Nicotine and Integrin Linked Kinase Interplay Modulates Synaptic Plasticity Mechanisms Required for Memory

by

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Abstract

Integrin Linked Kinase (ILK) has been associated with forms of synaptic plasticity required for memory. Nicotine, at low-concentration improves memory but higher concentrations impart learning and memory deficits. The relationship between nicotine and ILK, with regards to learning and memory, has yet to be investigated. In this study, I demonstrate the effect of different concentrations of nicotine on ILK and subsequent downstream signaling using H-19 rat hippocampal cells. In addition, I also show the differential modulation of synaptic plasticity by varying concentrations of nicotine is due to altered expression and function of synaptic nicotinic receptors. Our results indicate that nicotine affects cell viability, modulates ILK activity, micro-spine formation, and long term potentiation. Furthermore, nicotine also differentially modulated extracellular signal regulated kinase 1/2 (ERK1/2) required for synaptic plasticity. My data provides a novel mechanism by which nicotine modulates synaptic transmission and plasticity required for learning and memory.

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List of Abbreviations

AD	Alzheimer's Diseases
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaMKII	Calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding protein
ECM	Extra cellular matrix
ERK	Extracellular signal-regulated kinases
Gsk3β	Glycogen synthase kinase 3 beta
ILK	Integrin-linked kinas
ILKAP	ILK associated phosphatase
PI3K	Phosphatidylinositol-3 kinases
IPP3	Phosphatidylinositol 3-phosphate

IRs	Insulin receptor
LTP	Long Term Potentiating
nAchr-α7	Alpha-7 nicotinic receptor
NMDA	N-methyl-D-aspartate
PDK1	Phsphoinositide dependent kinase
PDK2	Pyruvate phsphoinositide dependent kinase
PINCH	Particularly interesting new cysteine-histidine-rich protein
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PPAR	Peroxisome proliferator-activated receptors
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
TBS	Tris buffered saline
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

CHAPTER I

Review of Literature

1.1. Integrin Structure and Function

Integrin receptors are a family of proteins that promote cell-cell and cell-Extracellular Matrix (ECM) interactions. In order to carry out proper differentiation, growth, function and proliferation, almost all cells require successive spreading and attachment into the ECM. Integrin provides biochemical and structural support essential for the processing of certain cellular functions (Ruoslahti 1997). The proper functions of tissues are maintained through the binding of cells to ECM (Hynes 2009), while these interactions are regulated by integrins. Integrins are also a key class of receptors that depend on the function of ECM components (Ghatak, Morgner et al. 2013).

Integrin contains a large number of cell surface proteins, composed of α/β non – covalent heterodimers, each owning signaling and specificity properties. In the case of mammals, research shows they possess 18 α- and 8 β- integrin genes that contribute to encoding of different integrin membrane proteins. On the other hand their short cytoplasmic domains regulate their enzymatic activity. In the case of signaling and scaffolding proteins, whenever a ligand such as Laminin, Fibronectin and Fibrinogen bind to them, they become activated (Morgan, Humphries et al. 2007, Zaidel-Bar, Itzkovitz et al. 2007, Campbell and Humphries 2011). However, when there are multidirectional signals, extracellular binding is regulated from inside the cell, while in

the case of ECM; the binding is regulated by signals conducted in the cells, as indicated in Figure 1.1 (Giancotti and Ruoslahti 1999).

Integrin is capable of forming a multitude of subunit compositions, with the β1 subunit being the most widely expressed in the brain. The β1 subunit is located in the brainstem, cortex and hippocampus. This subunit contributes to a wide array of biological functions that play a role in the growth and development of neurons. In addition, the signals also prevent cellular death of neurons, enhance synaptic plasticity and promote cell survival (Horwitz, Duggan et al. 1986, Wang, Butler et al. 1993, DeFreitas, Yoshida et al. 1995, Chan, Weeber et al. 2006, Shi and Ethell 2006).

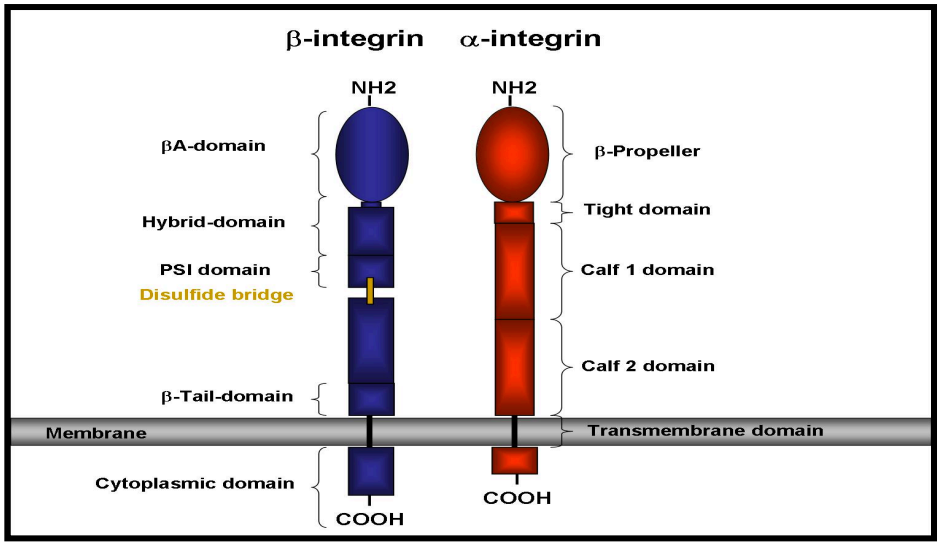


Figure 1.1: Structure of the heterodimeric transmembrane domains indicating how integrins play a bidirectional-signaling molecule between ECM and the Cytoplasm. Adapted from *The Role of Adhesion Receptors in Melanoma Metastasis and Therapeutic Intervention Thereof. (2011).*

1.2. Integrin Linked Kinase (ILK)

Integrin Linked Kinase (ILK), discovered in the 1996, is a serine/threonine kinase, with a molecular weight of 59 kDa, which binds to the β1 subunit of integrin. ILK consists of three domains (Hannigan, Leung-Hagesteijn et al. 1996). The N – terminal ankyrin repeats bind to various signaling molecules and adapter proteins, like the recently discovered cysteine-histidine-rich protein PINCH and the ILK associated phosphatase (ILKAP), which are important for ILK localization to focal adhesions and for regulating its signals. A central pleckstin-homology (PH) domain in the center connects the N-terminus with the C- terminal catalytic domain and binds to phosphoinositide lipids. The C- terminal forms a trigonal binding to facilitates ILK interaction with α- Parvin and β-Parvin (Hannigan, Troussard et al. 2005).

A cell culture study indicates that phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) is the ILK PH domain ligand (Legate, Montanez et al. 2006, McDonald, Fielding et al. 2008). The catalytic domain binds to parvins creating a ternary complex that later relocates to cell – ECM adhesions (Hannigan, Troussard et al. 2005). The PIP3 complex can serve as a platform for downstream signaling pathways engendered by integrin interfacing with the actin cytoskeleton (figure 1.2). Genetically, the importance of ILK has been demonstrated by embryonic lethality occurring in species that lack ILK, which leads to inhibition of embryonic body wall growth. ILK may was also suggested to be a scaffold protein or a pseudo-kinase. (Miller and Kaplan 2003, Hannigan, Troussard et al. 2005, Hannigan, McDonald et al. 2011).

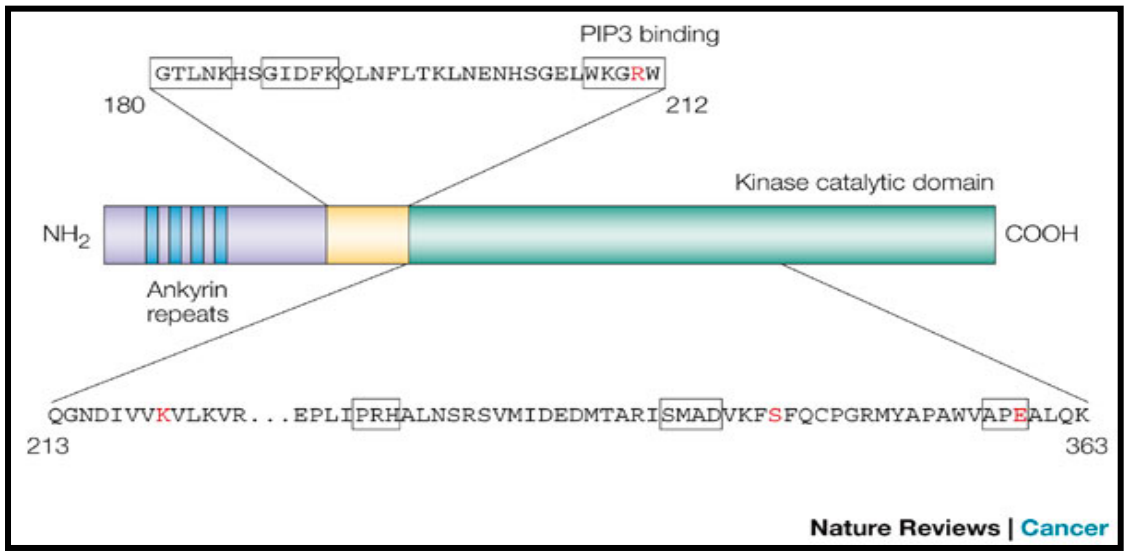


Figure 1.2: ILK structure and the amino acid responsible for its activity Adapted from *Hannigan, Troussard et al. Nature Reviews Cancer* 5, 51-63 (January 2005)

Research indicates that ILK can directly phosphorylate many proteins: myelin basic protein (Hannigan, Leung-Hagesteijn et al. 1996), β-parvin (Yamaji, Suzuki et al. 2001), glycogen synthase kinase-3β (Delcommenne, Tan et al. 1998), the regulatory light chain of myosin-II, MLC-20 (Deng, Van Lierop et al. 2001), Akt (Persad, Attwell et al. 2001), protein phosphatase inhibitory proteins CPI-17, PHI-1 (Deng, Sutherland et al. 2002). According to studies, ILK can be classified as a kinase since it can affect several downstream signaling pathways. Phosphorylation of these proteins plays an essential role in the proliferation, regulation, survival, and differentiation of different cell types (Legate, Montanez, et al. 2006).

