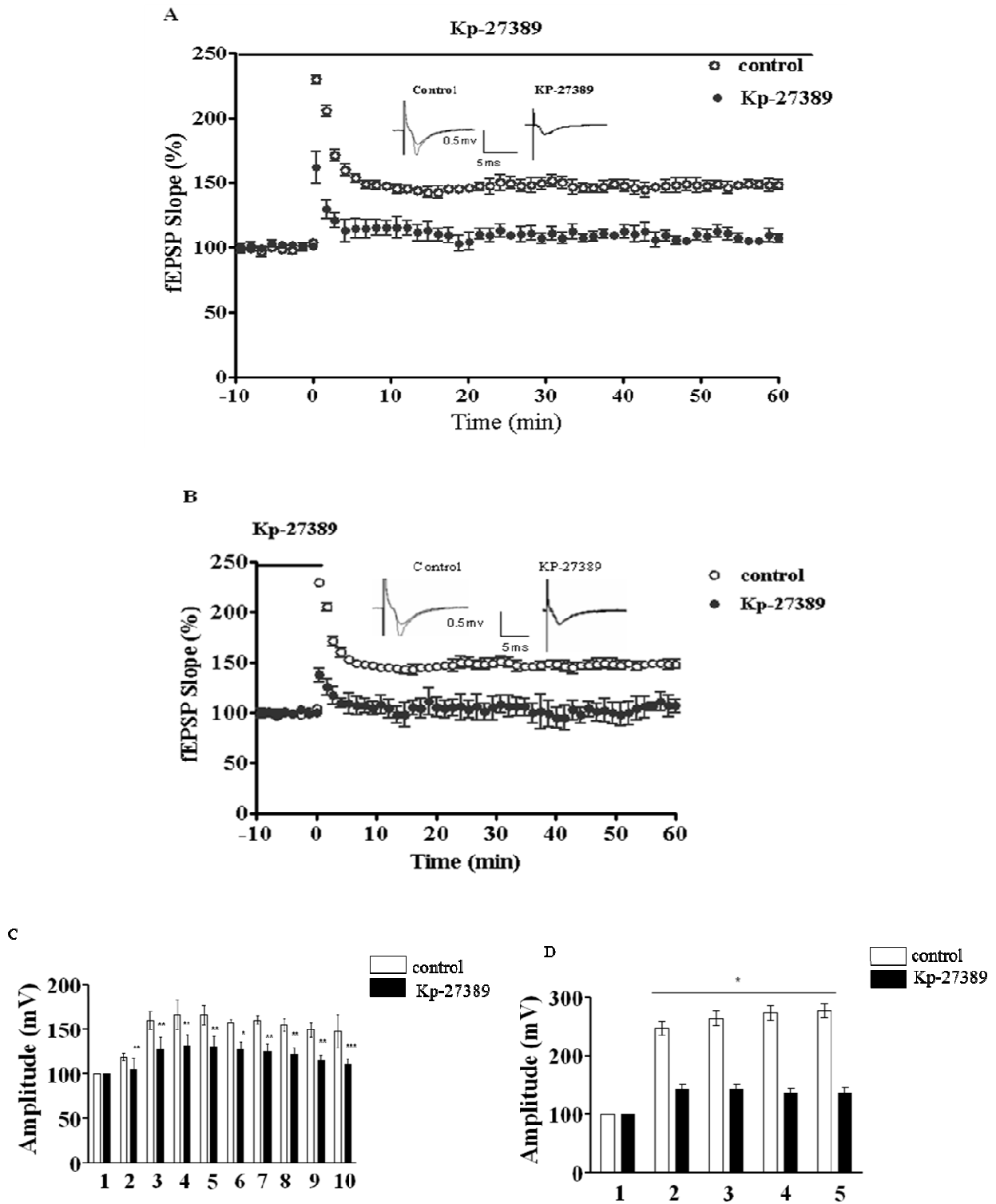


Fig. 3.2.1. Effect of Kp-27389 treatment on hippocampal LTP. (A) LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP in Kp-27389-treated acute hippocampal slices were reduced compared to control slices. Normalized fEPSPs slopes of 60 min post-TBS averaged $140.6\% \pm 3.288\%$ for control and $109.5\% \pm 1.187\%$ for Kp-27389-treated slices, ($p < 0.001$; $n = 6$). (B). LTP could not recover to a potentiated level even after Kp-27389 was withdrawn 5 minutes after TBS delivery. (C). Comparison of amplitudes of fEPSP responses within train and within successive trains. The fEPSP amplitudes were normalized to the amplitude of the first response. Within-train facilitation was significantly decreased in Kp-27389-treated slices ($p < 0.001$; $n = 6$). (D) In the control but not Kp-27389-treated slices, tetanic facilitation occurred with each successive train ($p < 0.001$; $n = 6$).

Fig.3.2.2

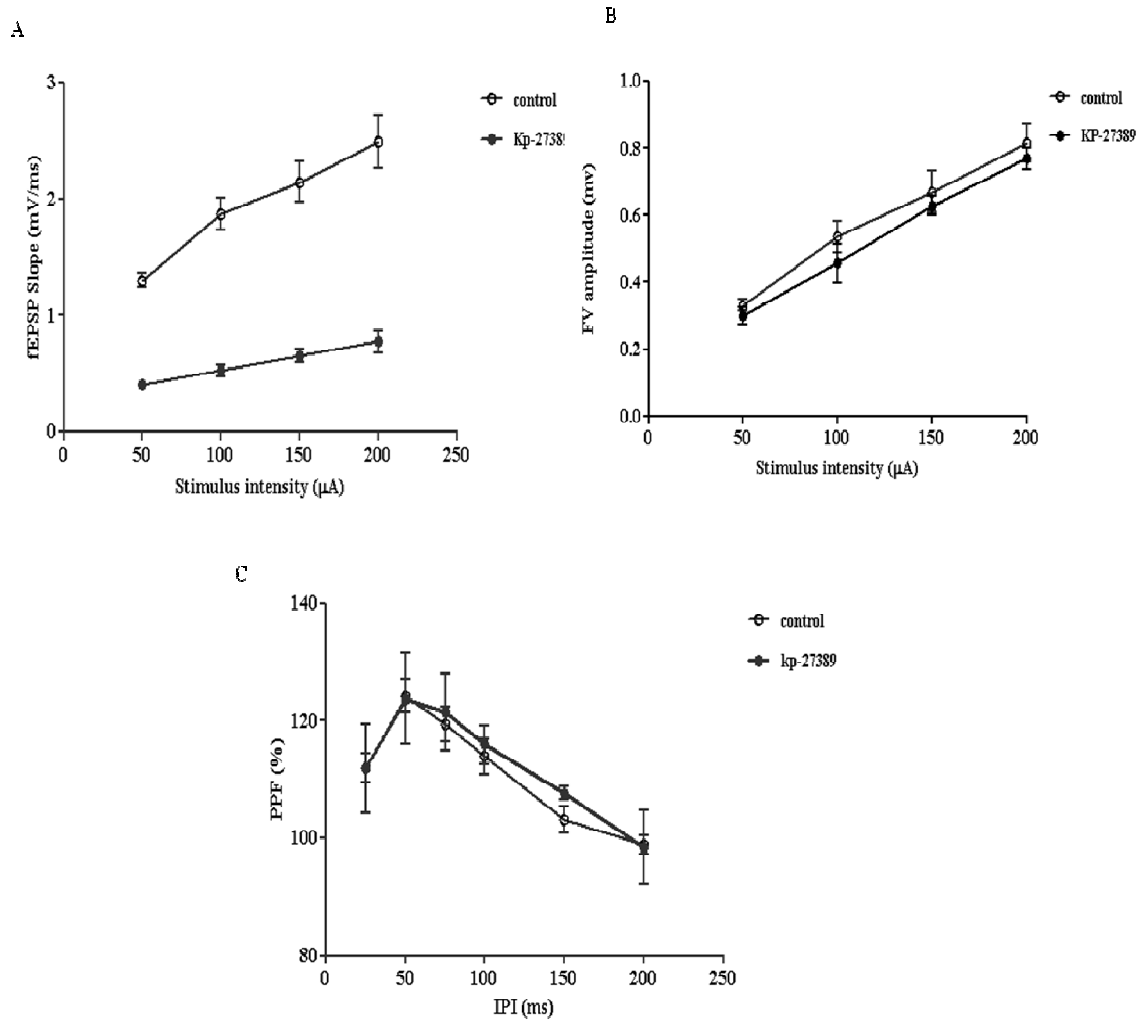


Fig. 3.2.2. Basal synaptic transmission is impaired by Kp-27389 treatment. (A) At all stimulus levels, the average fEPSP slope for Kp-27389 treated-slices was significantly reduced compared with control ($p < 0.01$; $n = 6$). (B) There was no statistical difference between control and Kp-27389-treated slices in the average fiber volley amplitude plotted at different stimulus intensities. (C) Comparison of PPF in control and Kp-27389-treated-slices. At different interpulse intervals, there was no statistical difference in the PPF ratio (slope2/slope1) between control and Kp-27389-treated slices.

Fig.3.3

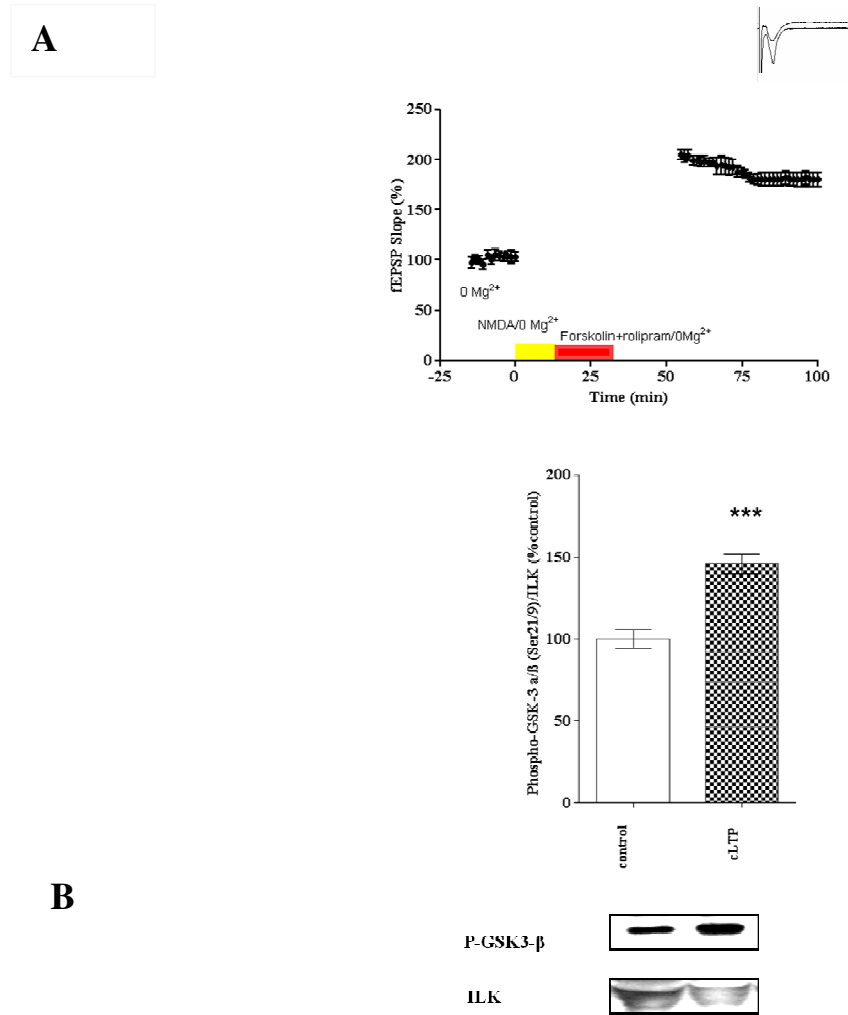
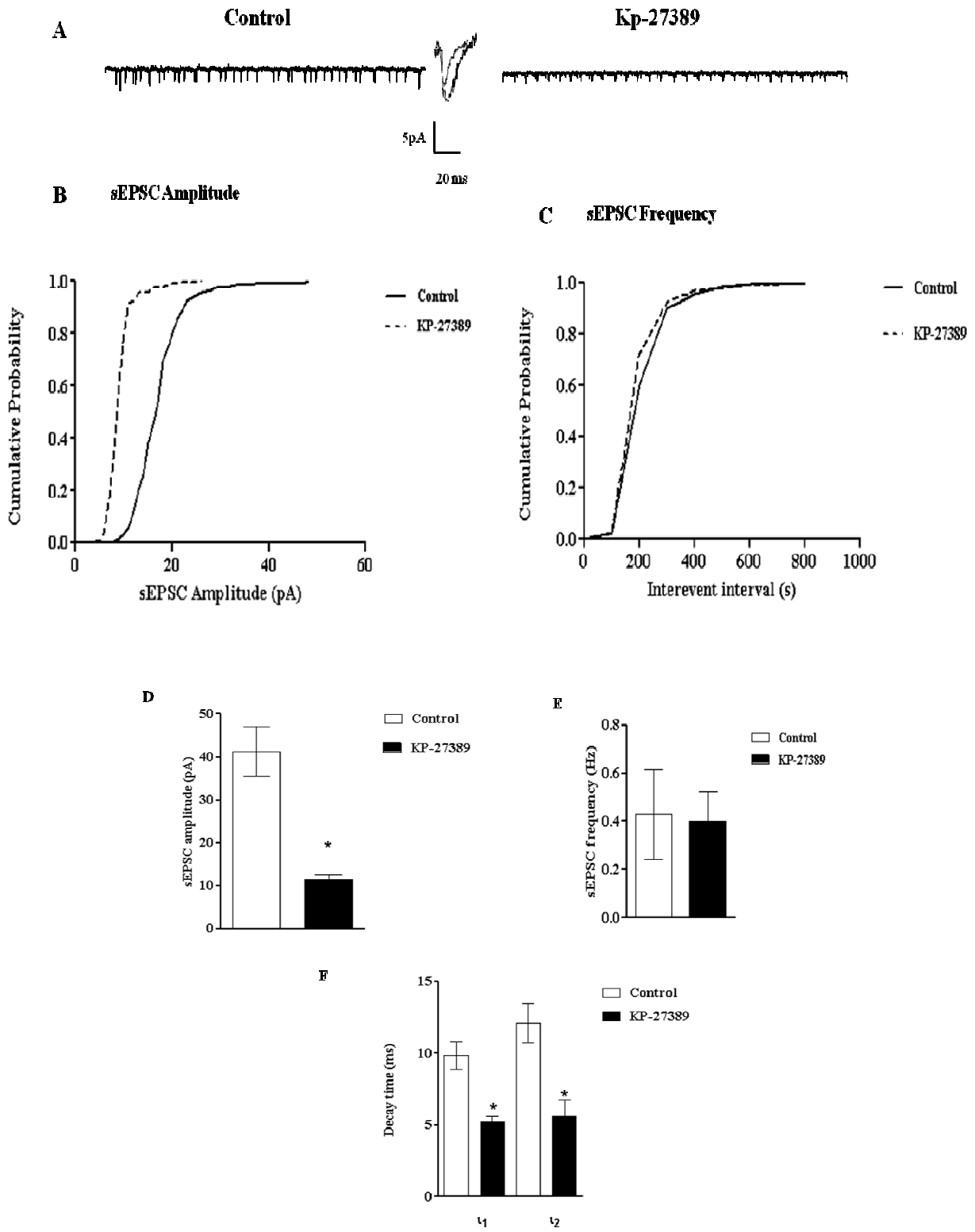


Fig.3.3. Molecular evidence for a role of ILK in hippocampal LTP. (A) Induction of cLTP with forskolin and rolipram. In OHC, application of forskolin/rolipram/0mM Mg^{2+} ACSF induced stable LTP. (B). ILK activity is increased during cLTP. ILK was immunoprecipitated from extracts of OHC with or without cLTP treatment and kinase assay was performed by incubating the immunoprecipitated material with GSK fusion protein. The reaction product was analysed by western blot with anti-GSK3 α/β S21/9 antibodies. The blot was stripped and reprobed with with anti-ILK antibodies. Quantification of p-GSK3 β S21/9 by densitometry revealed an increase of 45.6% \pm 4.532% relative to control in the cLTP samples ($p < 0.001$; $n = 5$).