1.3. Synaptic Transmission in the Brain

Synaptic transmission is a process by which neurotransmitters released presynaptically activate postsynaptic receptors sites thereby allowing neurons to communicate with each other. Synapses undergo extensive alterations in the amount of neurotransmitter released and the number of receptor sites resulting in changes of affinity between neurons. Therefore, synaptic transmission is a mechanism that is omnipresent and a source of the brain's plasticity. Numerous times per second, at the end of every one of our neurons, various sequence of events take place.

In the beginning, synthetic enzymes transform precursors into neurotransmitters, which are stored in synaptic vesicles that build up in the terminal button of the axon. Then, the nerve impulse reaches the terminal button of the axon and causes voltage sensitive calcium channels to open. Calcium ions enter into the cell, causing synaptic vesicles to amalgamate with the cell membrane, followed by release of neurotransmitter into the synaptic cleft.

Neurotransmitters then bind to receptors on the membrane of the postsynaptic neuron, thereby activating them. In turn, this causes the opening of channels that let certain ions cross the postsynaptic membrane establishing a synaptic potential (excitatory or inhibitory, depending on the type of ion involved). Lastly, the neurotransmitters dissociate from the receptors and return to the synaptic cleft, where they are either returned and recycled or metabolized (Bear, M., & Connors, B. 2007) figure 1.3.

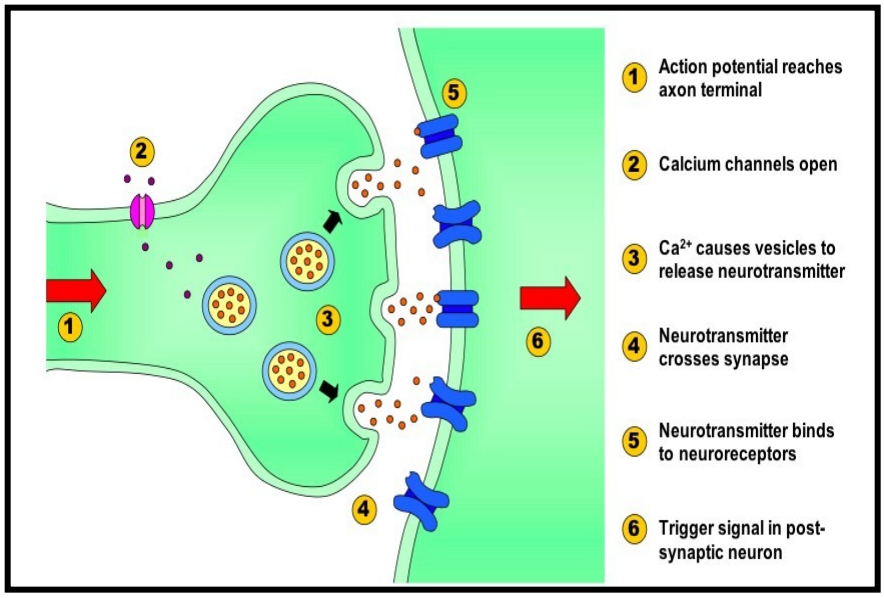


Figure 1.3.: Explanation of the synaptic transmission. Adopted from *http://www.ib.bioninja.com.au*

1.3.1. Cholinergic System

The Cholinergic system is an interconnection made up of well-structured and organized nerve cells. Cholinergic neurotransmission occurs throughout the body, but in the brain it is crucial for learning and memory.

The cholinergic system is made up of two types of receptors, nicotinic and muscarinic receptors, both displaying different functions. Muscarinic receptors are distributed through the CNS. They are G protein coupled receptors use secondary messenger system to coordinate tissue functions. Nicotinic receptors are ligand gated ions channels that are involved in many physiological processes that regulate neuronal muscular functions. However, both receptors are used in guiding cholinergic nerve function (Cuello, 1993).

1.3.2. Nicotinic Receptors

The hippocampus is accountable for learning and memory encoding, which is affected in many neurodegenerative conditions including Alzheimer's disease. Nicotinic receptors in the brain play a central role in memory formation and maintaining cognition (Levin 2013). Any defect in cholinergic transmission in the central nervous system is associated with attention reduction and cognitive impairment (Bartus, Dean et al. 1982), while increased activity results in depression (Janowsky, el-Yousef et al. 1974). Nicotine can either stimulate or desensitize nicotinic receptors, so understanding receptor action is vital to determine how nicotine effects cognitive function (Levin 2013). Nicotinic receptors are found both presynaptically and postsynaptically at most glutamatergic synapses in the hippocampus (Halff, Gomez-Varela et al. 2014).

The neuronal nicotinic acetylcholine receptors (nAChRs) are ligand gated ion channels in the plasma membrane, composed of different subunits α_2–α_{10} and β_2–β_4. The most abundant forms in the mammalian brain are the homomeric (α_7 nAChRs) and heteromeric ($\alpha_4\beta_2$nAChRs) (McGehee and Role 1995, Alkondon and Albuquerque 2001, Dani 2001) see figure 1.4. The (α_7nAChRs) receptors coordinate neuronal excitability in different regions of the brain by two mechanisms; either by modulation of neurotransmitter release from presynaptic terminals, or their location on pyramidal cells and somatodendritic sites of interneurons, where they can control the activity of the neuron (Pidoplichko, Prager et al. 2013).

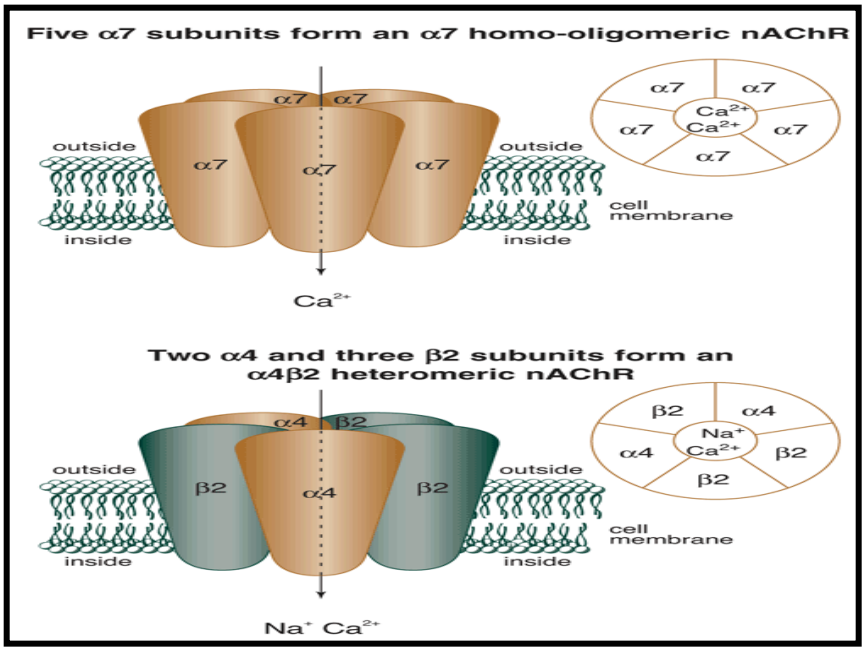


Figure 1.4: Nicotinic receptor subtype in the brain. *Adopted* from Davis, T. J. de Fiebre, C. M. Alcohol Res Health (2006)

The α4β2 nAChRs have lower calcium permeability, but higher acetylcholine affinity and slower desensitization rates (McGehee and Role 1995, Albuquerque, Pereira et al. 2009). α7nAChRs are found in pre-synaptic neurons and control neurotransmitter release, while post-synaptic α7nAChRs trigger intracellular signaling cascades and effect mechanism involved in learning and memory.

1.4. Long Term Potentiation

Nearly half a decade ago, Bliss and Lomo identified Long Term Potentiation (LTP). Both Bliss and Lomo described LTP as a long lasting increase in synaptic transmission, which is a product of transitory period of high frequency stimulation (Bliss and Lomo 1973). LTP is believed to be the cellular correlate of learning and memory. How LTP is generated and what changes occur in the synapse is not yet fully elucidated. LTP is induced by the generation of an action potential at the axon hillock of a neuron. A

dramatic propagation of that action potential travels down the axon to the axon terminal. At the axon terminal, the synaptic vesicles are fused to the cell membrane to releases glutamate (the major excitatory neurotransmitter in the brain implicated in learning and memory) by exocytosis.

This release of the neurotransmitter is caused by the activation of voltage gated Ca^{+2} channels in response to neuronal depolarization. After glutamate release from the presynaptic neuron, glutamate binds to two types of postsynaptic receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and the N-methyl-D-aspartate (NMDA) receptors. The glutamate binding to AMPA receptors causes a change in membrane potential leading to the removal of the Mg^{+2} blockade of the NMDA receptor. Then, NMDA allows the influx of Ca^{+2}, and it's binding to calmodulin leads to the activation of calcium-calmodulin-dependent kinase II (CaMKII) (Bear, M., & Connors, B. 2007).

The phosphorylation of AMPA receptors by CaMKII affects their ability to increase the flow of Na^{+} ions resulting in an increased sensitivity of the neuron to depolarize. CaMKII also regulates the trafficking of AMPA receptors to the cell membrane. Increased gene expression, protein synthesis and dendritic spine formation are ramifications of AMPA receptor trafficking. A plethora of biochemical signals are involved in increasing or decreasing LTP, and the alteration of ILK-GSK-3β signaling showed a decrease in postsynaptic trafficking of glutamate receptors, which resulted in synaptic loss and memory deficits (Shonesy, Thiruchelvam et al. 2012).

1.5 Role of ILK in Neuronal and Synaptic Transmission

ILK plays an important role in all cells types, but in neurons its ability to maintain and develop neuronal circuitry is crucial. ILK does so by regulating the initiation and growth of dendrites (Naska, Park et al. 2006), by increasing synaptic plasticity, and the cellular biological mechanism of learning and memory.

ILK activates the anti-apoptotic PI3K-Akt pathway. The activity of Akt depends on the formation of PIP3 and the triggering of PDK1 (phosphoinositide dependent kinase) and PDK2 (pyruvate phosphoinositide dependent kinase), a result of the phosphorylation of Akt at the threonine 308 and serine 473 residues. Once activated, ILK either prevents the activation of the pro-apoptotic pathway or initiates an anti-apoptotic effect (Vanhaesebroeck and Alessi 2000). ILK is also known for Akt-mediated neuroprotection (Gary, Milhavet et al. 2003).

Neuronal death induced by glutamate can be blocked by activation of integrin. This depends on the β1 subunit of integrin (Gary, Milhavet et al. 2003). However, α3 subunits are also found in the hippocampus. Tashiro and his colleges identified that α3β1 subunits are responsible for the neurite outgrowth observed in PC12 cells, indicating the importance of α3 in neuroprotection (Pinkstaff, Detterich et al. 1999, Tashiro, Monji et al. 1999).

1.6. Diabetes, Memory and ILK

Type 2 diabetes mellitus (T2DM) recently has been shown to be a contributing factor for the dementia and cognitive dysfunction seen in Alzheimer's disease (AD). Although, the precise pathophysiology of cognitive dysfunction in patients who suffer T2DM is still not completely defined.

It was shown that impairments in hippocampal LTP correlated well with alterations in ILK signaling (Martin, 2007). Although, a causal link between ILK and synaptic transmission has not yet been examined, current studies indicate that inhibition of ILK activity by using a specific and potent pharmacological modulator resulted in plasticity deficits and impaired synaptic transmission in the hippocampus.

Many studies indicate the role of insulin-signaling molecules in synaptic plasticity, which in turn have an effect on learning and memory formation (Plitzko, Rumpel et al. 2001, Hooper, Markevich et al. 2007). Insulin receptors (IRs) in the brain are expressed in astrocytes and neurons (Boyd, Clarke et al. 1985). In cortical and subcortical regions, the cortex and hippocampus respectively, synaptic IRs are of high density (Werther, Hogg et al. 1987). Therefore, it is not unreasonable to propose a correlation between insulin signaling and synaptic plasticity. In this manner activation of the insulin receptor leads to the activation of the PI3K/Akt signaling pathway, which is an essential signaling pathway for synaptic plasticity (Johnson-Farley, Patel et al. 2007, Sui, Wang et al. 2008).

Phosphorylation of Akt inactivates glycogen synthase kinase-3β (GSK-3β) by phosphorylation at the Serine 9 residue (Cross, Alessi et al. 1995). Peineau et al. report that (GSK-3β) coimmunoprecipitates with AMPA receptor subunits GluR1/2. Therefore, indicating the importance of (GSK-3β) in determining the direction of the synaptic plasticity. Inactive (GSK-3β) is essential for the induction of synaptic plasticity mechanisms including LTP (Hooper, Markevich et al. 2007, Peineau, Taghibiglou et al. 2007)

Current knowledge points to integrin-linked kinase (ILK) as the principal kinase in the hippocampus responsible for the inactivation of (GSK-3β). More specifically, the ILK/GSK3b signaling pathway controls the formation of dendrites in a developing hippocampus (Naska, Park et al. 2006).

Another study shows that intracranial STZ rodent model of AD have impaired synaptic transmission, LTP, learning, and memory deficits with an alteration in the ILK-GSK-3β signaling (Shonesy, Thiruchelvam et al. 2012).

1.7. Alzheimer's Disease and ILK

Alzheimer's disease (AD) is a neurodegenerative disease characterized by abnormal hyperphosphorylation of tau proteins and the accumulation of beta amyloid from the amyloid precursor protein (APP). Marked cholinergic neuronal loss in the basal forebrain, resulting in decreased acetylcholine in the hippocampus and cortex, reflects the cognitive deficits associated with Alzheimer's disease. This disease is the main form of dementia found all over the world, where individuals are primarily affected gradually, but harsh sudden losses of memory and other cognitive abilities can occur (Hyman, Damasio, et al. 1989). The occurrence and progression of AD is a complex pathological process which involves many pathways. Studies suggest that α7nAChR are involved in the progression of AD, because of its increased level of expression in the hippocampus. (Levin, McClernon et al. 2006). Aβ a high binding affinity for the alpha 7 nAChRs. Protein phosphorylation that is involved in the processes of synaptic plasticity is induced by the binding and activation of (α_7nAChRs) (Guan, Zhang et al. 2000; Kadir, Almkvist et al. 2006).

ILK can phosphorylate PIP3 which leads to the phosphorylation of AKT. There are two isoforms of GSK-3, α and β, which are encoded by different genes, which was initially purified from skeletal muscles (Woodgett 1991). Many studies have shown that GSK3-β can be phosphorylated in response to the phosphorylation of Akt. Hence, the most studied pathway for GSK3-β is regulated by Protein Kinase B (PKB/AKT) (Woodgett 1990) .

The GSK3-β mediated cAMP response element binding protein (CREB) regulates many cellular processes in neurons; such as synaptic plasticity, LTP, and cognition. In AD, GSK3-β inhibits transcription mediated by CREB (Cross, Alessi et al. 1995, Deisseroth, Bito et al. 1996). When GSK3-β is activated an increase in tau phosphorylation and Aβ production occurs, which leads to neuronal death.

CHAPER II

Materials and Method

2.1. Cell Culture

H19-7/IGF-IR rat hippocampal cells (American Type Culture Collection) were plated on poly-L-lysine (*S*igma, Aldrich) plates and grown at 34°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (American Type Culture Collection) supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biological), Gentamicin G418 (Sigma, Aldrich), 100 µl puromycin (Sigma, Aldrich), and 5 ml penicillin/streptomycin (Cellgro) for several days. When the cells were 90% confluent, they were differentiated into neuronal phenotype by changing the growth medium with differentiation medium that was supplemented with 100x N2 (Gibco), 1% Fetal Bovine Serum (FBS) (Atlanta Biological), and basic Fibroblast growth factor (bFGF) (Gibco). All experiments were done on cells seeded 24-72 hours in advance, allowing sufficient time for cell attachment to the surface of the cell culture plate and to reach the appropriate density to perform the experiment.

2.2. Nicotine Treatment

Cells were grown to 80% confluency in 10 cm plates (Falcon™ Tissue Culture Plates) with growth media containing 10% FBS at 34° C. Growth media was replaced with

differentiation media containing 1% FBS and incubated at 39° C for 12 hours. A stock solution (1mM) was prepared using 99.9% nicotine (Sigma, Aldrich) dissolved in deionized water. Cells were treated with different concentrations of Nicotine (5µM, 10µM and 15µM) and incubated another 12 hours before collecting proteins.

2.3. MTT Assay

Cell viability was determined using an MTT assay, in which living cells reduce the yellow water soluble MTT into purple insoluble formazan crystals, a function of mitochondrial activity. Cells were plated in 96 wells and left to grow to 70 % confluency. The cells were treated with different concentration of nicotine (5µM, 10µM, 15µM, 20µM) and incubated for another 24 hours at 37°C. A 12mM MTT (3-(4,5 dimethyiazol-2-yl)-2,5 diphenyltetrazolium bromide) stock solution was prepared by adding 1mL of sterile PBS to 5 mg of MTT and then further diluted with 9 mL PBS. After that the growth medium was removed and replaced with PBS containing 0.5 mg/ml MTT and incubated for 4 hours at 37°C. Next, the cells were solubilized by removing all but 25µL of PBS and adding 100µL of DMSO to each well followed by incubation at 37^{o}C for 10 minutes. The amount of formazan formed was determined by measuring the absorbance at 550nm.

2.4. Western Blot analysis

H-19 cells treated with nicotine were lysed using cell lysis buffer obtained from (Cell Signaling 10x). Protein concentration was determined using Bradford protein assay. The lysate was mixed with 2x laemmli buffer and then loaded into a 10% SDS-page gel.

The proteins were transferred to PVDF membranes (Immobilon-p Millipore, Germany) and then blocked for 1 hour with 5% non-fat dry milk dissolved in Tris Buffered Saline (TBS) containing 0.01% Tween. Membranes containing phosphorylated proteins were blocked with 1% BSA. The membranes were then washed three times for five minutes in TBST. Membranes were incubated with primary antibodies overnight at 4°C in β- actin (1:1000, Cell Signaling Technology, Danvers, Massachusetts), ILK (1:1000, Cell Signaling Technology, Danvers, Massachusetts), Akt (1:1000, Cell Signaling Technology, Danvers, Massachusetts), and Gsk-3β (1:1000, Cell Signaling Technology, Danvers, Massachusetts). Membranes were exposed to secondary anti-rabbit antibody (1:10000) that is conjugated with fluorophore DyLight 550 for 4 hours at room temperature. Next, membranes were visualized using an FLA-5100 imager with a 532 nM green laser and an LPG filter set (Fujifilm Inc., Tokyo, Japan). These scans were performed using excitation and emission wavelengths of λ_{532} nm and λ_{570} nm, respectively. Lastly, the band intensities were compared with β-actin for both nicotine treated cells and control.

2.5. Immunohistochemistry

The H19-7/IGF-IR cells were cultured on 12 well plates (Falcon™ Tissue Culture Plates). In the bottom of the wells microscope cover slips were treated with poly-L-lysine (Sigma, Aldrich). The cells were left to grow in the cover slips and then induced to differentiation. Then, they were espoused to an antibody solution affecting the nucleus of the cells and the structure of the actin cytoskeleton (1:10 – 1:50). Using a DAPI staining technique, the cell nucleus was labeled to confirm that the cell lysis had not

occurred. To label the F-actin in the cytoskeleton Phalloidin was used. While the cells were exposed to antibody, they were kept in humid and covered petri dishes. The dish was comprised of a wet sponge and a superficial pool of water. They were warmed before antibody treatment to increase the humidity in the dish. This was done to eliminate antibody solution evaporation. During antibody exposure the cells were incubated in a 37°C environment to block cell death. Once the immune-florescent labeling was done, the cells were photographed using an "AMG Evos fl" fluorescence microscope. Per each sample two images were recorded, then they were combined with the Image-J program. The program generates an overlay compound image that shows both DAPI fluorescence (blue) and phalloidin fluorescence (red).