Fig.3.4.1



3.4.1. Effect of Kp-27389 treatment on AMPARs-mediated whole cell synaptic currents. (A)

Sample traces show AMPARs-mediated sEPSC recorded at -70 mv membrane potential, which is reduced in amplitude, in Kp-27389-treated slices compared to control slices and with no change in frequency. (B) Cumulative probability of distributions of amplitude shows a significant shift of Kp-27389 curve to the left from the controls, suggesting decreased amplitude. (C) Cumulative probability of distributions of interevent interval did not exhibit any change of Kp-27389 curve compared to controls indicating no change in interevent interval. (D) Bar chart shows the significant reduction in mean amplitude of AMPARs-mediated sEPSCs in Kp-27389-treated slices than that of control ($p < 0.01$; $n = 6$). (E) Bar plot depicting the unchanged interevent interval of AMPARs-mediated sEPSCs in Kp-27389-treated slices compared to control (Kolmogorov-Smirnov Test). (F) The decay times of AMPARs-mediated sEPSCs were fitted with two terms. Bar plots also depict a significant decrease in AMPARs-mediated sEPSCs decay times in Kp-27389-treated slices than that of control (Student's t -test, $p < 0.05$; $n = 6$).

3.4.2.

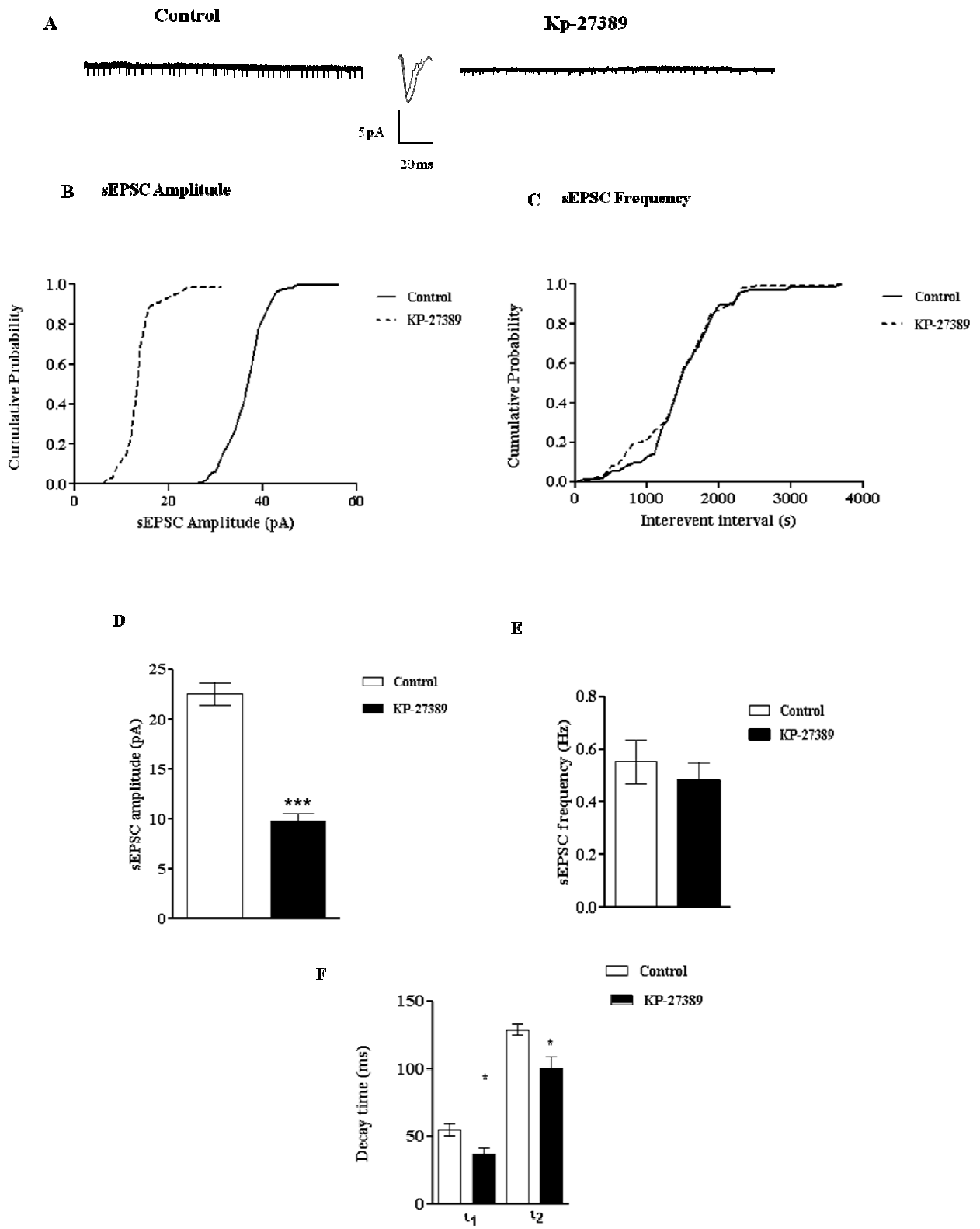
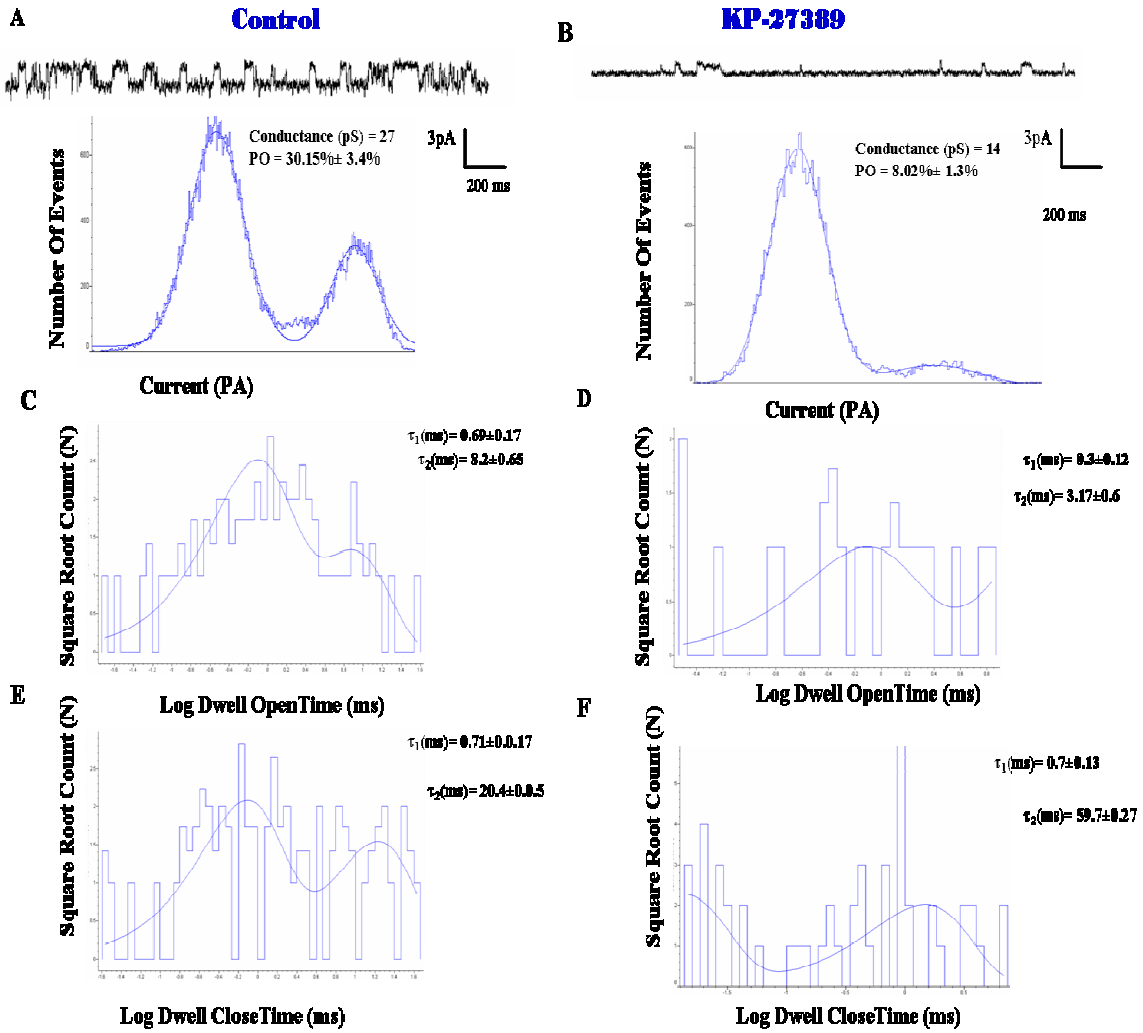


Fig.3.4.2. NMDARs-mediated currents were impaired in Kp-27389-treated slices.

A) Representatives of NMDARs-mediated sEPSC traces recorded at -40 mv membrane potential shows decrease in amplitude, in Kp-27389-treated slices compared to control slices and without any change in frequency. (B) Cumulative fraction curve of amplitude shows a significant decrease in amplitude Kp-27389-treated slices than that of control. (C) Cumulative fraction curve of interevent interval indicates no change in interevent interval in Kp-27389-treated slices compared to control. (D) Bar plot depicts the significant decrease in mean amplitude of NMDARs-mediated sEPSCs in Kp-27389-treated slices than that of control ($p < 0.001$; $n=6$). (E) Bar chart showing the unchanged interevent interval of NMDARs-mediated sEPSCs in Kp-27389-treated slices compared to control. (F) Bar plots also shows a significant reduction in NMDARs-mediated sEPSCs decay times in Kp-27389-treated slices than that of control ($p < 0.05$; $n=6$).

Fig. 3.5.1.



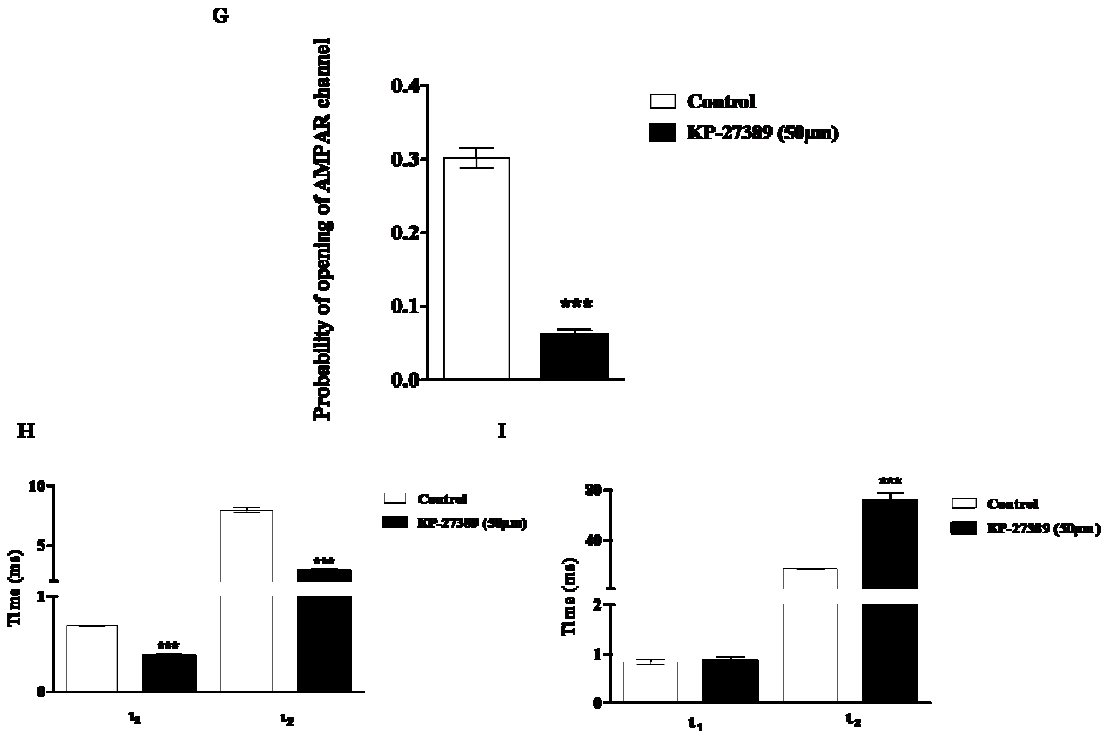
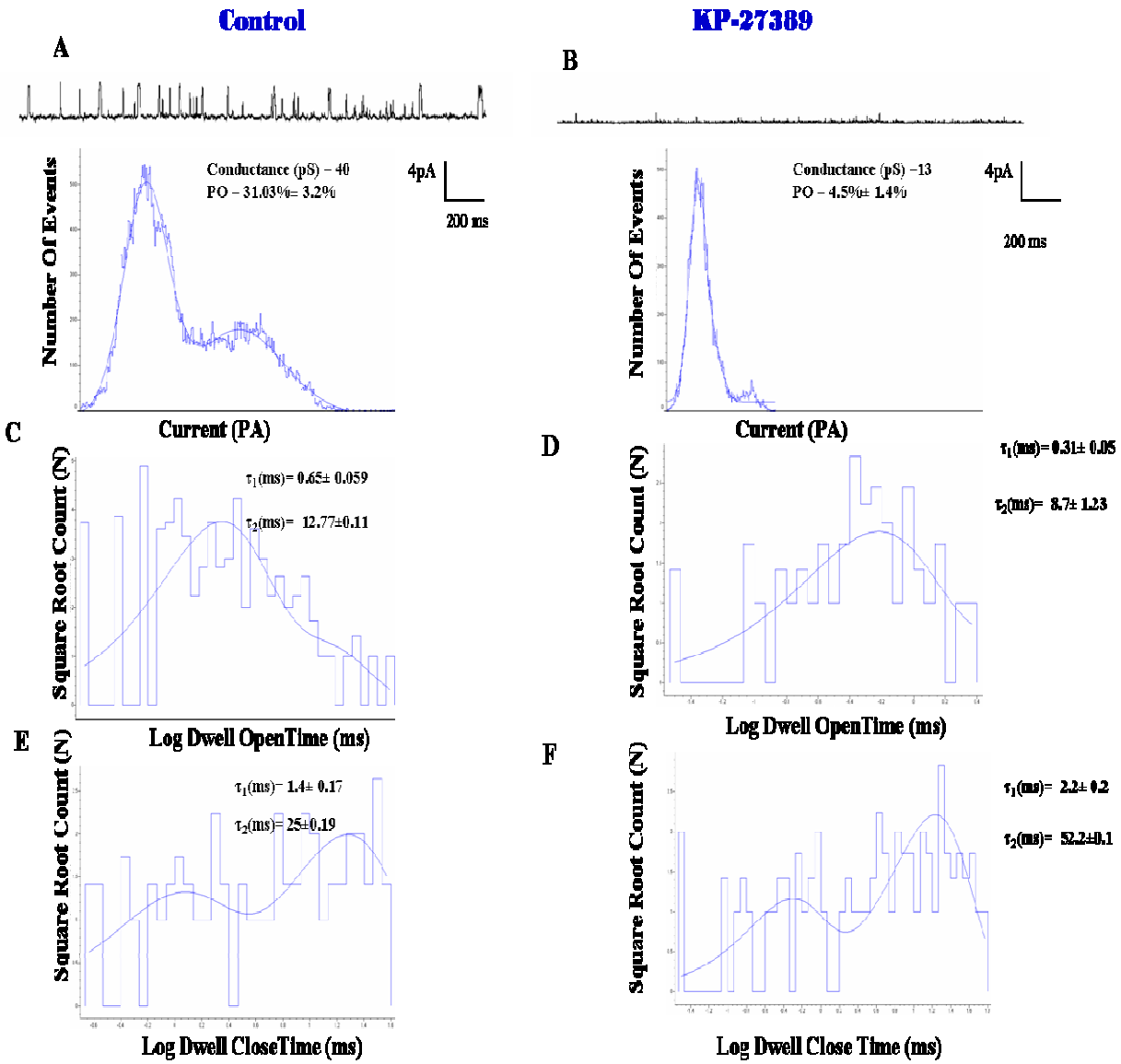


Fig.3.5.1. Kp-27389 treatment leads to alterations in single channel properties of synaptic AMPARs. Representative traces and corresponding current amplitude histograms are shown for (A) control synaptosomes and (B) Kp-27389-treated synaptosomes. The channel conductance of AMPARs currents were shifted from the higher conductance level 27 Ps in control synaptosomes to the lower conductance state 14 Ps in Kp-27389-treated synaptosomes. The channel open peak (right peak in each histogram) is decreased in Kp-27389-treated synaptosomes indicating a reduction in open probability. Single channel open and close time distributions for control (C) and Kp-27389-treated synaptosomes (D) and (E) and (F) respectively were fitted with 2 exponentials. (G) Bar chart illustrating the significant reduction of AMPARs channel open probability in Kp-27389-treated synaptosomes. (H) Bar plot showing significantly decreased open times τ_1 and τ_2 in Kp-27389-treated synaptosomes. (I) Bar chart showing significantly prolonged close time component (τ_2). Values are expressed as mean \pm SEM from 10 animals per group $p < 0.001$, $p < 0.01$, $p < 0.05$, two-tailed, unpaired Student's *t*-test).

Fig.3.5.2.



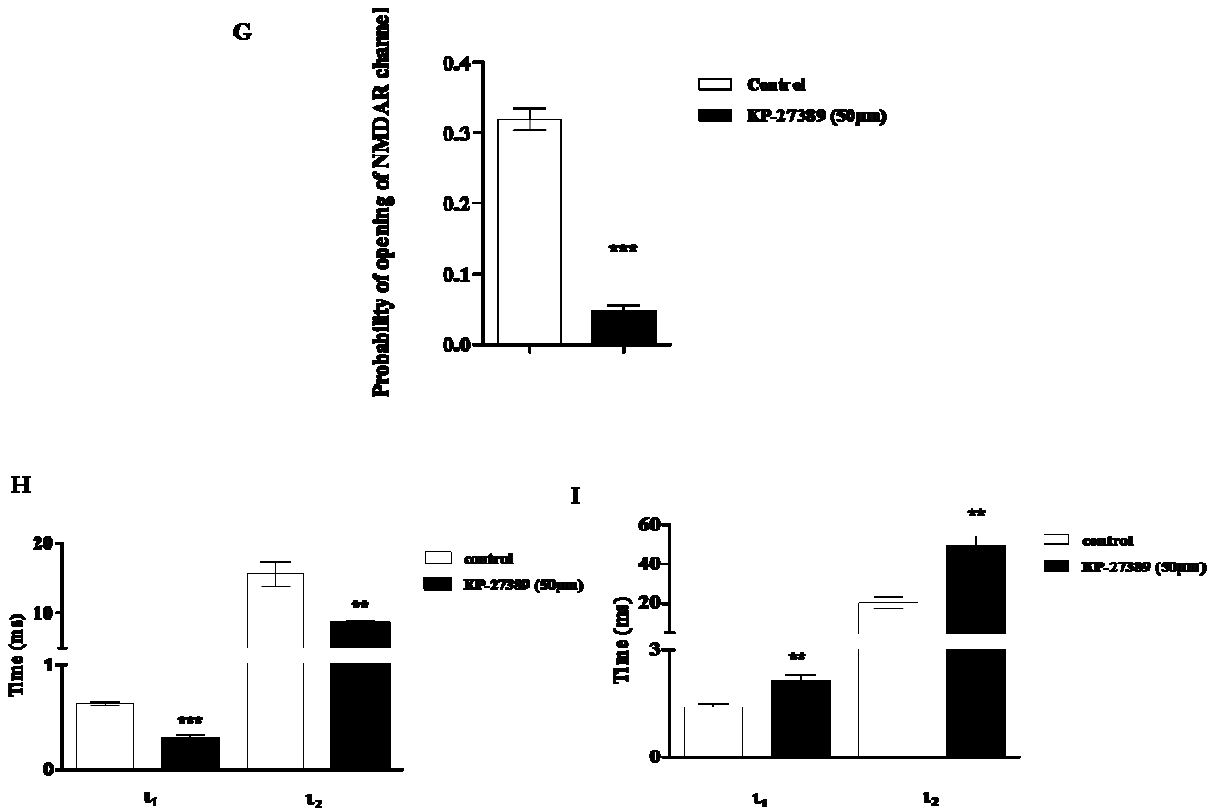


Fig.3.5.2. Single channel properties of synaptosomal NMDARs were altered in synaptosomes obtained from Kp-27389-treated hippocampal slices. Sample traces and corresponding current amplitude histograms are shown for (A) control synaptosomes and (B) Kp-27389-treated synaptosomes. The channel conductance of NMDARs currents were significantly reduced from 40 Ps in control synaptosomes to 13 Ps in Kp-27389-treated synaptosomes. The channel open peak (right peak in each histogram) is decreased in Kp-27389-treated synaptosomes indicating a reduction in open probability. Single channel open and close time distributions for control Kp-27389-treated synaptosomes (C) and (D) respectively and (E) and (F) respectively were fitted with 2 exponentials. (G) Bar plot illustrating the significant reduction of NMDARs channel open probability in Kp-27389-treated synaptosomes. (H) Bar chart showing the significant reduction in open times τ_1 and τ_2 in Kp-27389-treated synaptosomes. (I) Bar chart showing significantly increased close time component τ_1 and τ_2 .

Fig. 3.6.

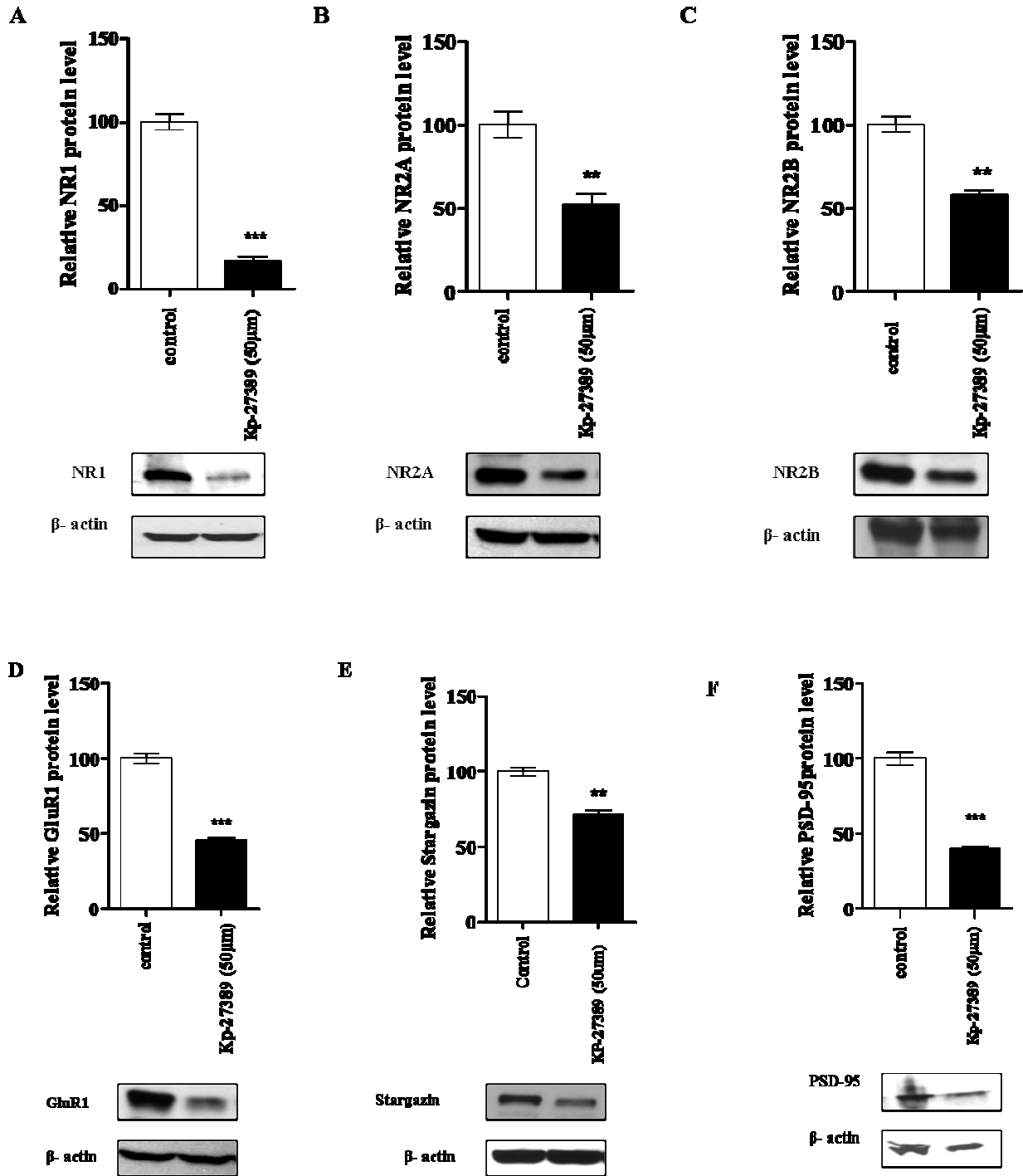


Fig.3.6. Synaptic expression of AMPAR, NMDAR subunits and their associated proteins were modified by Kp-27389-treatment. Protein levels of (A) GluR1 AMPAR subunit, (B) NR1, (C) NR2A, (D) NR2B NMDAR subunits, (E) the auxiliary AMPAR subunit stargazin and (F) the scaffolding protein PSD-95 were analysed by western blot experiments. For quantification, all protein levels were normalized to β -actin to control for gel loading errors. The quantitative protein concentrations are expressed as a percentage of the control group which was set as 100%. Representative bands are shown below the bar graphs. (n=10, $p < 0.001$, $p < 0.01$, $p < 0.05$, two-tailed, unpaired Student's *t*-test).

Fig.3.7.

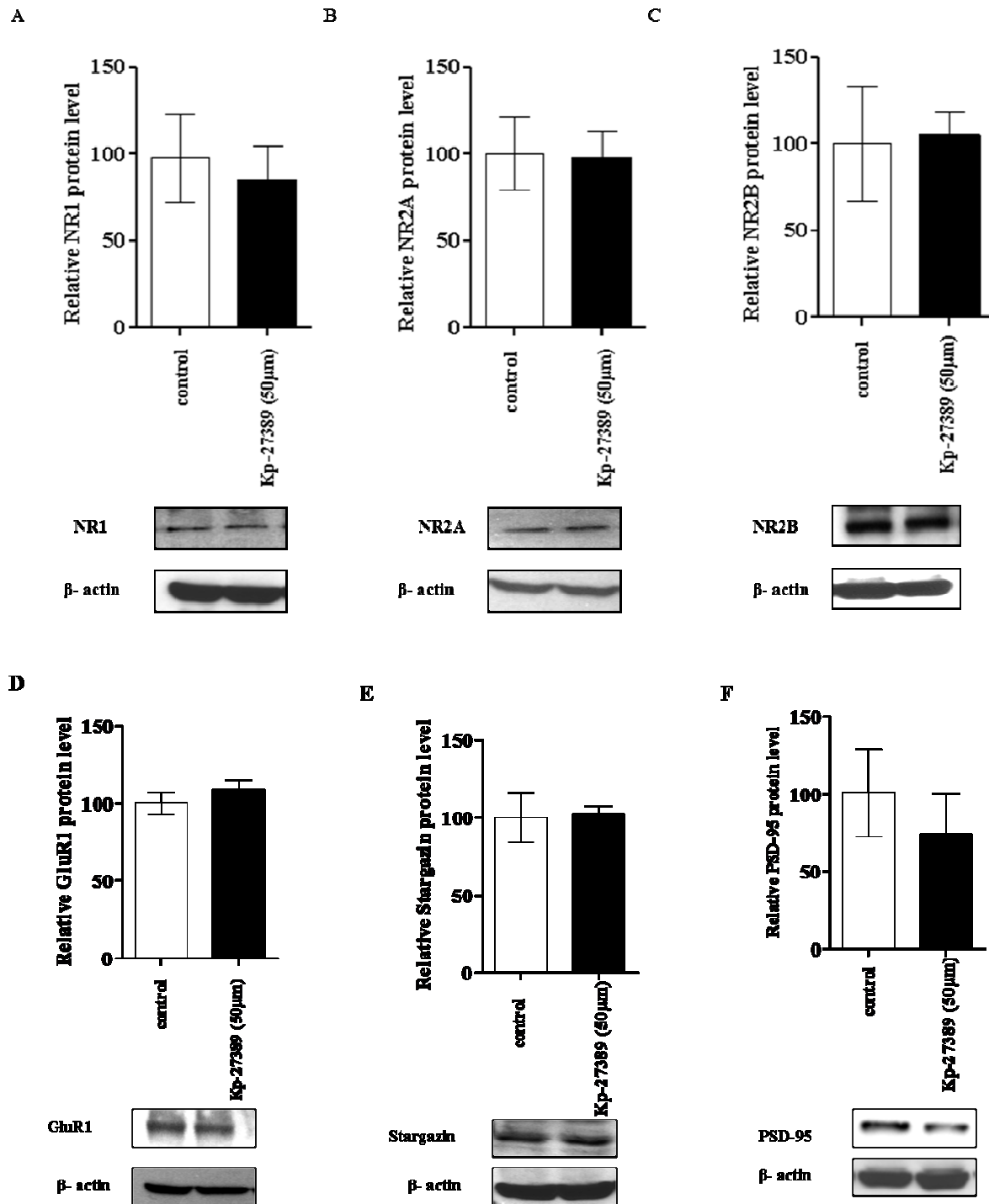


Fig.3.7. Effect of Kp-27389 treatment on the expression of AMPAR, NMDAR subunits and their associated proteins in whole hippocampus lysate. Western blot analysis were performed to quantify the protein levels of (A) GluR1, (B) NR1, (C) NR2A, (D) NR2B, (E) stargazin and (F) PSD-95 protein levels in the control and treated whole hippocampus lysate. Protein levels are normalized to β -actin and expressed as a percentage of the control group which was set as 100%. Representative bands are shown below the bar graphs. (n=10, $p<0.001$, $p<0.01$, $p<0.05$, two-tailed, unpaired Student's *t*-test).

Fig.3.8.