2.6. Animals

Sprague–Dawley female rats weighing 200–250 g (Charles River Laboratories, Wilmington, MA) were sedated with isoflurane and subcutaneous osmotic mini pumps (Alzet, Model 2004, Cupertino, CA) were implanted to deliver (-)-nicotine (6 mg/kg/day, free base) or physiological saline. The Osmotic mini pumps were removed after 4 weeks under brief isoflurane anesthesia and the wound was closed with surgical clips. All animals were held in a room at 22–24°C with 12 h light/dark cycles and unrestricted access to food and water. Animals were sacrificed and hippocampi were collected for the chronic nicotine exposure experiments. For acute nicotine exposure studies, non-treated rat hippocampal slices were incubated with 5μM, 10μM, and 15μM of nicotine for 1h before recording. Experiments were conducted in accordance with the Principles of Animal Care (NIH Publication 85–23, 1985) under animal protocols permitted by the

Auburn University Institutional Animal Care and Use Committee.

2.7. Single-channel Electrophysiology

The artificial lipid bilayer membrane formation was achieved by dehydrating the chloroform including 1,2 diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) using nitrogen, then the precipitate was dissolved in 1 mg/ml of anhydrous hexane (Sigma-Aldrich Co., Milwaukee, WI). A glass electrodes with 1.5 millimeter diameter, and 100 MΩ was pulled to generate a pipette with 1μm diameter, then was filled with intracellular fluids (ICF) comprises of 110 mM KCl, 4 mM NaCl, 2 mM NaHCO3, 1 mM MgCl2, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid with 7.4 pH. The pulled pipette was positioned to a reference electrode and dipped in a microbeaker containing extracellular fluids (ECF) composed of 125 mM NaCl, 5 mM KCl, 1.25 mM $NaH2PO_4$, and 5 mM Tris HCl, and 7.4 pH (Parameshwaran, Buabeid et al. 2012). Afterwards, 5μl of the phospholipids was applied to the ECF. The tip of the pipette, which contains the ICF, was dipped twice to form phospholipid bilayer (Figure 2.1.). When a stable membrane was formed synaptosomes were added to the microbeaker to let the receptors integrate with the artificial bilayer (Suppiramaniam, Vaithianathan et al. 2006) The synaptosomes preparation was done as described in (Johnson et al., 1997). After successful incorporation of the synaptosomes into the lipid bilayer different nicotine concentration was added, voltage was applied and the current was recorded.

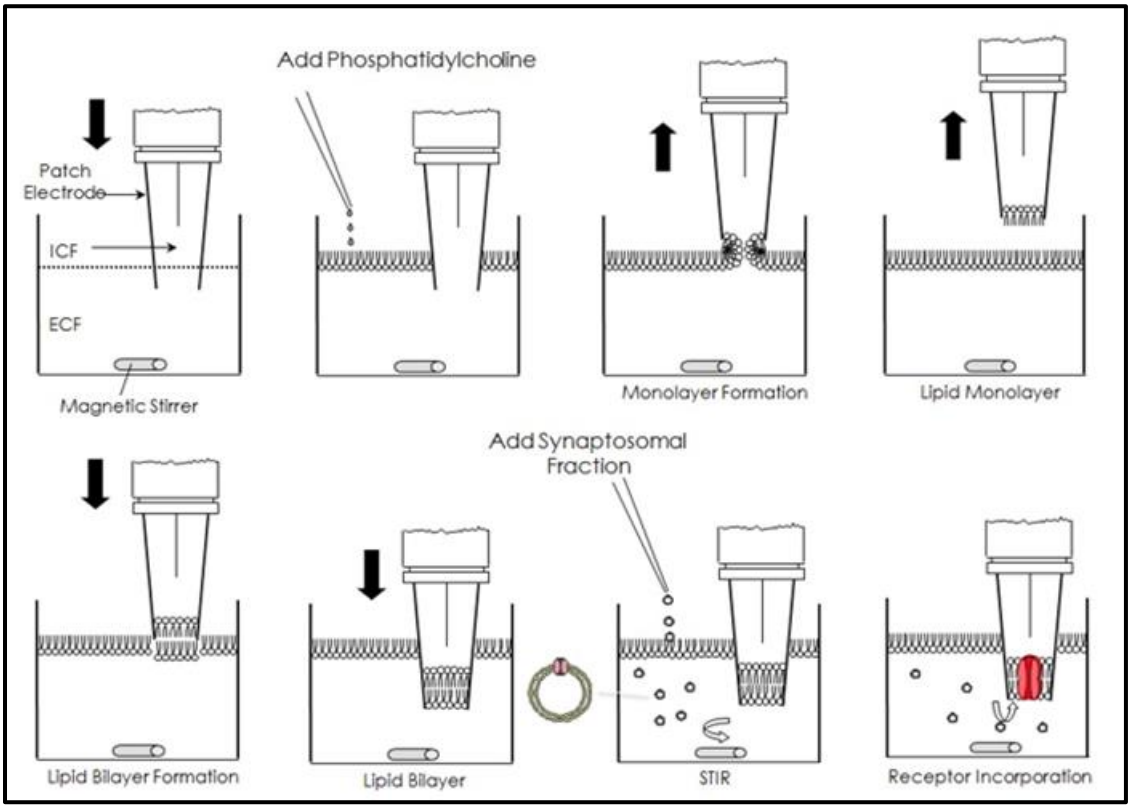


Figure 2.1: Artificial lipid bilayer membrane Preparation. Adapted from *Suppiramaniam et al., Methods Enzymol. (2006)*.

2.8. Preparations of Acute Hippocampal Slices

Animals were euthanized under CO_2 and immediately decapitated. The brain was washed with oxygenated cutting solution to remove blood. A vibratome Series 1000 tissue sectioning system (Technical products international Inc., St. Louis, MO,USA) was used with an oxygenated cutting solution (NaCl 85, KCl 2.5, $MgSO_4$ 4.0, $CaCl_2$ 0.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5) to slice the brain (350 micrometers). In a holding chamber the slices were preserved and

submerged in oxygenated-ACSF (artificial cerebral spinal fluid) for 1 hour at 30°C, before starting LTP recordings.

2.9. Extracellular Field Recordings

The brain slices were transferred into a submerged-type recording chamber, under the microscope (Nikon SMZ 745T microscope), and kept between two nylon nets. This chamber is constantly supplied with oxygenated ACSF (31°C) at a flow rate of 2–3 ml/minute. A bipolar platinum electrode was positioned on the CA3 region of the hippocampus. A glass microelectrode with a 1.5 mm outer diameter (World Precision Instruments, Sarasota, Florida), was pulled using a micropipette puller (Narishigie scientific instruments Lab, Tokyo) and filled with aCSF solution, approximately 200µl. The glass microelectrode is positioned on the CA1 region (the stratum radiatum in hippocampus) to record field excitatory postsynaptic potentials (fEPSPs) from the Schaffer collateral pathway. Two electrodes positioned in the middle of the stratum radiatum, with the Model 4D Digital Stimulus Isolation Amplifier (SIU), were inserted to stimulate the CA3 region (0.33Hz) for 10 minutes monitoring basal synaptic transmission. LTP induction was induced using theta burst stimulation (TBS). Four TBS were applied with an inter-TBS interval of 20 s. Each one of the TBS consists of 10 bursts carried at 5Hz, each burst containing 4 0.2 ms pulses at 100Hz. Field potentials were recorded with Axoclamp 2B (Axon Instruments, Foster City, CA) and analyzed using WinLtp software (Anderson et al., 2001).

2.10. Statistics

In this study, biochemical and electrophysiological statistical data was preformed using Prism software and the results were analyzed by one-way ANOVA (Tukey's Multiple Comparison Test and kruskal-walis test). Protein levels were normalized to β-actin and quantified as a percentage of the control, which was set as 100%. All experiments were repeated 6 times and a p-value lower than 0.05 was considered significant.

CHAPTER III

Results

ILK signaling is a critical feature that impacts learning and memory. In this study, I demonstrate that nicotine interacts with ILK signaling regulating micro-spine formation, a known function of ILK. Nicotine mediates altered expression of ILK via the nicotinic receptor modulation. I also demonstrated that chronic or higher concentrations of nicotine (10 -15μM) exposure down-regulated ILK expression and decreased basal synaptic transmission and LTP whereas, lower concentrations (5μM) of nicotine enhanced ion channel function of nicotinic receptors and increased synaptic transmission and LTP.

3.1. Nicotine modulates cell proliferation in H-19 hippocampal cell line

In order to evaluate the concentration dependent effects of nicotine on cell proliferation, I used H-19 hippocampal cell lines and applied various concentrations of nocotine. These cell lines have been previously shown to express nicotinic receptors (CITE). The sensitivity of the H19-7 cell line exposed to nicotine for 12 hours was measured by the MTT cytotoxicity assay, as shown in Figure 3.1. Cells were grown for four days and differentiated for 12 hours in the absence and presence of various concentrations of nicotine (5μM to 20μM). Increasing nicotine concentrations 5μM showed 12%

decrease (**p<0.01) in cell viability compared to the control, 10μM nicotine exhibited 18% decrease when compared to the control (***p<0.001). 15μM nicotine showed 19% decrease compared to the control (***p<0.001). On the other hand, 20μM induced 28% decrease in cell viability compared to control (***p<0.001), representing the effect of nicotine in hippocampal cell lines. The statistical significance difference was performed using one way ANOVA (Tukey's Multiple Comparison Test) *p<0.05, **p<0.01,and ***p<0.001,n=6. * Compared to control, # compared to 5μM.

3.2. Nicotine alters the expression of ILK

Our laboratory has demonstrated that exposure to nicotine during development altered expression of ILK (unpublished data). I performed Immunohistochemical analysis on H19-7/IGF-IR cells exposed to nicotine. When the cells were exposed to 5μM concentration of nicotine there was 30% increase the expression of ILK, while in 10μM showed 50% decrease compared to the control (*p<0.05). 15μM concentrations down regulated the expression of ILK 30% when compared to control and control (**p<0.01). The statistical difference between 5μM and 10μM, 15μM was done using one way ANOVA (Tukey's Multiple Comparison Test) *p<0.05, **p<0.01 n=6.