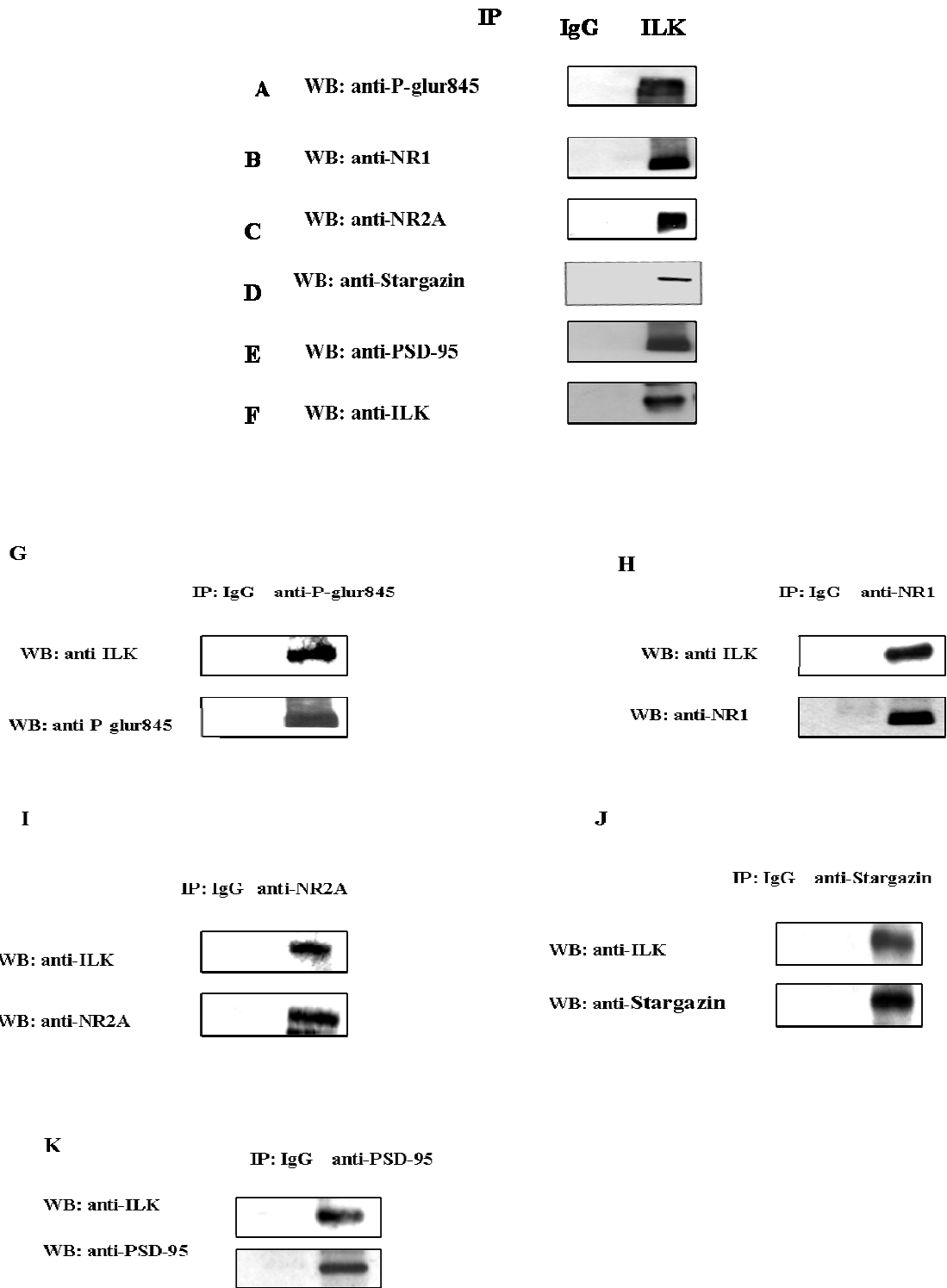
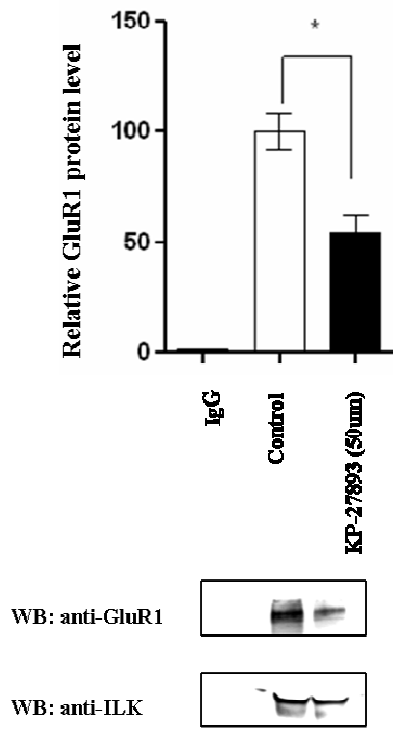


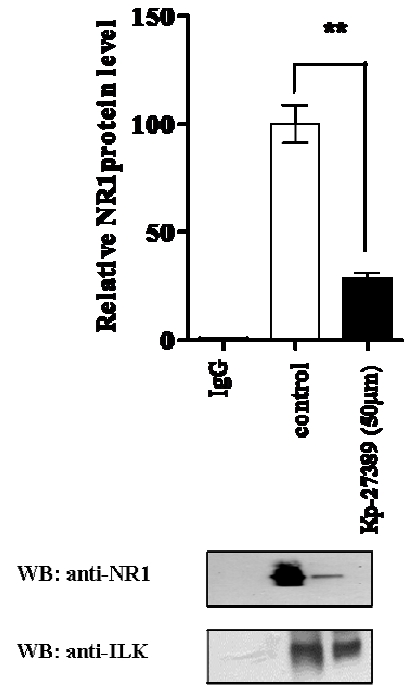
Fig.3.8. Co-immunoprecipitation of ILK with P-GluR1 Ser⁸⁴⁵, NR1, NR2A, stargazin and PSD-95 in the hippocampus homogenate. In the hippocampus, anti-ILK antibody co-immunoprecipitated (A) p-GluR1 Ser⁸⁴⁵, (B) NR1, (C) NR2A, (D), stargazin and (E) PSD-95. Detection was done by performing western blot analysis using anti-p-GluR1 Ser⁸⁴⁵, anti-NR1, anti-NR2A, anti-stargazin and anti- PSD-95 antibodies respectively. (F) Blots were stripped and reprobed with anti-ILK antibodies. The IgG antibody served as a negative control (n=6). (G) anti- p-GluR1 Ser⁸⁴⁵, (H) anti-NR1, (I) anti-NR2A, (K) anti-stargazin and (L) anti- PSD-95 antibodies were used to co-immunoprecipitate ILK from the hippocampus homogenate. Detection was done by performing western blot analysis using anti-ILK antibody. Blots were stripped and reprobed with anti- p-GluR1 Ser⁸⁴⁵, anti-NR1, anti-NR2A, anti-stargazin and anti- PSD-95 antibodies respectively. The IgG antibody served as a negative control.

Fig. 3.9.

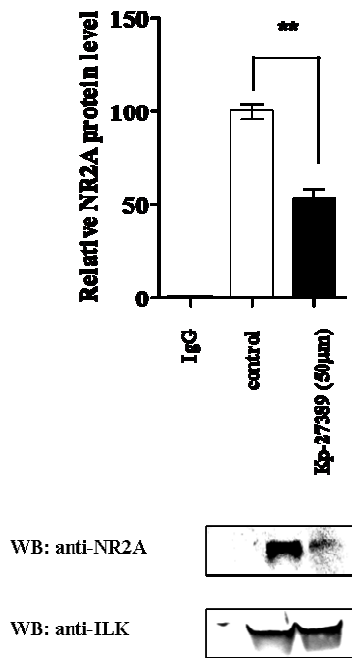
A



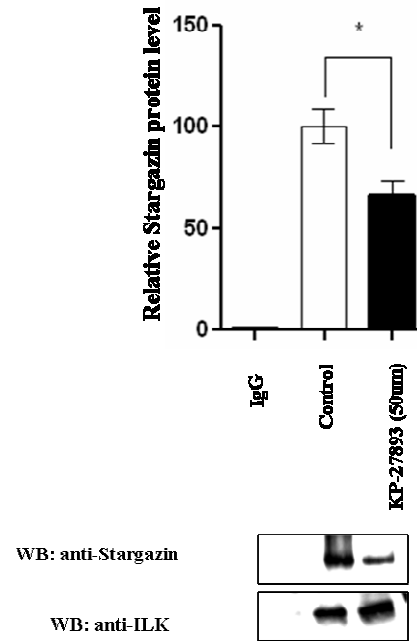
B



C



D



E

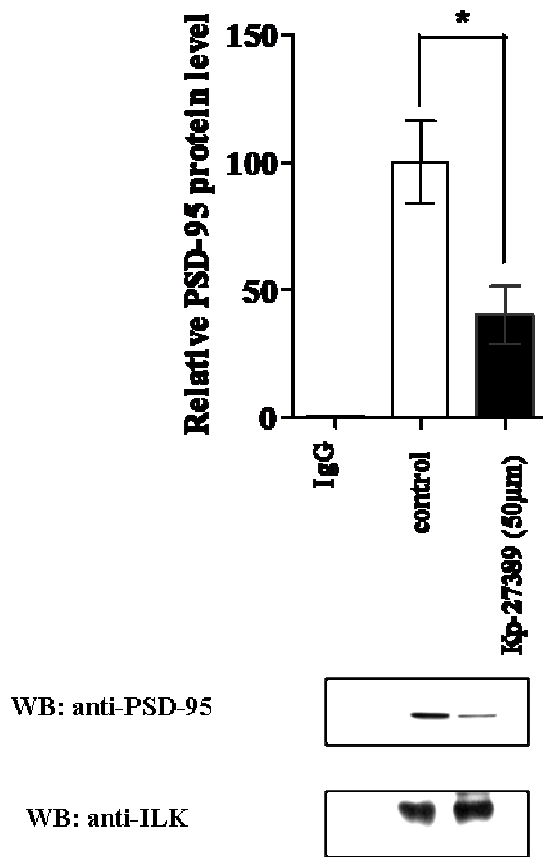


Fig.3.9. Co-immunoprecipitation of ILK with (A) GluR1, (B) NR1, (C) NR2A, (D), stargazin and (E) PSD-95.in the hippocampus was disrupted by Kp-27389 treatment. We quantified protein amounts of GluR1, NR1, NR2A, stargazin and PSD-95 co-immunoprecipitated with anti-ILK antibody in the homogenates prepared from control and Kp-27389-treated hippocampal by performing western blot analysis. Protein levels are normalized to ILK and expressed as a percentage of the control group which was set as 100%. Quantity of GluR1, NR1, NR2A, stargazin and PSD-95 were significantly reduced in Kp-27389-treated hippocampal homogenates as compared with control ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.05$ and $p<0.05$ respectively, two-tailed, unpaired Student's *t*-test).

DISCUSSION

In order to define a role of a protein in LTP induction, either the inhibition of activity of this protein should result in LTP deficits or the activity of the protein should be enhanced during LTP induction (Citri and Malenka, 2008). In this context, the present study utilized a potent and specific ILK inhibitor, KP-27389 to inhibit the kinase domain of ILK without affecting other domains. Our results demonstrate that LTP was not induced by TBS protocol in KP-27389 treated hippocampal slices. Assessment of basal synaptic transmission at various stimulus intensities revealed that fEPSP slope was significantly attenuated in KP-27389-treated slices. These findings established causality between ILK, basal synaptic transmission and LTP in the hippocampus. Consistent with our results, another recent study showed that ILK is implicated in the regulation of neural plasticity in the NAc core in response to cocaine sensitization (Chen et al., 2010).

To investigate whether induction of LTP leads to enhanced activity of ILK, we induced chemical LTP in organotypic hippocampal slice culture by application of the adenylatecyclase activator (Forskolin) and the phosphodiesterase inhibitor (Rolipram). We observed that ILK activity is increased during induction of cLTP in organotypic slice culture. The increase in ILK activity during cLTP further strengthens the role played by ILK in the regulation of hippocampal synaptic plasticity. The cLTP induced by these agents is similar to electrically induced LTP, however, molecular studies after electrical stimulation of slices tend to be time consuming and labor intensive. In addition, only a subset of the synapses is modified by electrical stimulation resulting in difficulty in detecting molecular changes. In contrast, a large proportion of the synapses are modified by cLTP and therefore, changes can be readily detected (Makhinson et al., 1999, Otmakhov et al., 2004). Induction of cAMP-dependant LTP in acute slices requires low

frequency stimulation of presynaptic fibers. This is because the majority of CA3-CA1 connections are severed during acute slice preparation preventing bursting of CA3 cells (Otmakov et al., 2004). Burst activity of CA3 cells is required for cLTP induction (Mackinson et al., 1999). Induction of cAMP-dependent LTP in slice culture, although it is still NMDARs-dependant, does not require electrical stimulation. Damaged axons in slice culture preparations are replaced by collaterals from CA3 cells. Therefore, CA3 activity can potentially activate all CA1-CA3 synapses. Thus, in slice cultures, application of forskolin/rolipram is a useful protocol for a detailed examination of the morphological changes and the biochemical pathways implicated in NMDARs-dependant LTP (Otmakov et al., 2004 and Gobert et al., 2008).

Deficits in LTP in KP-27389 treated slices could be due to altered axonal depolarization, presynaptic release and/or postsynaptic mechanisms. Therefore, we assessed the presynaptic fiber volley amplitude at various stimulus intensities. We found the fiber volley amplitude did not differ significantly among the control and treated slices at various stimulus intensities, indicating that the conversion of the presynaptic stimulus into axonal depolarization was not affected by KP-27389 treatment. In addition, no change has been detected in PPF% in the KP-27389-treated slices. PPF is a presynaptic form of synaptic plasticity that is likely mediated by an additional neurotransmitter release (Wu and Saggau, 1994, Zuker and Regehr, 2002). Overall, our results suggest that KP-27389-treatment does not impair presynaptic mechanisms. Most of the excitatory neurotransmission in the hippocampus is mediated by AMPARs (Dingledine et al., 1999, Malinow and Malenka, 2002). In this respect, we investigated the spontaneous excitatory postsynaptic currents (sEPSCs) mediated by AMPARs. Analysis of the within TBS facilitation revealed a significant reduction of potentiation in Kp-27389 -treated slices compared to control slices. In addition, there was significant facilitation with subsequent

TBS, in the control, whereas such facilitation was largely impaired in the KP-27389-treated slices, suggesting that excitatory postsynaptic potentials (EPSPs) mediated by the activation of NMDARs is diminished in KP-27389-treated slices (Larson and Lynch, 1988). In addition, the form of cLTP investigated in this study is NMDAR-dependent. Therefore, we assessed the (sEPSCs) mediated by NMDARs. Our results demonstrate a significant reduction in AMPA and NMDARs-mediated current amplitude in KP-27389-treated slices. However, no significant change in the frequency of AMPA or NMDARs-mediated sEPSCs has been detected in KP-27389-treated slices. The frequency events of sEPSCs, most often correlates to the probability of release from the presynaptic terminal. Therefore, the lack of change in the frequency of AMPA and NMDARs-sEPSCs further confirm our previous finding that deficits in LTP caused by KP-27389 are not due to presynaptic dysfunction.

The mean current amplitude of single channel currents is a product of single channel conductance (g) and probability of opening. Therefore, a reduction in conductance or open probability will reduce the mean current amplitude of the single synaptic receptors. If this is the case, the amplitude of the spontaneous currents will be decreased if any one of these parameters is reduced for single synaptic receptors (Kanju et al., 2007). Therefore, we performed single channel recordings for AMPARs and NMDARs current using synaptosomes isolated from control and KP-27389-treated acute hippocampal slices. Interestingly, our studies revealed that, KP-27389 treatment caused a reduction in single channel AMPA and NMDARs open probability and open times coupled with increased close times. In addition, a decline in the single channel AMPA and NMDARs conductance was observed in KP-27389-treated synaptosomes.

The decline in AMPA and NMDARs-sEPSCs amplitude could be also due to decreased synaptic expression of AMPA and NMDARs (Conti and Weinberg, 1999). We noted a

significant reduction in the protein levels of GluR1, NR1, NR2A and NR2B subunits in KP-27389-treated synaptosomal fraction. In addition, we observed a significant decrease in the protein level of stargazin, a TARP that helps to anchor AMPARs to the surface of the postsynaptic density (Bats et al., 2007). Stargazin also influences the biophysical properties of GluR1-AMPARs by enhancing their channel conductance and slowing glutamate-induced deactivation and desensitization (Nicoll et al., 2006). The decrease in stargazin levels in KP-27389-treated synaptosomes provides an explanation of the decreased synaptic expression and single channel conductance of AMPARs. Moreover, a reduction in the protein level of PSD-95 in KP-27389-treated synaptosomal fraction was noted. PSD-95 is a scaffolding protein that promotes clustering and synaptic expression of AMPA (Bats et al., 2007) and NMDARs. PSD-95 can also affect NMDAR channels properties; it reduces the desensitization of NMDARs responses and enhances NMDAR channel openings (Lin et al., 2004; Lin et al., 2006, Lau and Zukin, 2007). Similarly, the reduction in the protein level of PSD-95 in KP-27389-treated synaptosomal fraction can explain the decrease in the synaptic expression of GluR1 AMPARs and NR1, NR2A and NR2B NMDAR subunits. Moreover, it could explain the reduction in single channel NMDARs open probability and open time.

Alteration in the synaptic expression of GluR1, NR1, NR2A and NR2B, stargazin and PSD-95 caused by KP-27389 treatment could be due to disruption of protein synthesis. In order to address this possibility, we performed western blot experiments to measure the protein level of GluR1, NR1, NR2A, NR2B, stargazin and PSD-95 in the whole hippocampal homogenates. Surprisingly, we could not find any significant difference in the KP-27389-treated whole hippocampal homogenates, indicating that KP-27389 did not affect GluR1, NR1, NR2A, NR2B, stargazing and PSD-95 proteins synthesis. Consistent with the results from the present study, our

previous report showed, in i.c. STZ brains, the synaptic expression of GluR1 and NR2A is decreased without affecting the protein synthesis (Shonesy et al., 2012). However, the lack of effect of ILK on the synthesis of these proteins reported in our study is in contrast to the previously reported role of ILK in the regulation of GluR1 and PSD-95 genes transcription in the NAc core of cocaine sensitized rats (Chen et al., 2010). This discrepancy between the results of the two studies may be attributed to the short duration of treatment with the ILK inhibitor KP-27389 (2 to 3) hours. This few hours may not be sufficient to induce any change in the transcription of genes.

ILK is a multi domain focal adhesion protein that not only participates in signal transduction pathways necessary for cell survival and gene expression, but also serves as an important molecular scaffold at the cell-extracellular matrix adhesion sites (Wu, 1999; Dedhar, 2000; Wu, 2001). Through its two separate domains, the N-terminal ankyrin repeat domain and the C-terminal kinase domain, ILK can interact with a variety of proteins (Dedhar, 2000; Wu, 2001, Dobрева et al., 2008). Therefore, it is possible that ILK can interact with these proteins at their synaptic sites and affect their synaptic localization. In order to test this possibility we studied the interaction between ILK and p-GluR1ser845, NR1, NR2A, PSD-95 and stargazin by performing co-ip studies in the hippocampus. Our data revealed that ILK forms complexes with p-GluR1ser845, NR1, NR2A, PSD-95 and stargazin. Interestingly, our results are in line with results of a previous study that demonstrated that in the NAc core ILK is co-immunoprecipitated with PSD-95, p-GluR1ser845 and GluR1 (Chen et al., 2010).

Next, we tested the effect of KP-27389 treatment on the interaction of ILK with p-GluR1ser845, NR1, NR2A, PSD-95 and stargazin. The results of our study revealed that KP-27389 treatment significantly disrupted ILK interaction with these proteins. Therefore, it is

reasonable to presume that impaired ILK activity is sufficient to inhibit the interaction of ILK with AMPARs and NMDARs and their associated proteins, resulting in impaired synaptic expression. Moreover, P-GluR1-845 enhances the probability of channel opening and the surface expression of AMPARs (Boehm and Malinow, 2005, Wang et al., 2005, Jiang et al., 2006, Derkach et al., 2007, Citri and Malenka et al., 2008). ILK-P-GluR1-845 interaction may also explain the decrease in the probability of opening and the synaptic expression of AMPARs by inhibiting ILK activity.

A recent study reported that ILK/PSD-95 and GluR1 interaction is mediated by the N-terminal ankyrin repeat domain (Chen et al., 2010). Chen et al used an ILK silencing approach that targeted all the 3 domains of ILK. It is interesting to note that in our study, inhibition of kinase domain alone was sufficient to disrupt ILK/PSD-95 and GluR1 interaction. This raises the possibility that kinase domain may have influence over the functionality of N-terminal ankyrin repeat domain that interacts with the PDZ domain of PSD-95. Another possibility is that ILK interacts with these proteins through its C-terminal domain, as it has been reported that through its C-terminal kinase domain, ILK can interact with multiple proteins including integrins, CHILKP, actopaxin, α -parvin, affixin, β -parvin and paxillin. Mutation in ILK C-terminal kinase domain abolishes these interactions (Wu and Dedhar, 2001). In addition, a recent study reported that GluR1 is colocalized with paxillin (Piao et al., 2009) which is an ILK C-terminal kinase domain binding protein, supporting a role of C-terminal kinase domain in ILK interaction with GluR1 and other proteins.

In summary, our findings indicate a novel role for ILK in regulating hippocampal synaptic plasticity. First, our results demonstrate that suppression of ILK activity impaired hippocampal basal synaptic transmission and LTP. Second, the activity of ILK is increased in the

hippocampus during cLTP. Third, inhibition of ILK activity resulted in alteration in the synaptic expression and function of AMPA and NMDARs and their associated proteins. Finally, ILK complexes in the hippocampus with AMPA and NMDARs and their associated proteins and inhibition of ILK activity disrupted such interaction. Taken together, our data indicate an essential role for ILK in the regulation of hippocampal synaptic plasticity. In addition, the results of this study provide new information on the potential mechanisms by which ILK exerts its regulatory effect on basal synaptic transmission and synaptic plasticity in the hippocampus.

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4. Peroxisome proliferator activated receptors delta ameliorates diabetes induced cognitive dysfunction

ABSTRACT

The increased risk of cognitive dysfunction in patients with type II diabetes increases the need to develop novel therapeutic targets for diabetes-induced cognitive deficits. The peroxisome proliferator activated receptors delta (PPAR delta) are highly expressed in the brain and shown to improve spatial memory and hippocampal neurogenesis in control rodents. However, the effect of PPAR delta agonist on diabetes-induced dementia has not yet been explored. In this study, the effects of selective PPAR delta agonist, GW0742, on hippocampal synaptic transmission, plasticity and spatial memory were investigated in db/db mice model of type II diabetes. Oral administration of GW0742 for 2 weeks significantly attenuated deficits in hippocampal long-term potentiation (LTP) observed in db/db mice. In addition, GW0742 effectively prevented impairments in spatial recognition memory (hippocampal-dependant task) reported in db/db mice in the Y-maze test. The amelioration of LTP and spatial memory deficits in db/db mice caused by GW0742 treatment was accompanied by improvement in AMPA and NMDARs-mediated synaptic transmission in the hippocampus. A recent study reported that, phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity, is upregulated in the fat tissue of T2DM patients and db/db mice. In addition, activation of PPAR delta has been shown to induce ILK transcription and enhance its activity. Interestingly, we found that integrin-linked kinase (ILK) activity is significantly reduced in the hippocampus of db/db mice and that activation of PPAR delta rescued ILK activity. A recent study demonstrated that PPAR delta ameliorated amyloid burden and inflammation in a transgenic mouse model of Alzheimer's disease (AD). Our results demonstrated that ILK expression is significantly reduced in the

human AD hippocampus. Our findings suggest that activation of PPAR delta could rescue the activity of ILK and thereby ameliorate cognitive dysfunction in AD and type II diabetes.

INTRODUCTION

Several lines of evidence suggest a relationship between cognitive dysfunction such as Alzheimer's disease (AD) and type II diabetes (Leibson et al., 1997, Janson et al., 2004, Biessels et al., 2006, Kulstad et al., 2006, Zhao et al., 2009, Park, 2011). In a clinical context, patients with type II diabetes appear to have two to three fold increased relative risk for AD (Stolk et al., 1997, Ott et al., 1999, Petot et al., 2003, Zhao et al., 2009), and more than 80% of AD patients appear to have type II diabetes, or show high plasma and low cerebrospinal fluid (CSF) insulin levels as well as disturbance in brain insulin signaling that are associated with heavy deposition of β -amyloid plaques and neurofibrillary tangles (Craft et al., 1998, Messier, 2003, Janson et al., 2004, Cole and Frautschy, 2007, Zhao et al., 2009, Park, 2011). Cognitive impairments have also been reported in experimental models of type II diabetes. For example, impaired hippocampus-dependant long-term potentiation and spatial memory have been demonstrated in the type II diabetic mouse strain db/db that is characterized by hyperinsulinemia and hyperglycemia (Li et al., 2002, Sharma et al., 2010, Dinel et al., 2011). Similar deficits have been also reported in the intracerebroventricular streptozocin-injected rats (ic.STZ), a model of sporadic AD with central insulin resistance and brain insulin deficiency (Biessels et al., 1996, de la Monte et al., 2006, Shonsey et al., 2012). However, the mechanisms of diabetes-induced cognitive impairments have not been fully elucidated (Janson et al., 2004).

Peroxisome proliferator-activated receptor (PPAR) alpha, gamma and delta represent the PPAR subfamily of the nuclear hormone receptor superfamily. These receptors regulate gene transcription in response to ligand binding to specific promoter regions of target genes

(Schoonjans et al., 1996, Blumberg et al., 1998, Willson et al., 2000, Chawla et al., 2001). PPARs play a role in regulation of lipid and glucose metabolism and they have been successfully exploited to be clinically useful in the treatment of metabolic diseases like hyperlipidemia and type-2-diabetes (Robinson and Grieve, 2009, Bilsen and Nieuwenhoven, 2010). Activation of PPARs has been also demonstrated to exert additional benefits to the central nervous system. In this regard, Pedersen et al., reported that treatment of Tg2576 mice with the PPAR gamma agonist rosiglitazone significantly enhanced spatial working and reference memory and also decreased brain level of β -amyloid 1-42 (Pedersen et al., 2006). Clinical trials of rosiglitazone in AD patients reported that rosiglitazone therapy significantly improved cognition in AD patients (Chalimoniuk et al., 2004, Watson et al., 2005, Risner et al., 2006). In addition to the beneficial effects exerted by PPAR gamma activation in the brain, early treatment with PPAR delta agonists prevented neurodegeneration and associated cognitive deficits in an experimental model of sporadic AD (de la Monte et al., 2006). Activation of PPAR delta also decreased brain inflammation and amyloid burden in transgenic animal model of AD (Kalinin et al., 2009). However, it remains unclear if activation of PPAR delta which is the predominant isotype in the brain (Braissant et al., 1998, Woods et al., 2003) will improve cognitive dysfunction caused by type II diabetes. Therefore, in view of the neuroprotective action of PPAR delta agonists and due to the fact that PPAR delta is the most prevalent PPAR in the brain, we investigated the effect of the high affinity PPAR delta agonist GW0742 on hippocampal long-term potentiation (LTP) and spatial memory in the db/db/ mice. Our findings provide direct evidence of the therapeutic potential role of GW0742 in diabetes-induced cognitive dysfunction.

In the hippocampus, AMPA and NMDA glutamate receptors play complementary roles in synaptic plasticity and memory (Huang and Stevens, 1998; Kleshchevnikov, 1998;

Malenka and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir 2002; Brecht and Nicoll, 2003; Derkach et al., 2007). Therefore, we investigated the effect of PPAR delta activation on AMPA and NMDAR-mediated synaptic currents. Our results demonstrated that activation of PPAR delta ameliorates the deficits in AMPA and NMDARs-synaptic transmission in db/db/ mice.

Our recent study reported that integrin-linked kinase (ILK), which is a serine-threonine kinase that regulates cell growth and differentiation (Dedhar et al., 1999), play a crucial role in synaptic transmission and LTP in the hippocampus (unpublished data). In addition, we have demonstrated previously that ILK is downregulated in ic. STZ brains and this downregulation is well correlated with deficits in synaptic transmission and plasticity in the ic STZ rodents (Shonesy et al., 2012). A recent study reported that, phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity, is upregulated in the fat tissue of T2DM patients and type 2 diabetic mouse model db/db mice (Yu et al., 2011). In addition, activation of PPAR delta has been shown to induce ILK transcription and to enhance its activity (Di-Poï et al., 2002). Our findings suggest that ILK activity is modulated in the hippocampus of db/db mice and that activation of PPAR delta with a selective PPAR delta agonist could rescue the activity of ILK and thereby ameliorate cognitive dysfunction induced by type II diabetes.

MATERIALS AND METHODS

Animals:

Male C57BL/KsJ-db/db mice (8 weeks old) and their age-matched non-diabetic m/m littermates were purchased from Jackson laboratories, PA. All mice were housed in plastic cages with free access to food and water and maintained in temperature-controlled room (22-23°C) on a 12-h light/dark cycle. The db/db mice were randomly divided into 2 groups. One group received a

daily oral administration of GW0742 (10 mg/kg) that continued for 2 weeks and the other group received saline. The age-matched non-diabetic m/m mice served as control and also divided into 2 random groups and received either saline or GW0742 daily for 2 weeks. All experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Auburn University.

Human brain Lysates:

AD and age-matched control (normal human brain) (hippocampus) tissue lysate were obtained from Novus Biologicals (Littleton, CO). The samples were as closely aged-matched as possible.

Y-maze test:

A Y-maze was used for this study. The maze consisted of 3 arms at a 120-degree inclination from each other. Each arm measured 7.5 cm x 38 cm x 12 cm (w x l x h). The maze was constructed of opaque beige plastic. The arms were elevated a further 2 cm to prevent animals from climbing up the walls and escaping the maze. The arms were randomly labeled as Arms A, B, and C. Extra-maze cues were provided by a cylinder covered in a checkerboard pattern (Arm A), a triangle (Arm B), and a 3-dot domino pattern (Arm C). All extra-maze cues elevated about 15 cm above the arm back wall. The maze was thoroughly cleaned with a 35% (v/v) alcohol solution between subjects to eliminate odor cues, and it was located in a dimly illuminated room with a white noise background. All sessions were recorded using a camera located above the center of the maze. All animals were subject to a training (exploration) and testing sessions. During the training session, one of the arms was closed. Thus, subjects could only navigate the remaining two arms, the arm in which they were started in the maze (Start Arm) and another arm (Other arm). The identity of the Start and Other arms were randomized within groups but remained constant within subjects. Subjects were released into the Start arm and allowed to

explore the Start and Other arm for 15 min, upon which time they were removed from the maze and returned to their home cages. Three hours later, all animals were returned to the maze and allowed to explore all three arms for 6 min. Because of rodents' natural curiosity, it was expected that they would spend a large proportion of the test session exploring the previously occluded arm (Novel arm). Upon completion of the test period, all animals were returned to their home cages. Recordings of the test session were scored by three independent observers who were blind to the group and identity of the Novel arm for all subjects. All scores were averaged across observers with the constraint that if a given observer's score deviated by more than 10 s or 1 entry from the ratings of the other two observers, that observer's score was not included in the average. This criterion is very stringent, but it ensures high interpreter reliability. All scores were obtained from at least two independent observers. Measures for the Start and Other arm were averaged for all variables, and they will be referred as the Familiar arms. Number of alternations and dwell time into each arm were recorded. Due to the body condition of db/db mice subjects (i.e., increased weight and reduced mobility), the extent to which exploratory behavior may be compromised due to decreased general activity or increased anxiety in a novel environment was analyzed by looking at the number of alternations animals made within the maze; this provides a rough measure of general exploratory behavior. Dwell time was analyzed by contrasting the proportion of the total time spent in the maze's arms that subjects spent in the Novel and Familiar arms.

Electrophysiological experiments:

Acute hippocampal slice preparations:

Acute hippocampal slices were prepared from db/db mice and their matched controls using standard experimental procedure, previously described by (Zeng et al., 1995) and (Zeng and

Tietz, 1999). Mice were decapitated under CO₂ anesthesia. After rapid dissection, the brains were immediately placed in ice-cold oxygenated (95% O₂, 5% CO₂) dissection buffer consisting of (in mM): NaCl 85, KCl 2.5, MgSO₄ 4.0, CaCl₂ 0.5, NaH₂PO₄ 1.25, NaHCO₃ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5). Coronal hippocampal slices of 350 μm thickness were obtained using a vibratome Series 1000 tissue sectioning system (Technical products international Inc., St. Louis, MO, USA). These slices were incubated for 2 hours at 30°C in holding chamber of artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 119, KCl 2.5, MgSO₄ 1.3, CaCl₂ 2.5, NaH₂PO₄ 1.0, NaHCO₃ 26, dextrose 11.0, and continuously bubbled with 95% O₂/5% CO₂.

Extracellular field recordings:

For electrophysiological recordings a single slice was then transferred to a submerge-type recording chamber and held between two nylon nets. The submersion chamber was continuously perfused with oxygenated ACSF (30°C) at a flow rate of 2–3 ml/min. A platinum bipolar electrode was placed in the stratum radiatum to deliver test pulses (0.033 Hz) to the Schaffer-collateral commissural pathway of the hippocampal CA1 region. Glass microelectrodes (1–4 MΩ) were pulled from 1.5-mm-outer diameter glass tubing with the use of a micropipette puller (Narishige scientific instruments Lab, Tokyo) and filled with (ACSF). The glass electrodes used for recording field excitatory postsynaptic potentials (fEPSPs) from the CA1. Recordings of field EPSPs (fEPSPs) were made in the middle of the stratum radiatum with an Axoclamp 2B (Axon Instruments, Foster City, CA) (Shonsey et al., 2012). Test intensities were set to obtain fEPSP slopes of 50% of those at which population spikes were detectable as determined from input–output curves for each slice. Following stable baseline recordings of at least 20 minutes, theta burst stimulation consisted of one episode of the following: 10 bursts of stimuli, each of four

pulses at 100 Hz; interburst interval, 200 msec was applied to the Schaeffer-collaterals and fEPSPs were measured in *stratum radiatum*. 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 Win-LTP acquisition software (Anderson and Collingridge, 2007). The data are presented as means \pm SEM. For plasticity experiments, significance was determined using 2-way repeated measures ANOVA, and Tukey's post-hoc analysis. Results are presented as mean \pm SEM.