3.3. Nicotine decreases the expression of T-Akt and the p-Akt

In order to evaluate effect of various concentrations of nicotine on the down stream-signaling cascade of ILK, I measured the expression of Akt using H-19 cells. Results suggest that nicotine alters the expression of total Akt and phosphorylated Akt, as shown in Figure 3.2. When H-19 hippocampal cells were exposed to 5μM there was no significant decrease in the t-Akt, but with10μM there was a significant 40% decrease in the expression of the t-Akt (***p<0.001). In 15μM levels of t-Akt was 50% decreased

(***p<0.001) compared to the control. The levels of p-Akt with 5μM showed 40% decreased (**p<0.01) compared to the control. In 10μM and 15μM p-Akt, a 50% decrease was shown compared to the control (***p<0.001). The Changes in Akt phosphorylation and expression are essential for regulation of synaptic plasticity in the hippocampus. One way ANOVA (Tukey's Multiple Comparison Test) was used to determine the statistical difference between 5 μM and 10 μM, 15 μM, n=6.

3.4. Nicotine decreases the expression of t-Gsk3α/β and p-Gsk3β

I demonstrate that increased nicotine exposure to H-19 hippocampal cells, showed expression of t-Gsk3β. In 5μM and 10μM there was no significant decrease, but in 15μM decreases expression of t-Gsk3β was seen in 40% (*p<0.05). Likewise, 5μM of nicotine showed no significance decrease in the expression of p-Gsk3b from the control but when the cells were exposed to 10μM the decrease was 40% (**p<0.01) and 15μM 30% (***p<0.001) compared to the control (Figure 3.4). The statistical significance difference between control and nicotine treated was analyzed using one way ANOVA (Tukey's Multiple Comparison Test) *p<0.05, **p<0.01, ***p<0.001 n=6.

3.5. Nicotine impacts micro-spine formation in H-19 hippocampal cell line

Treatment of H-19 hippocampal cells with different concentrations of nicotine revealed nicotine's effect on micro-spine formation. When cells were treated with 5μM concentrations of nicotine micro-spine growth were boosted compared to the control. However, with 10μM and 20μM there was no micro-spine development when compared to control and the 5μM treatment group and control (Figure 3.5.).

3.6. Nicotine differentially modulates single-channel properties of synaptic nicotinic receptors

Single-channel recording indicated that nicotine treatment alters the kinetic properties of nicotinic receptor in the synapses. We used synaptosomes that were isolated form rat hippocampi, and we blocked all the receptors by cocktail antagonists except the nicotinic receptor. Our results in Figure 3.7 exhibit a comparison between the properties of the nicotinic receptors in control and nicotine treated animals. The amplitude histograms constructed for the sample traces. In control recordings the open probability of single channel nicotinic receptors were 32%. The channel open probability increased to 48% with 5μM of nicotine. The 10μM and 15μM nicotine treatment decreased channel open probability to 24% and 18% respectively. The nicotinic receptors antagonist MLA at 1 μM concentration completely inhibited the synaptic nicotinic receptor channel activity, indicating that the channel activity elicited by nicotine are due to synaptic nicotinic receptors.

3.7. Effects of nicotine treatment (in vivo) on ILK, basal synaptic transmission and LTP

The changes in ILK expression show that nicotine has direct effects on synaptic function. By measuring extracellular field recordings, we studied the effects of different concentration of nicotine treatment on glutamatergic neurotransmission in the Schaeffer Collateral pathway of the hippocampus. Our results indicate that there is a significant alteration of LTP in nicotine treated rats (Figure 3.7.). In figure 3.7 C the chronic nicotine

exposure reduced the LTP compared to the control. In figure 3.7 D 5μM treated group showed an increase in LTP compared to the control, 10μM and 15μM، $P<0.001$ between groups, ANOVA and kruskal-walis test.

3.7.1. Input/output curves

To study the synaptic function in nicotine-exposed animal, we looked into the input-output correlation of Schaffer collateral-CA1 synapses in response to single electrical stimuli. Stimulating and recording electrode was placed in the CA1 region of stratum radiatum in the hippocampus. The stimulus was reduced till there was no field excitatory post-synaptic potential (fEPSP). Then, the stimulus was increased in increments, six responses was collected and averaged at each increments. In each group (control and nicotine treated) rodents, the data were averaged to establish input-output curves (Figure 3.7 F). There were significant difference between the nicotine treated group and the control. There is a significant difference between the control and the 5μM, 10μM and 15μM. To examine the mechanisms underlying the excitatory effect and the strength of synaptic transmission seen in the 5μM treated, we measured the size of the presynaptic fiber volley, which is a minor deflection of the field response that precedes the postsynaptic potential and relates with the amount of presynaptic afferents triggered by the stimulation pulse at different input current intensities. Figure 3.7 G shows that 5μM nicotine resulted in an increase in the EPSP slope of the presynaptic fiber volley across different stimulation intensities compared with control. Application of 10μM and 15μM decreases the EPSP slope of the presynaptic fiber volley $P<0.001$ between groups, alpha value=1, ANOVA and kruskal- walis test.

3.7.2. Paired pulse facilitation (PPF)

It is accepted that the changes in the amplitudes of the first and the second potentials represent a modification occur in the presynaptic synapses component (Chen et al., 1996). To examine whether change in fEPSP slope in nicotine-exposed slices was due to altered probability of glutamate release, I evaluated the PPF across a range of inter-stimulus intervals. PPF is known as short-lasting form of synaptic plasticity of the presynaptic neurons, causing in increase in synaptic response to the second stimulus relative to the first stimulus (Wu and Saggau, 1994). It is believed that this phenomenon might be controlled by additional neurotransmitter release during the second stimulation triggered by remaining calcium after the first action potential. As the release of the neurotransmitter is associated with to paired-pulse ratio, so if the nicotine exposed slices showed decrease in the EPSC amplitude, then what expected is an increase in paired-pulse ratio. Our result showed with 5μM nicotine, there was a decrease in the PPF ratio. Conversely, with increased concentration of nicotine 10μM and 15μM an increased paired-pulse ratio was seen proposing that high concentration of nicotine reduced probability of glutamate release (Figure 3.7. H) $P<0.05$ between groups, alpha value=1.0, ANOVA and Tukeys test.

3.8. Nicotine alters the expression of extracellular signal regulated kinase 1/2 (ERK1/2)

Our laboratory has previously demonstrated that exposure to nicotine during development altered the expression of ERK (Parameshwaran et al., 2013). I performed Immunohistochemical analysis on H19 7/IGF-IR cells exposed to nicotine.

When the cells were exposed to 5μM concentration of nicotine there was 28% increase the expression of ILK, while in 10μM and 15μM nicotine showed 21% and 24% decrease compared to the control. The statistical significance difference between control and nicotine treated was significantly validated using one way ANOVA (Tukey's Multiple Comparison-Test)*$p<0.05$,**$p<0.01$,n=3.

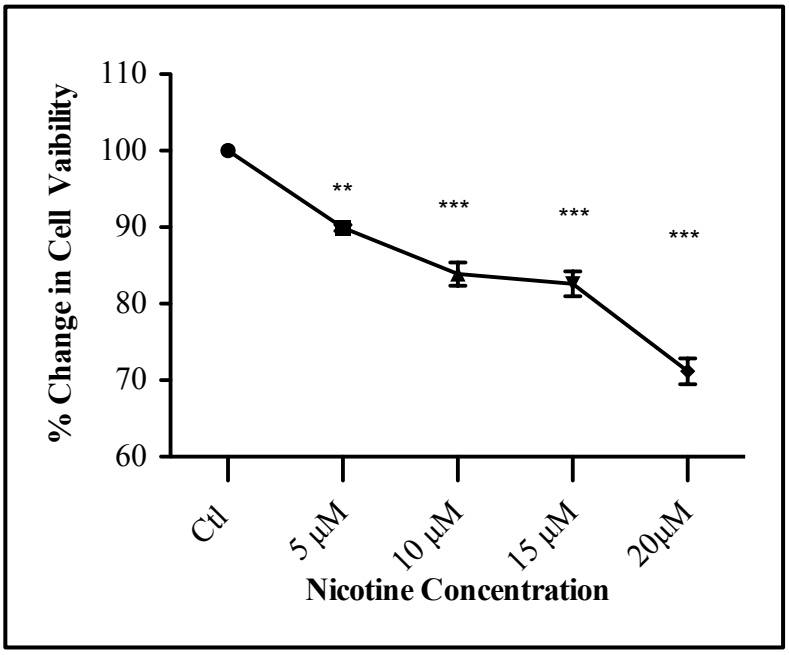


Figure 3.1. : Effect of Nicotine treatment on a H-19-α-7-IGFR cell line. MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide data indicate the effect of different nicotine concentrations on cell viability. Treating the cells with different concentrations of nicotine 5µM, 10µM, 15µM, and 20µM, showed a decrease in cell viability when compared to the control. The Data was significantly validated using one way ANOVA (Tukey's Multiple Comparison Test) **$p<0.01$,and ***$p<0.001$,n=5.

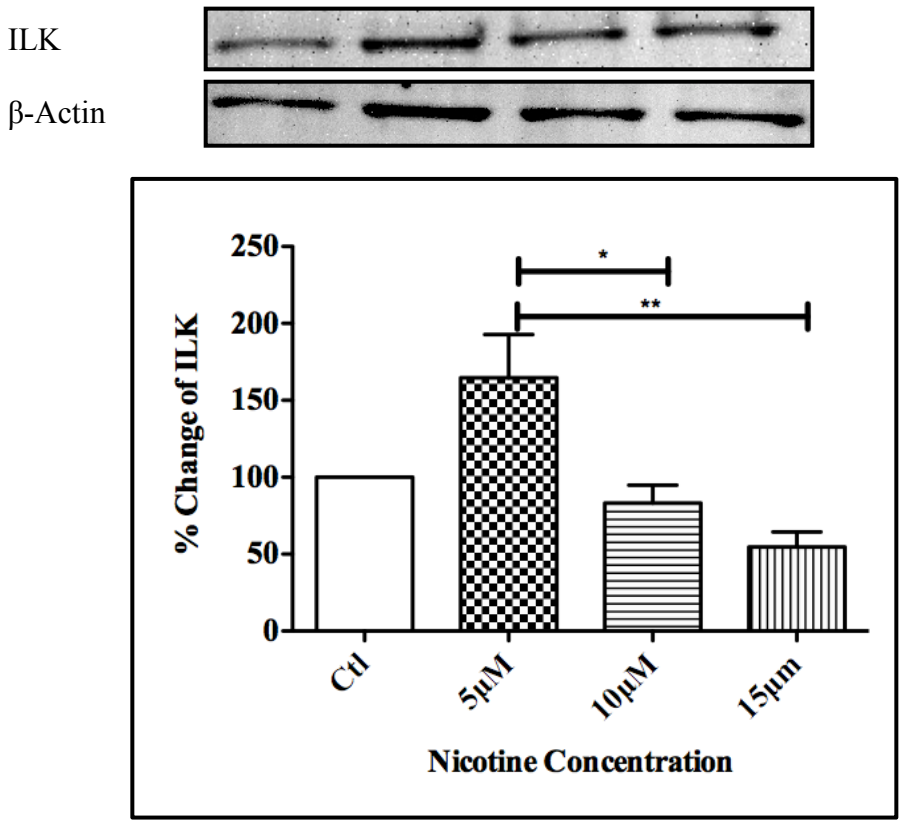


Figure 3.2. : Effect of nicotine on ILK expression levels on H-19-α-7-IGFR cell line. Western blot analysis shows ILK levels slightly increase with 5μM nicotine exposure, but with 10μM and 15μM concentrations the levels decrease when compared to the control and the 5μM treated group. The difference in the statistics between 5μM and 10μM, 15μM was significantly validated using ANOVA (Tukey's Multiple Comparison Test) *$p<0.05$, **$p<0.01$ n=6.