Whole cell patch clamp recordings:

After the 2 hours incubation period, slices were transferred to a recording chamber perfused with ACSF solution saturated with 95% O₂/5% CO₂ at a rate of 2 ml/min Individual hippocampal slices were visualized by Nomarski differential interference contrast optics with Olympus BX51WI (Olympus,USA) equipped with a water immersion lens. Recordings from CA1 pyramidal neurons were made with patch pipettes (5–7 M Ω), pulled from borosilicate glass capillaries (nonfilamented, 1.5 mm OD, World Precision Instruments, Sarasota, FL) on a Sutter P-2000 Puller (Sutter Instruments, Novato, CA) and filled with a solution containing (in mM): 122.5 C-gluconate, 10 HEPES, 1.0 EGTA, 20 KCl, 2.0 MgCl₂, 2.0 Na₂·ATP, 2 QX-314, 0.25 Na₃·GTP·3H₂O, pH 7.3 (adjusted with KOH), 280–290 mOsm. To conserve G protein mediated responses GTP was added in the pipette solution, ATP was included to supply energy for other intracellular phosphorylation reactions and to prevent rundown of calcium channels and Cs⁺ was added to eliminate K⁺ currents. By applying negative pressure to the pipette during approach to the cell, tight seals (\geq 2 G Ω before breaking into whole cell mode) were achieved. Using the whole cell voltage-clamp technique, AMPA receptor mediated quantal events (sEPSCs) were isolated from CA1 pyramidal neurons in the presence of 50 μ M aminophosphonopentanoic acid

(APV) and 30 μ M bicuculline methiodide (BMI). Axopatch 200-B amplifier (Axon Instruments, Inc., Foster City, CA) was used to voltage-clamp ($V_h = -70$ mV) neurons. Current output was low-pass filtered (2 kHz) and sampled at 10 kHz. The signal was continuously monitored on-line (Clampfit 9 Software, Axon Instruments), and digitized by using (Digidata, 1200, Axon Instruments). Baseline sEPSC activity was recorded in each neuron for at least 10 min. Using the Mini-Analysis program (Synaptosoft, Decatur, Ga), recorded events ≥ 4 pA, with a faster rise than decay were detected and analyzed. sEPSCs amplitude and decay kinetics were measured using a single exponential function. Data were compared using ANOVA with Tukey's post-hoc analysis. Results are presented as mean \pm SEM.

ILK kinase activity assay :

ILK kinase activity was determined as previously published (Eke et al., 2009). Briefly, hippocampi were homogenized in ice –cold cell lysis buffer (Cell Signaling, Danvers, MA) supplemented with complete protease inhibitor cocktail. The extracts were centrifuged for 15 minutes at 10,000 $\times g$, at 4 $^{\circ}$ C to remove cellular debris, and the protein content of the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL). Anti-ILK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with gentle roking at 4 $^{\circ}$ C overnight with protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). 500 μ g of total protein were incubated with immobilized anti-ILK antibody cross-linked to the A/G plus agarose beads with gentle roking at 4 $^{\circ}$ C for 4 hours. After ILK was selectively immunoprecipitated from the cell lysates, the immunoprecipitated products were washed twice in lysis buffer and twice in kinase assay buffer (Cell Signaling, Danvers, MA), containing (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM β -glycerolphosphate, 0.1 mM sodium orthovanadate, and 2 mM dithiothreitol). The samples were then resuspended according to the manufacturer's protocol

(Cell Signaling, Danvers, MA) in 50 μ l of kinase assay buffer containing 200 μ M ATP and 1 μ g of GSK-3 β fusion protein. After 30 min incubation at 30 C $^{\circ}$, the kinase reaction was terminated by the addition of Laemmli SDS sample buffer (Bio-Rad, Hercules, CA). ILK kinase activity was measured subsequent to SDS-PAGE and Western blotting, by detection of phosphorylated GSK fusion protein by using anti-GSK3 α/β S21/9 antibodies.

Western Blot Analysis.

For western blotting, hippocampal lysates were resolved by 10% SDS-PAGE and blotted to PVDF membranes (immobilon- P; Millipore, Bedford, MA). Membranes were blocked with 5% non-fat dry milk in PBS containing 0.01% Tween 20 for 1 h and then incubated with anti-ILK (1:1000; Cell Signaling Technology) and anti- β -actin (1:1000; Cell Signaling Technology) overnight at 4 $^{\circ}$ C. Membranes were then probed with corresponding anti-rabbit or anti-mouse horseradish peroxidase conjugated second antibodies for 1 h (1:5000; Cell Signaling Technology, 1:5000; Cell Signaling Technology) and developed using the enhanced chemiluminescence (SuperSignal West Femto ECL reagent (Pierce Biochem)). All immunoreactive bands were scanned with a desktop scanner and were subsequently quantified by densitometric analyses using Quantity 1 analysis software (Bio-Rad, Hercules, CA, USA). The densities of each band which represented individual animals were normalized to β -actin and then compared with control levels for both control and treated groups. The data represent the mean \pm SEM. Significance was determined using ANOVA with Tukey's post-hoc analysis.

RESULTS

4.1) GW0742 treatment ameliorates deficits in spatial working memory performances in db/db mice:

Previous study indicated that spatial working memory assessed in the Y-maze test was impaired in db/db mice (Dinel et al., 2011). In this study, we investigated the effect of GW0742 treatment on the spatial memory deficits in db/db mice. We measured the time spent into each maze's arm by all groups of mice. Although, there were no between-group differences in total time spent in the arms (as opposed to dwelling in the center of the apparatus), $F(2, 11) = 2.67$, $MSE = 327$, dwell time into novel arm was significantly different in vehicle-treated db/db mice. For example, a one-way ANOVA conducted on the proportion of time spent in the novel arm, $F(2, 11) = 7.08$, $MSE = 159.77$, $p < 0.05$, followed by a series of planned comparisons, revealed that control db/db mice dwelled in the novel arm significantly less than control m/m mice, $p < 0.01$. GW0742-treated db/db mice dwell time in the novel arm was significantly higher than that of vehicle-treated db/db mice $F(1, 11) = 5.31$, $p < .05$. Furthermore, the GW0742-treated m/m mice's dwell time did not differ from that of control m/m mice, $F(1, 11) < 1.0$ (Figure 4.1.A). We then analyzed dwell time by contrasting the proportion of the total time spent in the maze's arms that animals spent in the novel and familiar arms. The control db/db mice spent significantly more time in the familiar arms (average of the start and other arms) than the novel arm, $t(5) = 7.39$, $p < .001$. However, there were no differences in dwell time between the novel and familiar arms in either, GW0742-treated db/db mice $t(2) = 3.17$, or control m/m mice, $t(4) = 0.63$, or GW0742-treated m/m mice $t(4) = 0.85$ (Figure 4.1.B). Due to the body condition of db/db mice (i.e., increased weight and reduced mobility), the possibility that the extent to which exploratory behavior may be compromised due to decreased general activity or increased anxiety

in a novel environment was analyzed by looking at the number of alternations animals made within the maze; this provides a rough measure of general exploratory behavior. The number of alternations was analyzed with a one-way analysis of variance (ANOVA), $F(2, 10) = 26.79$, $MSE = 29.87$, $p < .001$ (Fig. 4.1.C). Control m/m mice produced more alternations than the vehicle-treated db/db mice, $F_s(1, 10) = 48.21$, $p < .001$. However, the control db/db mice and GW0742-treated db/db mice did not differ in this measure, $F(1, 10) < .10$. Thus, although the non-diabetic m/m mice showed more activity and general exploratory behavior than vehicle-treated and GW0742-treated db/db mice, mobility was not a concern when comparing the two db/db mice groups.

4.2) Treatment with GW0742 improves diabetes-induced LTP deficits in db/db mice:

Since LTP is a form of synaptic plasticity that is widely accepted now as a cellular correlate of memory processing, we next examined LTP in the shaffer collateral synapses of hippocampal slices of vehicle-treated and GW0742 treated m/m and db/db mice. Long-term potentiation was induced with theta burst stimulation (TBS) protocol that involves 5 trains of 10 bursts of 4 pulses at 100 HZ; with interburst interval 200 msec. A two-way repeated-measures ANOVA showed that LTP, measured 55-60 min following TBS, was significantly impaired in 5 slices from vehicle-treated db/db mice, (Fig.4.2 A; $p < 0.01$; $n = 5$) with an average of $105.7 \% \pm 1.419 \%$, compared with control m/m mice in which LTP was induced in five of five slices with an average of $(153.3 \% \pm 4.510 \%; p < 0.01 ; n = 5)$. However, LTP significantly induced in GW0742-treated db/db mice with an average of $140.4\% \pm 3.408 \%$ (Fig. 4.2 B. $p < 0.001$; $n = 5$). In addition, the LTP average percent induced in the GW0742-treated m/m mice was not significantly different than that induced in control m/m mice ($155.7 \pm 3.444 \%; n = 5$). Treatment with GW0742 did not affect LTP average in control m/m mice to any significant extent ($153.9 \pm$

4.5 %; n=5). These results demonstrate that treatment with GW0742 rescued LTP deficits caused by diabetes in db/db mice, and thereby ameliorate impairment in spatial memory performances in db/db mice. Analysis of the within TBS facilitation was performed by normalizing the amplitudes of sEPSPs with the amplitude of the first fEPSP. A two-way repeated measures ANOVA revealed a significant reduction of potentiation in slices obtained from db/db mice compared to control slices (Fig.4.2. C; $p < 0.01$; n=5), whereas potentiation is rescued in GW0742-treated db/db mice (Fig. 4.2. C; $p < 0.001$; n=5). We then normalized the first pulse of fEPSPs from 2nd, 3rd, 4th and 5th TBS to that of the 1st TBS to assess whether subsequent TBS resulted in facilitation in db/db mice. Our results revealed that there was significant facilitation with subsequent TBS, in the control, whereas such facilitation was largely impaired in the vehicle-treated db/db mice slices (Fig.4.2 D; $p < 0.001$; n=5), suggesting that excitatory postsynaptic potentials (EPSPs) mediated by the activation of NMDA receptors is diminished in vehicle-treated db/db mice. Facilitation is rescued in GW0742-treated db/db mice (Fig. 4.2.D; $p < 0.001$; n=5).

4.3. Impairments in AMPAR-sEPSCs are rescued by GW0742 treatment in db/db mice:

Most of the excitatory neurotransmission in the hippocampus is mediated by AMPARs. In order to test whether alterations in AMPAR-mediated currents contribute to the LTP deficits observed in db/db mice, we performed whole cell patch clamp recordings in CA1 pyramidal neurons of the hippocampus. We recorded sEPSCs that occur without inhibition of the Na⁺ channels. The NMDAR activity was blocked by APV (50 μ m). Our results indicate that the mean current amplitude of AMPAR-sEPSCs was significantly reduced from (9.650 ± 0.3844 pA) in control to (5.470 ± 0.1837 pA) in db/db mice (Fig.4.3.C; $p < 0.001$; n=5). Next we examined the effect of GW0742 on altered AMPAR-sEPSCs in db/db mice. Our data showed that the mean current

amplitude of AMPAR-sEPSCs was significantly increased from (5.470 ± 0.1837 pA) in vehicle-treated db/db mice to (7.654 ± 0.5554 pA) in GW0742-treated db/db mice (Fig. 4.3.C; $p < 0.01$; $n = 5$). In addition, the mean current of AMPAR-sEPSCs in the GW0742-treated m/m mice was not significantly different than that in control m/m mice ($42.9 \pm 2.3\%$ $p <$; $n = 6$). The mean current frequency of AMPAR-sEPSCs was significantly reduced from (2.620 ± 0.1281 pA) in control to (0.5993 ± 0.1246 pA) in db/db mice (Fig. 4.3. C. 5; $p < 0.001$; $n = 5$). Next we examined the effect of GW0742 on altered AMPAR-sEPSCs in db/db mice. Our data showed that treatment of db/db mice with GW0742 did not affect the deficits in mean current frequency of AMPAR-sEPSCs to any significant extent, the mean current frequency of AMPAR-sEPSCs was (0.5993 ± 0.1246 pA) in db/db mice, and (0.9 ± 0.44 pA) in GW0742-treated db/db mice (Fig 4.3.D; $n = 5$). In addition, the mean current of AMPAR-sEPSCs in the GW0742-treated m/m mice was not significantly different than that in control m/m mice (2.623 ± 0.2537 ; $n = 5$).

4.4. ILK expression is not altered in the hippocampus of db/db mice:

Recently, we reported an essential role for integrin-linked kinase (ILK), which is a serine-threonine kinase that functions downstream of growth factor and β -integrin receptors in synaptic transmission and LTP in the hippocampus (unpublished data). In addition, our previous study demonstrated that in an animal model of sporadic Alzheimer's disease and central insulin resistance ILK expression in the hippocampus is decreased and this downregulation is well correlated with deficits in synaptic transmission and plasticity in these animals (Shonesy et al., 2012). Based on these findings, we assessed ILK protein levels in the hippocampus of db/db mice. Our results revealed no significant alteration in the protein level of ILK expression in the hippocampus of all mice groups ($90.16 \pm 9.830\%$ in vehicle-treated db/db mice, 95.83 ± 10.55

% in GW0742-treated db/db mice, and 99.56 ± 20.64 % in GW742-treated m/m mice relative to control, Fig.4.4, n=5).

4.5. ILK activity is attenuated in the hippocampus of db/db mice and treatment with GW-0742 rescued the inhibition of ILK activity in db/db mice:

Since, our recent study demonstrated that inhibition of ILK activity was sufficient to impair LTP and AMPA and NMDA receptor mediated basal synaptic transmission in the hippocampus (unpublished data), we then verified if ILK activity is modified in the hippocampus of db/db mice. Kinase assay for ILK was performed, in which we quantified the amount of P-GSK-3 β at serine-9. Surprisingly, ILK activity estimated by P-GSK3- β Ser21/9, is significantly reduced in the hippocampus of db/db mice ($62.5\% \pm 2.988\%$ relative to control, Fig. 4.5. ; $p < 0.001$; n=5). Moreover, treatment with GW-0742 significantly increased ILK activity) in GW-0742-treated db/db mice ($89.9\% \pm 1.5\%$). ILK activity in the GW-0742-treated m/m mice was not significantly different than that in control m/m mice ($101.3\% \pm 3.39\%$; n= 5). These results suggest that GW-0742 could rescue the deficits of ILK activity in db/db mice.

4.6. ILK expression is significantly reduced in the human AD hippocampus:

Our previous study demonstrated that in an animal model of sporadic Alzheimer's disease, ILK expression in the hippocampus is decreased and this downregulation is well correlated with deficits in synaptic transmission and plasticity in these animals (Shonesy et al., 2012). Based on these findings, we assessed ILK protein levels in the human AD hippocampal lysate. Our results revealed significant reduction in the protein level of ILK expression in the human AD hippocampus ($27.02\% \pm 6.113\%$, relative to age-matched control (normal human brain) (hippocampus) tissue lysate, $100\% \pm 7.19\%$, Fig.4.6, n=3).

FIGURES AND FIGURE LEGENDS

Fig.4.1

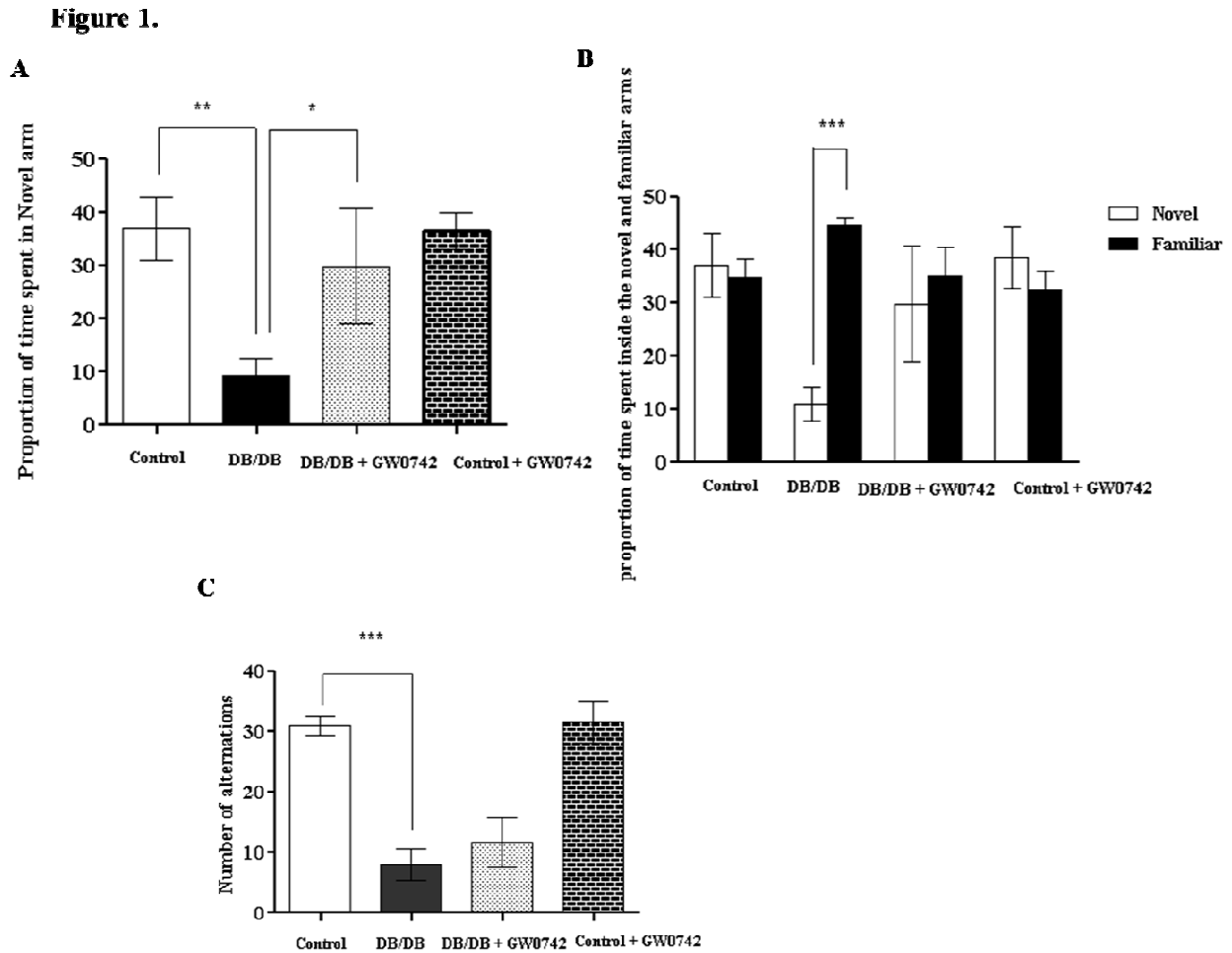


Fig.4.1. Treatment with GW0742 improves diabetes-induced spatial memory performances deficits in db/db mice. (A) Bar chart shows the significant reduction in the time spent by vehicle-treated db/db mice in exploring the novel arm in the y-maze than in the control non-diabetic m/m mice $p < 0.01$ or in GW0742-treated db/db mice $p < 0.05$. GW0742 treatment did not affect the time spent in exploring the novel arm in the control non-diabetic m/m mice. (B) Bar plot showing drastic reduction of time devoted to explore the novel arm when compared to the familiar arm by vehicle-treated db/db mice $p < .001$. No significant difference in the dwell time in the novel arm from that of familiar arm in both non-diabetic m/m mice and GW0742-

treated db/db mice. Treatment with GW0742 did not affect the time spent in exploring the novel or the familiar arm in the control non-diabetic m/m mice. (C) Bar plot depicts the significant decrease in activity and general exploratory behavior in db/db mice relative to the control non-diabetic m/m mice $p < .001$. GW0742 treatment didn't have any differential effect on the activity and general exploratory behavior of db/db mice or control m/m mice.

Fig. 4.2.

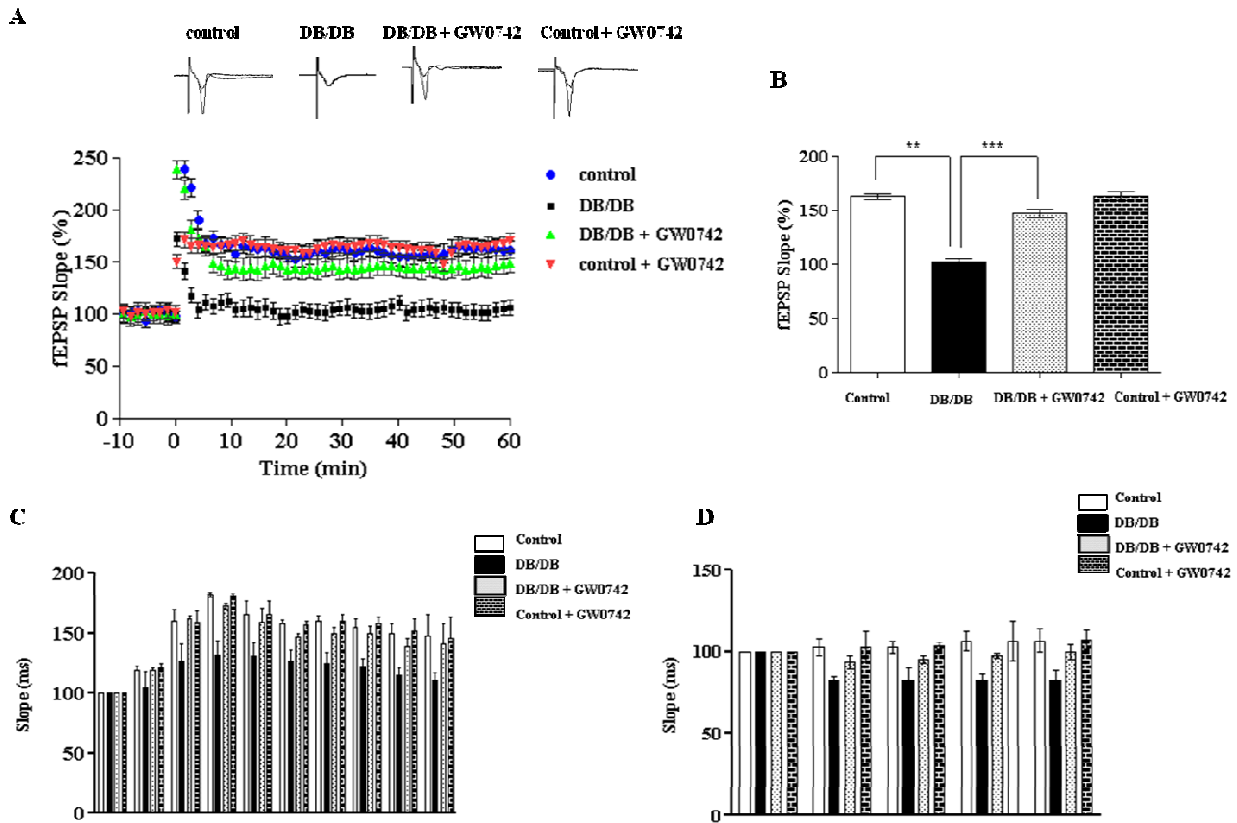


Fig. 4.2. Effect of GW0742 treatment on hippocampal LTP in db/db mice. (A) LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP was significantly impaired in acute hippocampal slices from vehicle-treated db/db mice, normalized fEPSPs slopes of 60 min post-TBS averaged $105.7 \% \pm 1.419 \%$ for vehicle-treated db/db mice and $153.3 \% \pm 4.510 \%$ for control m/m mice, $p < 0.001$; $n = 5$). GW0742-treatment significantly improved LTP deficits in db/db mice, (LTP averaged $140.4 \% \pm 3.408 \%$). GW0742-treatment didn't affect the fEPSPs slopes average % in control m/m mice to any significant extent ($155.7 \pm 3.444 \%$; $n = 5$). (B). Bar plot showing drastic reduction of LTP in vehicle-treated db/db mice compared to control m/m mice. LTP recovered to a potentiated level in GW0742 treated db/db mice, whereas LTP in control m/m mice was not affected by GW0742 treatment.

Fig.4.3

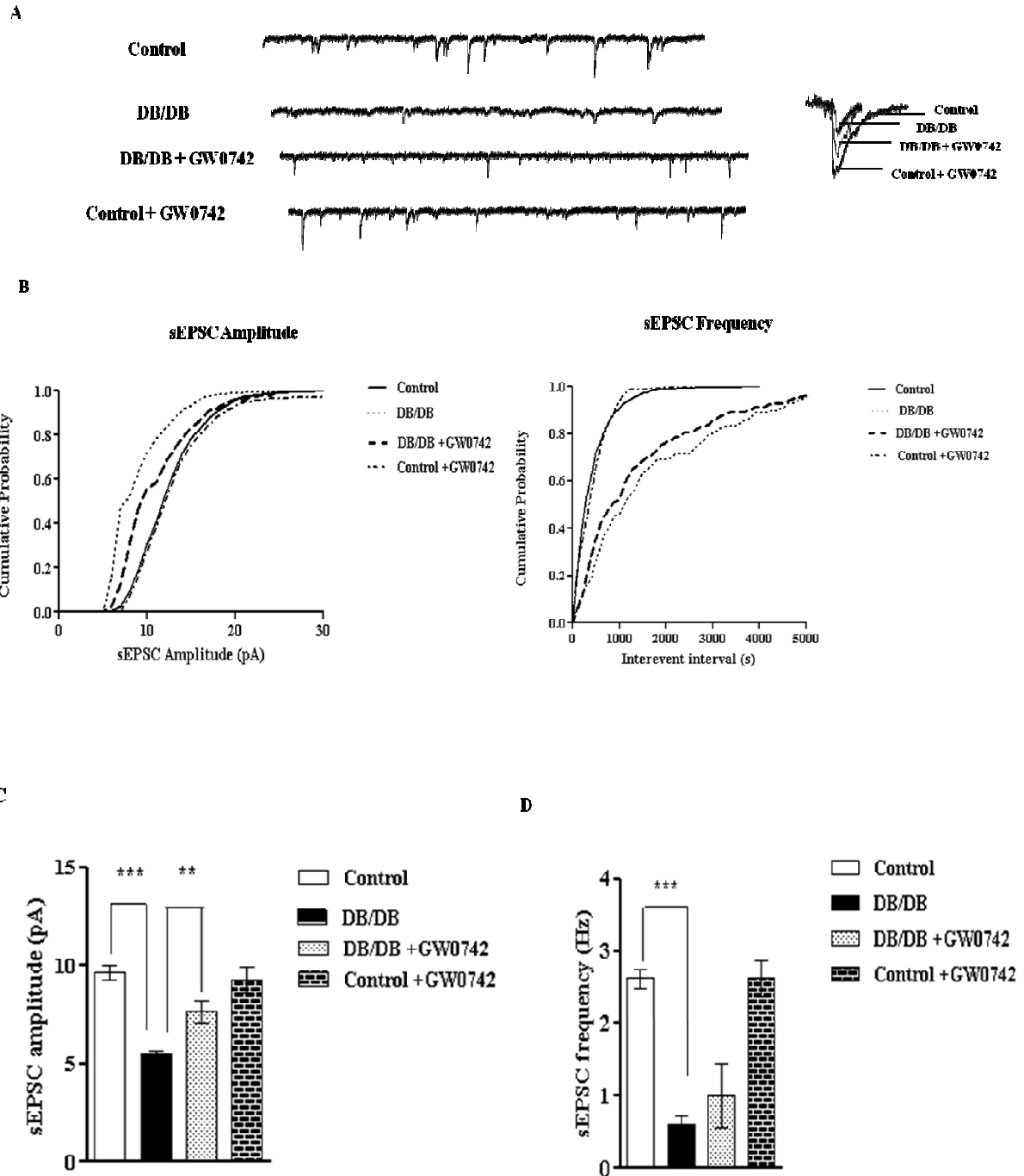


Fig. 4.3. Effect of GW0742 treatment on AMPARs-mediated whole cell synaptic currents.

(A) Sample traces show AMPARs-mediated sEPSC recorded at -70 mv membrane potential, which is reduced in amplitude and frequency, in vehicle-treated db/db mice compared to control mice. Treatment with GW0742 rescued the deficits in AMPAR-sEPSCs amplitude but not frequency in db/db mice. Treatment with GW0742 did not affect AMPAR-sEPSCs amplitude or frequency in control mice (B) Cumulative probability of distributions of amplitude and frequency shows a significant shift of vehicle-treated db/db mice curve to the left from the controls, suggesting decreased amplitude and frequency. Cumulative probability of distributions of amplitude, but not frequency exhibits significant shift of GW0742 -treated db/db mice curve to the right from the vehicle-treated db/db mice, suggesting increased amplitude without any effect on frequency. Treatment with GW0742 did not affect AMPAR-sEPSCs amplitude or frequency in control mice (C) Bar chart shows the significant reduction in mean amplitude of AMPARs-mediated sEPSCs in vehicle-treated db/db mice than that of control ($p < 0.001$; $n = 5$). Treatment with GW0742 significantly increased AMPAR-sEPSCs amplitude in vehicle-treated db/db mice ($p < 0.01$; $n = 5$). Treatment with GW0742 did not affect AMPAR-sEPSCs amplitude in control mice (D) Bar plot depicting the decrease in interevent interval of AMPARs-mediated sEPSCs in vehicle-treated db/db mice compared to control (Kolmogorov-Smirnov Test) ($p < 0.001$; $n = 5$). Treatment with GW0742 did not exert any apparent effect on the deficits in interevent interval of AMPARs-mediated sEPSCs in vehicle-treated db/db mice. Treatment with GW0742 did not affect AMPAR-sEPSCs interevent interval in control mice.

Fig. 4.4.

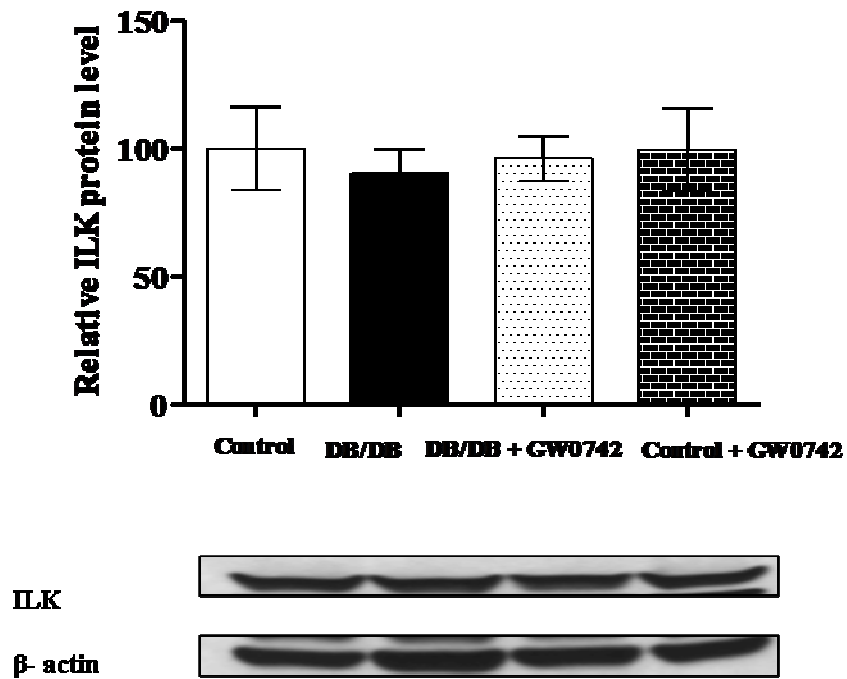


Fig. 4.4. ILK expression in db/db mice. Western blot analysis was performed to quantify the protein level of ILK in control m/m mice and vehicle-treated db/db mice. Protein levels are normalized to β -actin and expressed as a percentage of the control group which was set as 100%. Representative bands are shown below the bar graphs. ILK Quantity were not changed in vehicle-treated db/db mice hippocampal compared with control m/m mice (n=5).

Fig. 4.5.