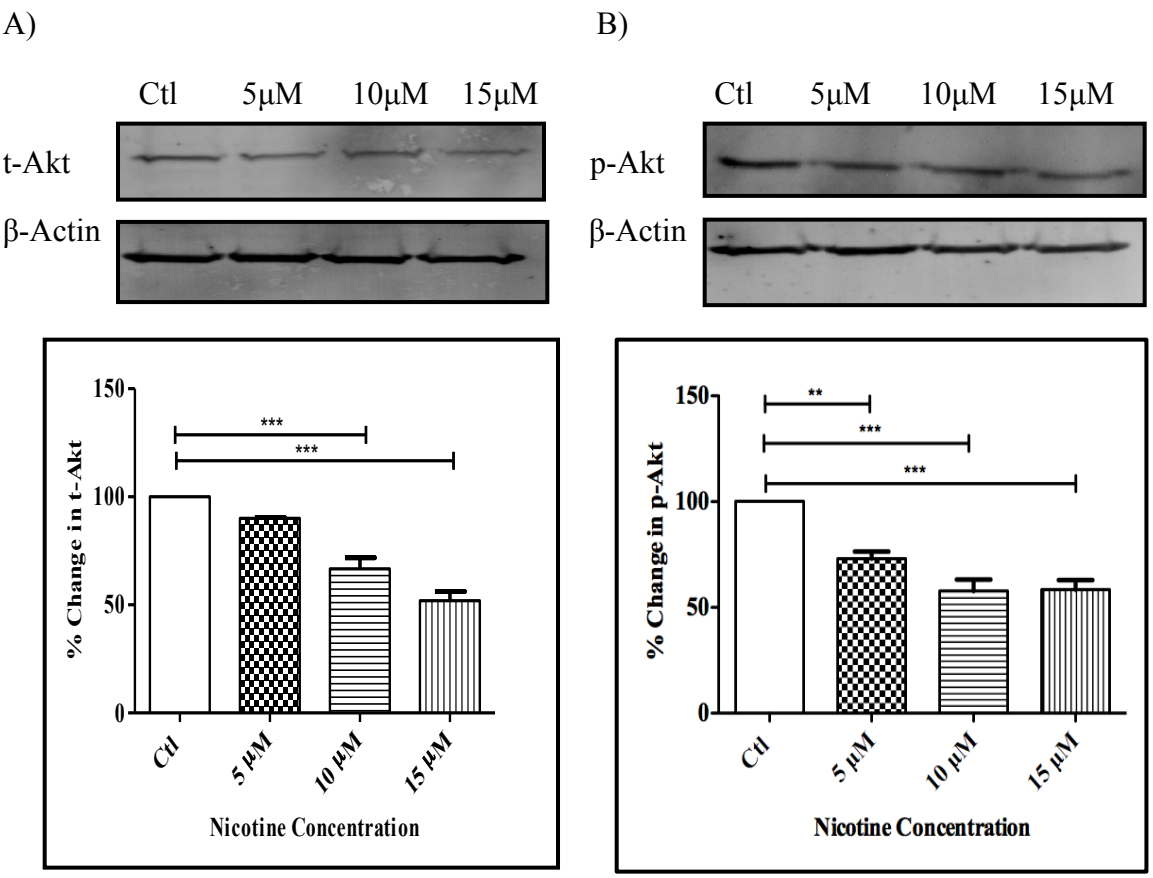


Figure 3.3. : Effect of different concentrations of nicotine on p-Akt and T-Akt. Western blot analysis indicating the down regulation of A) T-Akt and B) p-Akt with increased concentrations of nicotine when compared to β-Actin. A) There was no significant decrease in the T-Akt with 5µM compared to the control, but with 10µM and 15µM there is a significant decrease. B) p-Akt was decreased in 5µM, 10µM and 15µM compared to the control. The statistical significance difference between control and nicotine treated was significantly validated using one way ANOVA (Tukey's Multiple Comparison Test) *p<0.05, **p<0.01, ***p<0.001 n=6.

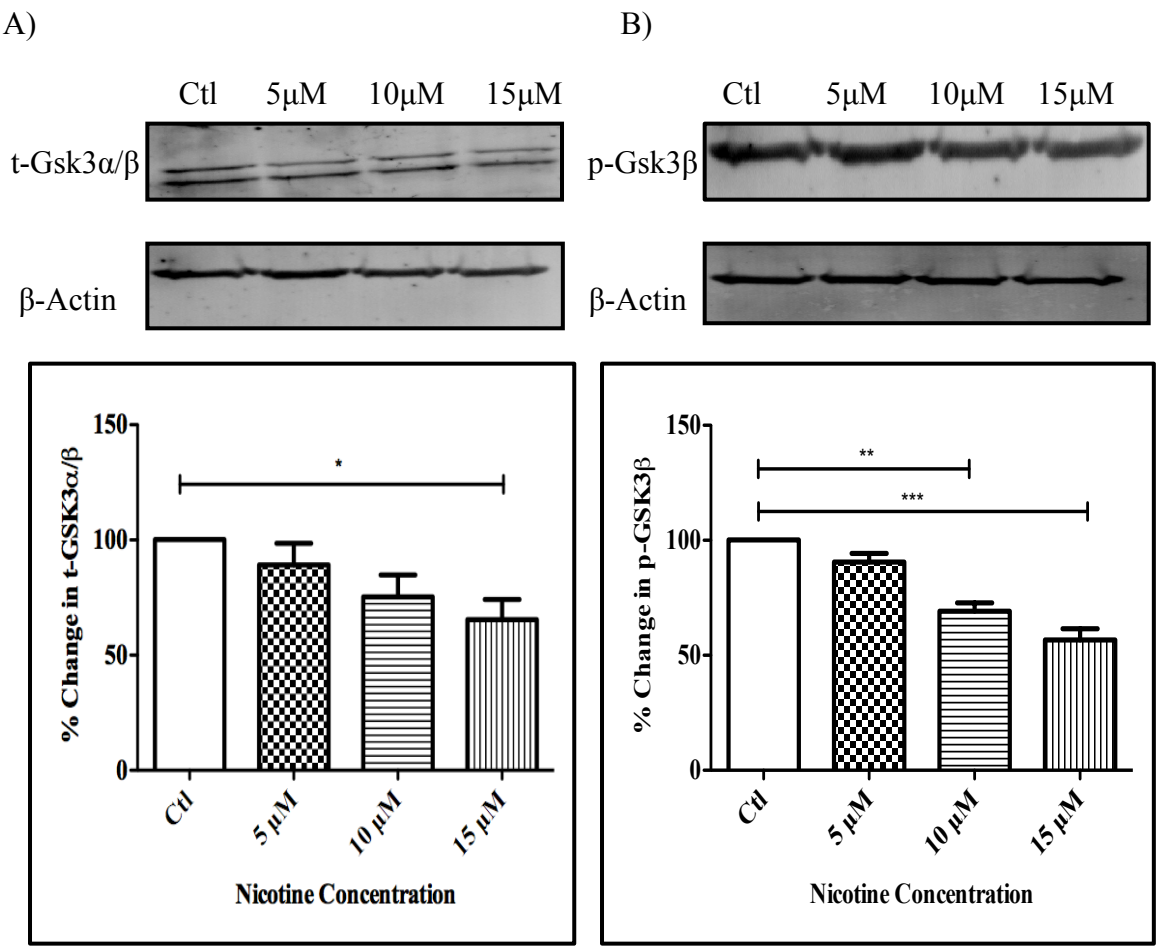


Figure 3.4. : Effect of different concentrations of nicotine treatment on t-Gsk3α/β and p-Gsk3β. The down regulation of T-Gsk3α/β and p- Gsk3β with increased concentration of nicotine when compared to β-Actin. A) T-Gsk3α/β was decreased with 5µM, 10µM and 15µM when compared to the control. B) The effect of nicotine on p-Gsk3β indicating that 5µM show no significant decrease compared to the control. However, with 10µM and 15µM the p- Gsk3β decreased. The statistical significance difference between control and nicotine treated was significantly validated using one way ANOVA (Tukey's Multiple Comparison Test) *p<0.05, **p<0.01, ***p<0.001 n=6.