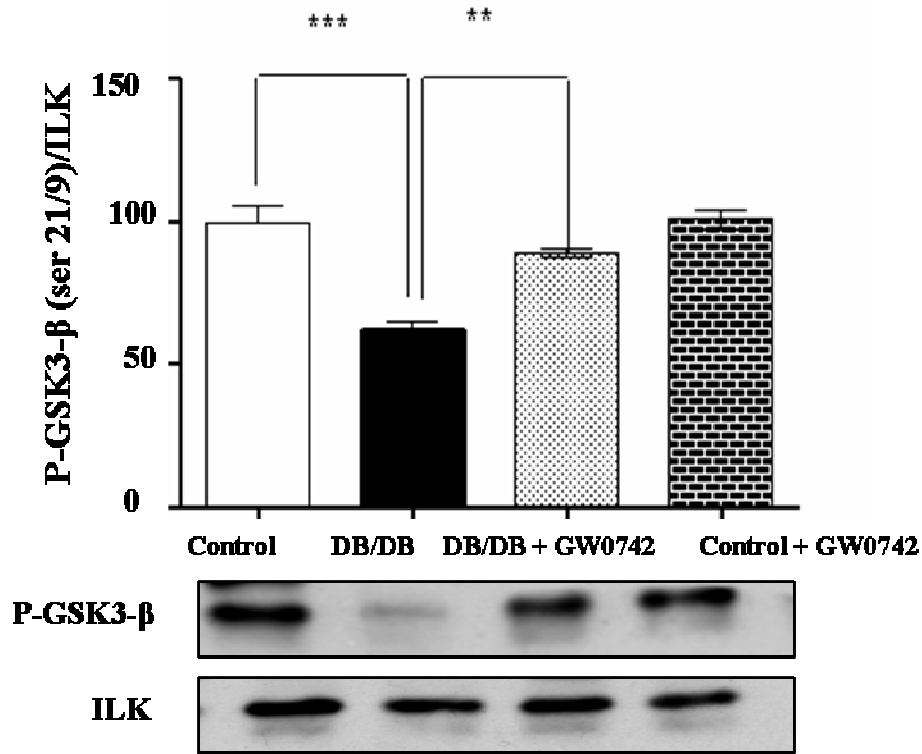


Fig. 4.5. ILK activity in db/db mice. ILK was immunoprecipitated from hippocampal extract. Kinase assay was performed by incubating the immunoprecipitated material with GSK fusion protein. The reaction product was analysed by western blot with anti-GSK3-β S21/9 antibodies. The blot was stripped and re-probed with anti-ILK antibodies. Quantification of p-GSK3β S21/9 by densitometry revealed a significant reduction in ILK activity in the hippocampus of db/db mice relative to control, ($p < 0.001$; $n = 5$). Moreover, treatment with GW-0742 significantly increased ILK activity in GW-0742-treated db/db mice ($p < 0.01$; $n = 5$). ILK activity in the GW-0742-treated m/m mice was not significantly different than that in control m/m mice.

Fig. 4.6.

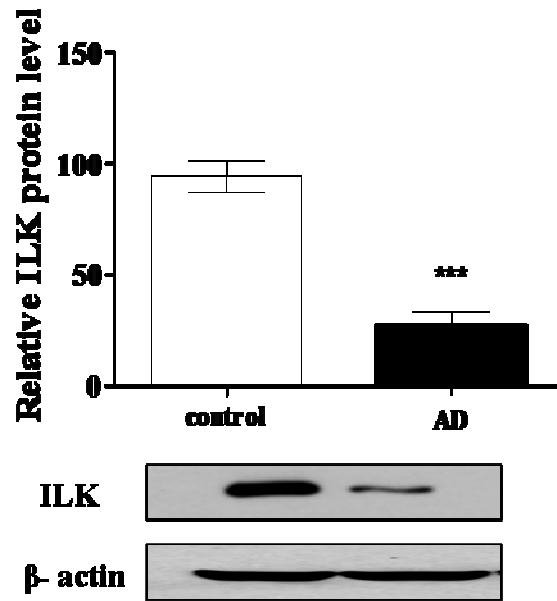


Fig. 4.6. ILK expression in AD hippocampus. Western blot analysis was performed to quantify the protein level of ILK in control m/m mice and vehicle-treated db/db mice. Protein levels are normalized to β -actin and expressed as a percentage of the control group which was set as 100%. Representative bands are shown below the bar graphs. Our results revealed significant reduction in the protein level of ILK expression in the human AD hippocampus relative to age-matched (normal human brain) (hippocampus) ($p < 0.001$, $n = 3$).

DISCUSSION

The present study aims to investigate whether activation of PPAR delta, the most abundantly expressed PPAR in the brain, can restore cognitive dysfunction relevant to type II diabetes. Our results demonstrate that treatment with the selective PPAR delta agonist GW0742 ameliorated cognitive dysfunction in type II diabetes db/db mice.

In this study we found that the time spent by vehicle-treated db/db mice in exploring the novel arm in the y-maze test was significantly shorter than in the control non-diabetic m/m mice or in GW0742-treated db/db mice. In addition, the time devoted to explore the novel arm was significantly less than the familiar arm in control db/db mice. However, the dwell time in the novel arm did not differ from that of familiar arm in both non-diabetic m/m mice or in GW0742-treated db/db mice indicating that db/db mice displays impaired spatial memory and that administration of GW0742 ameliorated diabetes-induced spatial memory deficits in db/db mice. Treatment with GW0742 didn't have any significant effect on spatial memory performance of control m/m mice in the y-maze test. Although there is a contradictory report concerning spatial working memory performance deficits in db/db mice (Sharma et al., 2010), the impaired spatial memory performance of db/db mice reported in our study is consistent with previous findings (Li et al., 2002, Dinel et al., 2011, Oomura et al., 2010). However, the deficits in spatial memory performance in db/db mice may be due to increased body weight and subsequent reduction in mobility of these animal compared to the non-diabetic control m/m mice (Sharma et al., 2010, Zhao et al., 2011). Based on this finding, we assessed the physical activity and general exploratory behavior of all mice groups by measuring the number of alternations between different maze's arms. Our results revealed that control m/m mice displayed more activity and general exploratory behavior than vehicle-treated db/db mice. However, treatment with GW0742

did not have any differential effect on the activity and general exploratory behavior of db/db mice indicating that the mobility is not a contributing factor in the ameliorative effect of GW0742 on spatial memory deficits in db/db mice.

Next we investigated whether administration of GW0742 to db/db mice affects hippocampal LTP, the prevalent cellular correlate of memory (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008). In agreement with the data obtained from other reports (Li et al., 2002), vehicle-treated db/db mice showed LTP deficits in the CA1 hippocampal region. The results of our study indicate that treatment with GW0742 ameliorated the deficits in LTP observed in db/db mice. Moreover, no significant difference in LTP average percent was noted when GW0742 was administered to non-diabetic m/m mice. Taken together, the present findings provide a direct evidence of the ameliorative effect of GW0742 on synaptic plasticity and memory deficits induced by type II diabetes.

In the hippocampus, AMPA and NMDARs play complementary roles in LTP. Therefore, we next assessed AMPA and NMDARs-mediated synaptic currents in db/db mice. Our results revealed that the mean current amplitude of AMPARs-mediated sEPSCs were significantly attenuated in vehicle-treated db/db mice. In addition, analysis of the within TBS facilitation revealed that excitatory post synaptic potentials (EPSPs) mediated by NMDARs were impaired in vehicle-treated db/db mice. Interestingly, administration of GW0742 significantly restored the impairment in the AMPARs-mediated sEPSCs mean current amplitude and NMDARs-mediated EPSPs, whereas the AMPA and NMDARs-mediated synaptic transmission in control m/m mice were not affected by this treatment. Taken together, these results indicate that disruption of AMPA and NMDARs-mediated glutamatergic neurotransmission in this hippocampus may be accelerated in diabetic db/db mice resulting in deficits in LTP and spatial memory. In addition,

GW0742 treatment attenuates diabetes-induced glutamatergic dysfunction resulting in amelioration of LTP and spatial memory deficits in db/db mice.

The mechanisms underlying the ameliorative effects of GW0742 on glutamatergic dysfunction caused by diabetes remain to be elucidated. Our recent study implicated a protein kinase known as integrin linked kinase (ILK) in AMPA and NMDARs-mediated synaptic transmission and plasticity in the hippocampus (unpublished data). Another recent study conducted by our group demonstrated that ILK expression was decreased in the hippocampus of an animal model of sporadic Alzheimer's disease with central insulin resistance, and ILK down-regulation was correlated well with synaptic deficits observed in these animals (Shonesy et al., 2012). PPAR delta has been reported to stimulate the transcription of ILK by binding to a PPAR response element in the second intron of ILK (Di-Poi et al., 2002). Therefore, it is likely to presume that the expression of such kinase may be altered in the hippocampus of db/db mice leading to synaptic deficits, and that rescuing ILK expression by PPAR delta agonist GW742, ameliorates these deficits in db/db mice. However, our results revealed no significant change in ILK expression in the hippocampus of vehicle-treated db/db mice relative to control m/m mice. The lack of change of ILK expression in the hippocampus of db/db mice may be because these changes in protein expression occur in a manner dependant on aging. This is consistent with a recent study reported that ILK protein levels were unchanged in 8 weeks old diabetic rats, however, ILK protein leveles were significantly reduced in the spinal cord of 20 weeks old diabetic (Jiang et al., 2010).

We previously found that inhibition of ILK activity is sufficient to induce deficits in hippocampal LTP and basal synaptic transmission and that ILK activity in the hippocampus was increased by induction of LTP (unpublished data). Moreover, a recent study reported that

phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity (Morimoto et al., 2000; Persad et al., 2001; Persad et al., 2000), is upregulated in the fat tissue of T2DM patients and type 2 diabetic mouse model db/db mice (Yu et al., 2011) and suppression of PTEN activity has been reported to increase glutamatergic neurotransmission and synaptic strength (Datta et al., 1997). Di Pooi et al. have reported that PPAR delta stimulates ILK activity by modulation of PTEN activity. In view of the above findings, we investigated whether ILK activity is modified in the hippocampus of db/db mice. Surprisingly, we found significant reduction in ILK activity in the hippocampus of vehicle-treated db/db mice. Moreover, administration of GW0742 significantly enhanced ILK activity in db/db mice, whereas, ILK activity in control m/m mice was not affected by this treatment. Therefore, it is likely that disruption of ILK activity contributes to diabetes-induced cognitive deficits.

In addition, our previous study demonstrated that in an animal model of sporadic Alzheimer's disease, ILK expression in the hippocampus is decreased and this downregulation is well correlated with deficits in synaptic transmission and plasticity in these animals (Shonesy et al., 2012). Based on these findings, we assessed ILK protein levels in the human AD hippocampal lysate. Our results revealed significant reduction in the protein level of ILK expression in the human AD hippocampus relative to age-matched control. These results further strengthen our hypothesis that alteration in ILK is involved in the pathogenesis of cognitive dysfunction.

In conclusion, this study suggests that alteration of ILK activity may be involved in diabetes-induced deficits in glutamergic neurotransmission in db/db mice, resulting in cognitive deficits, and that rescuing the activity of ILK by of PPAR delta activation ameliorates cognitive

dysfunction induced by type II diabetes. Therefore, ILK provides a link between T2DM and AD pathology.

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SUMMARY AND CONCLUSIONS

This study was inspired by the finding that type 2 diabetes mellitus (T2DM) is a risk factor for cognitive dysfunction and dementia such as Alzheimer's disease (AD). Clinical studies demonstrated that, patients with type II diabetes appear to have two to three fold increased relative risk for AD. Cognitive impairments have also been reported in type II diabetic mouse strain db/db and in intracerebroventricular streptozocin-injected rats (ic. STZ), a model of sporadic AD with central insulin resistance and brain insulin deficiency. However, the exact pathophysiology of cognitive dysfunction in patients who suffer T2DM remain to be clearly understood.

Our recent study using ic. STZ rodents, revealed that hippocampal synaptic impairments and deficits in Long term potentiation (LTP) correlated well with alterations in the integrin linked kinase (ILK) signaling. ILK is a serine-threonine kinase that functions downstream of growth factor and β -integrin receptors to regulate cell growth and differentiation. Following its activation, ILK phosphorylates PKB/AKT on serine-433 residue. ILK is also the dominant kinase for GSK-3 β phosphorylation and subsequent inactivation in the hippocampus. The role of ILK in hippocampal synaptic transmission and plasticity has not yet been investigated.

In the current study, we investigated the synaptic transmission and plasticity in the hippocampus after inhibition of ILK activity by using a potent and specific ILK inhibitor, KP-27389. Our results demonstrated that LTP was not induced by TBS protocol in KP-27389-treated hippocampal slices. Assessment of basal synaptic transmission at various stimulus intensities revealed that fEPSP slope was significantly attenuated in KP-27389-treated slices. Additionally, we assessed ILK activity in the hippocampus after induction of chemical LTP (cLTP) in organotypic slice culture. We observed that ILK activity is increased during induction of cLTP in

organotypic slice culture. The increase in ILK activity during cLTP further strengthens the role played by ILK in the regulation of hippocampal synaptic plasticity. In this study, we demonstrated for the first time, the existence of a causal link between ILK and hippocampal synaptic plasticity.

We further explored the role of ILK in mediating hippocampal synaptic plasticity at a single receptor level to the more complex mechanisms that affect the plasticity in the hippocampus. The AMPA and NMDARs play complementary roles in the expression and induction of long-term potentiation (LTP), respectively in the hippocampus. Our results demonstrate a significant reduction in AMPA and NMDARs-mediated current amplitude in KP-27389-treated slices. Reduction in the mean current amplitude of the single synaptic receptors could be due to modifications of the kinetics and/or the number of functional AMPA and NMDARs at the membrane of post synaptic density. Our results revealed that, KP-27389 treatment altered single channel properties of synaptic AMPA and NMDARs. In addition, we noted a significant reduction in synaptic expression of AMPA and NMDARs in KP-27389-treated slices. Stargazin and post synaptic density-95 (PSD-95) are members of MAGUKs (membrane-associated guanylate kinase), a family of proteins that play an important role in anchoring AMPA and NMDARs to the membrane of postsynaptic densities. In addition, reports showed that stargazin and PSD-95 affect AMPA and NMDARs biophysical properties respectively. A reduction in the protein level of stargazin and PSD-95 in KP-27389-treated synaptosomal fraction was noted in our study. However, we could not find any significant difference in the protein level of GluR1, NR1, NR2A, NR2B, stargazin and PSD-95 in KP-27389-treated whole hippocampal homogenates, indicating that KP-27389 did not affect their proteins synthesis.

It is possible that ILK can interact with these proteins at their synaptic sites and affect their synaptic localization. Our data revealed that ILK forms complexes with p-GluR1ser845, NR1, NR2A, PSD-95 and stargazin. Treatment with KP-27389, significantly disrupted ILK interaction with these proteins. Therefore, it is reasonable to presume that impaired ILK activity is sufficient to inhibit the interaction of ILK with AMPARs and NMDARs and their associated proteins, resulting in alteration in function and expression of synaptic AMPA and NMDARs and subsequent synaptic deficits.

A recent study reported that, phosphatase and tensin homolog (PTEN), a negative modulator of ILK activity, is upregulated in fat tissue of T2DM patients and type 2 diabetic mouse model db/db mice. Cognitive impairments have also been reported in db/db mice. Interestingly, we found that ILK activity is significantly reduced in the hippocampus of db/db mice. In addition, our results revealed significant reduction in ILK expression in the human AD hippocampus. These results further strengthen our hypothesis that alteration in ILK signaling is involved in the pathogenesis of cognitive dysfunction.

Activation of peroxisome proliferator activated receptors delta (PPAR delta) has been shown to induce ILK transcription and enhance its activity. PPAR delta are highly expressed in the brain and shown to improve spatial memory and hippocampal neurogenesis in control rodents. However, the effect of PPAR delta agonist on diabetes-induced dementia has not yet been explored. In this study, the effects of selective PPAR delta agonist, GW0742, on hippocampal synaptic transmission, plasticity and spatial memory were investigated in db/db mice. Our results revealed that, activation of PPAR delta, by GW0742 significantly ameliorated cognitive dysfunction and enhanced ILK activity in db/db mice.

In conclusion, this study suggests that alteration of ILK activity may be involved in diabetes-induced cognitive deficits. Therefore, ILK provides a link between T2DM and AD pathology. Activation of PPAR delta rescued ILK activity and ameliorated cognitive dysfunction induced by type II diabetes. Thus, PPAR delta agonists could be a new therapeutic regimen for T2DM-induced cognitive deficits.