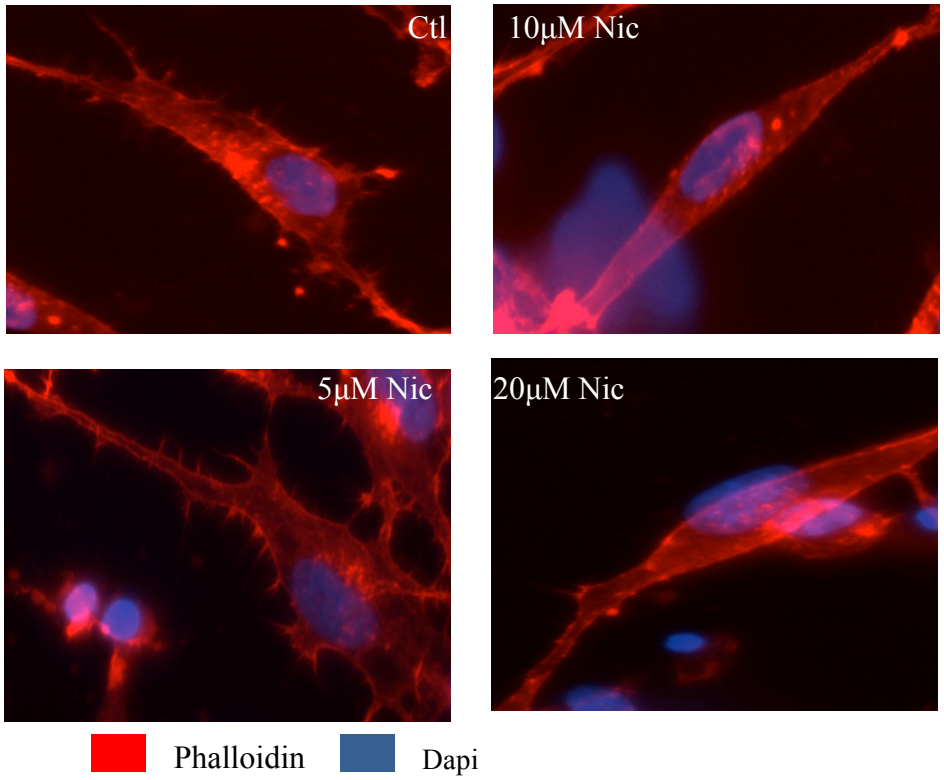


Figure 3.5. Effect of nicotine on micro-spine formation. Control neurons were not exposed to nicotine. Normal neurite growth is visible. 5µM neurons, exposed to low levels of nicotine, exhibited increased neurite growth when compared to control neurons. 10µM nicotine neurons, exposed to moderate levels of nicotine, have almost no neurite growth compared to control neurons. 20µM nicotine neurons, exposed to high levels of nicotine, continue to have no neurite growth. The DAPI fluorescence in all figures (in blue) verifies that the cell is alive.

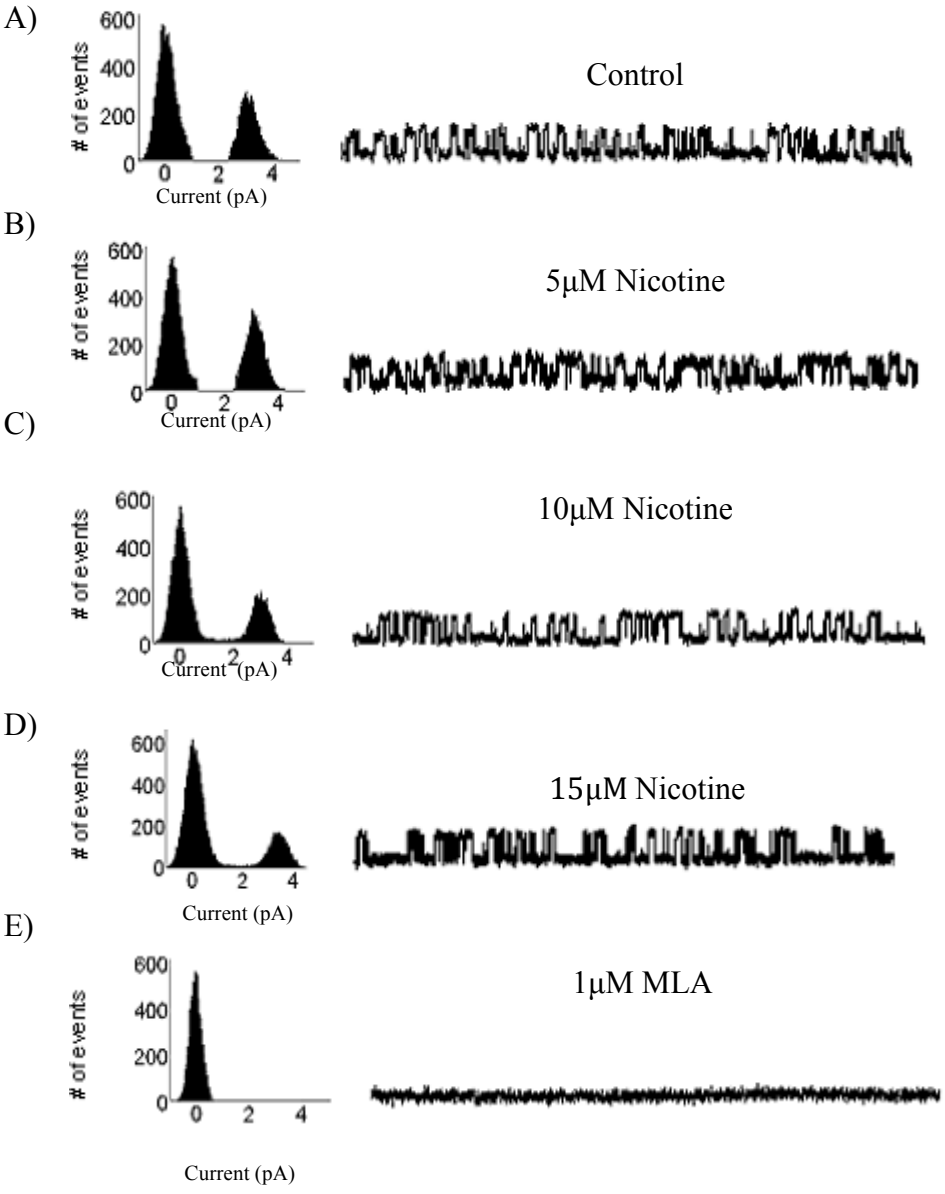
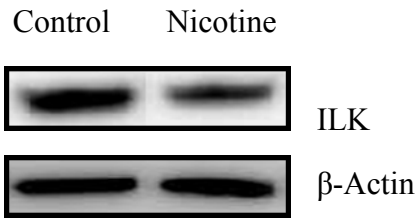
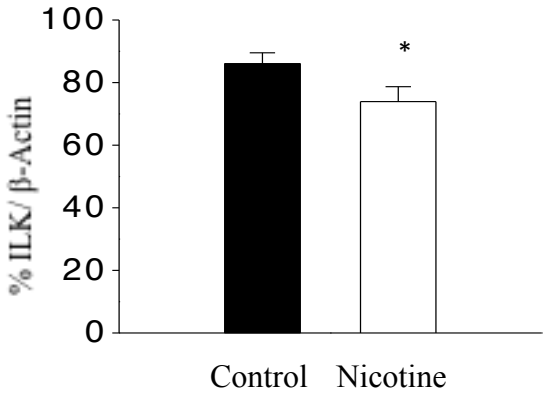


Figure 3.6: Nicotine treatment altered the single channel open probability of hippocampal synaptic nicotinic receptors. Amplitude of the histograms shows two separate peaks for close and open states. A) Representing the open probability (32%) of the control. B) Channel open probability increased to 48 +/- 8% with 5μM of nicotine. C & D) 10μM and 15μM nicotine decreased channel open probability to 24 +/- 6% and 18+/-9% % respectively. E) The nicotinic receptors antagonist MLA at 1 μM concentration completely inhibited the synaptic nicotinic receptor channel activity n=6.

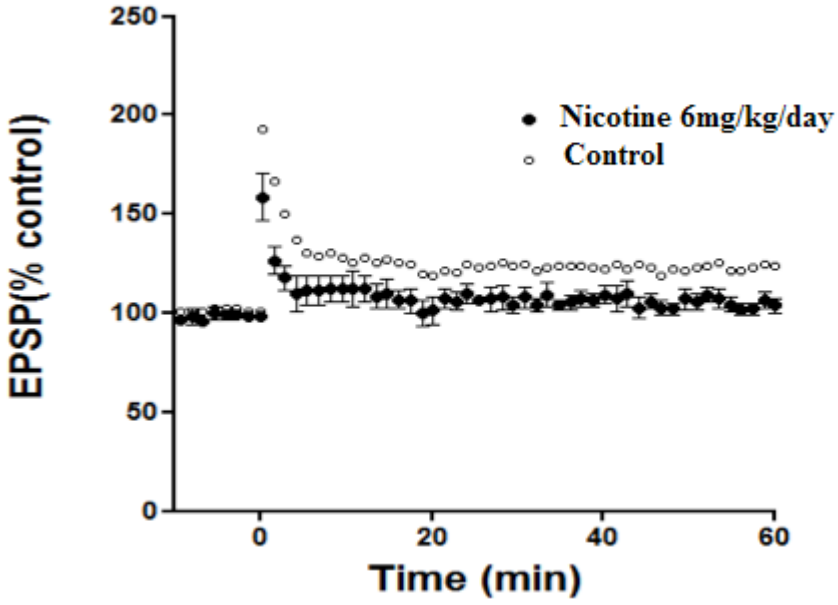
A)

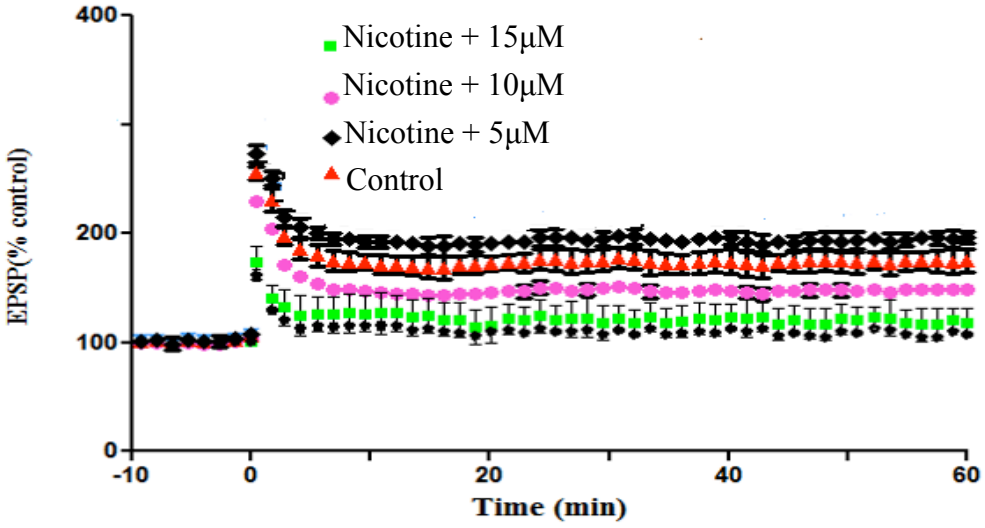

Control
Nicotine
ILK
β-Actin

B)


100
80
60
40
20
0
% ILK/ β-Actin
*
Control
Nicotine

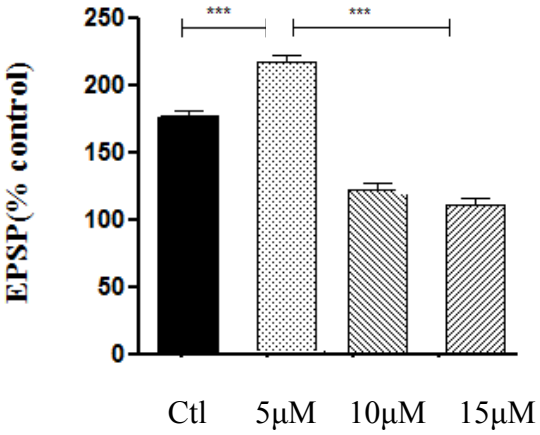
C)


250
200
150
100
50
0
EPSP(% control)
Nicotine 6mg/kg/day
Control
0
20
40
60
Time (min)

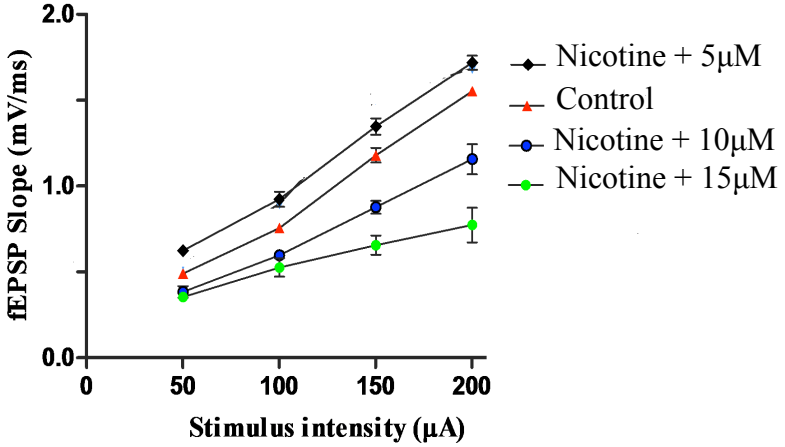

400
200
100
0
EPSP(% control)
Nicotine + 15μM
Nicotine + 10μM
Nicotine + 5μM
Control
-10
0
20
40
60
Time (min)

D)

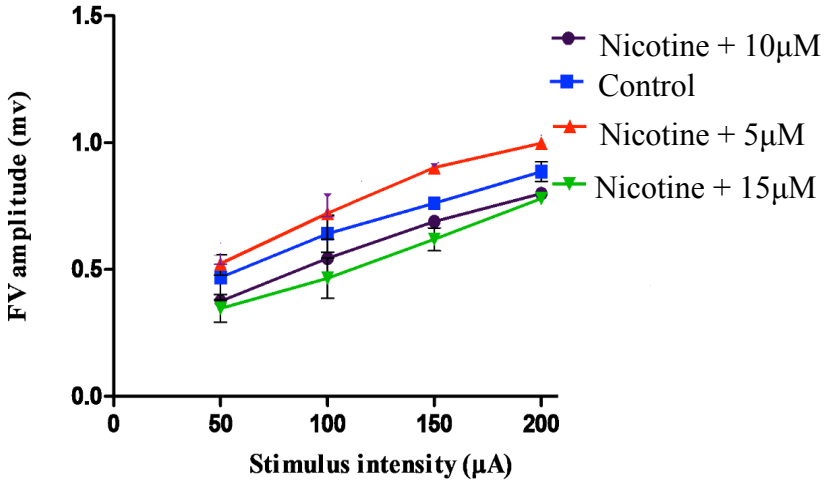
E)


***
***
250
200
150
100
50
0
EPSP(% control)
Ctl
5μM
10μM
15μM

F)


2.0
1.0
0.0
fEPSP Slope (mV/ms)
0
50
100
150
200
Stimulus intensity (μA)
Nicotine + 5μM
Control
Nicotine + 10μM
Nicotine + 15μM

G)


1.5
1.0
0.5
0.0
FV amplitude (mv)
0
50
100
150
200
Stimulus intensity (μA)
Nicotine + 10μM
Control
Nicotine + 5μM
Nicotine + 15μM

H)

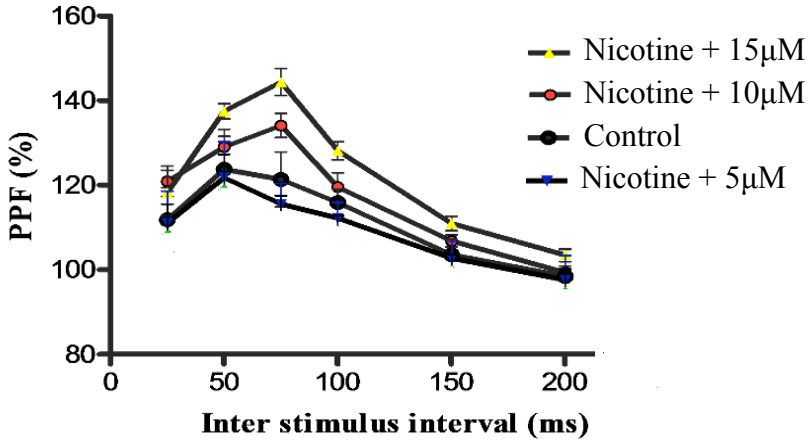


Figure 3.7. Nicotine treatment alters ILK expression and LTP. LTP induction was done through heta burst stimulation (TBS). This data depicts field excitatory postsynaptic potentials (fEPSP) of hippocampi in nicotine treated rodents. A & B) western blot analysis showing decreased ILK expression compared to the control in animals with chronic nicotine exposure. C) Decrease in LTP after chronic nicotine exposure to rodents. D & E) Synaptic responses recorded after acute application of varying concentrations of nicotine to hippocampal slices indicate altered LTP expression. Synaptic responses recorded indicate that LTP was increased in the hippocampus of nicotine 5µM treated hippocampal slices when compared to control, while 10µM and 15µM showed significant reduction in the LTP, $P<0.001$ between groups, ANOVA and kruskal-walis test. F) input/output plot of fEPSP slope versus stimulus intensity, which represent difference in I/O response between 5µM, 10µM, 15µM and saline exposed groups, $P<0.001$ between groups, alpha value=1, ANOVA and kruskal-walis test. G) Plot represents

presynaptic fiber volley amplitude (FV) versus different stimulus intensity, $P<0.001$ between groups, alpha value=1, ANOVA and kruskal-walis test. H) Comparison of PPF (higher PPR indicates reduced probability of release) in saline and nicotine slices, $P<0.05$ between groups, alpha value=1.0, ANOVA and Tukeys test n=6.

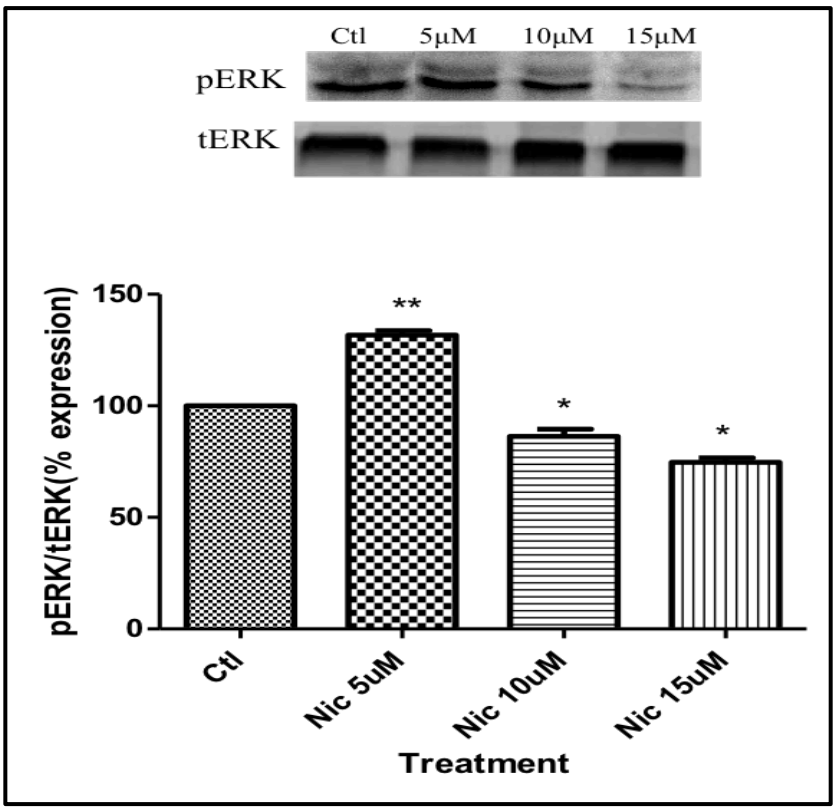


Figure 3.8. : Effect of different concentrations of nicotine on p-ERK and t-ERK. Western blot analysis indicating the altered expression of t-ERK and p-ERK with increasing concentrations of nicotine when compared to β-Actin. There was no significant change in the t-ERK with varying concentrations of nicotine compared to the control. The ratio of p-Akt/t-Akt indicates that with 5μM nicotine there was significant increase. However, with 10μM and 15μM nicotine there was significant decrease in the ratio of p-Akt/t-Akt. The statistical significance difference between control and nicotine treated was significantly validated using one way ANOVA (Tukey's Multiple Comparison Test) *$p<0.05$, **$p<0.01$ n=3.

CHAPTER V

Discussion

This study determined the effect of nicotine exposure using hippocampal cell line (H-19) and acutely isolated rodent hippocampal slices. Previous studies have indicated that acute/lower concentrations of nicotine exposure can enhance learning and memory, but high concentrations of nicotine can lead to memory deficits (Levin 2002). This study aimed to identify the possible hippocampal neuronal signaling cascades affected during nicotine exposure at various concentrations. Previous studies in our laboratory illustrated the importance of ILK signaling in cognition and synaptic plasticity (Shonesy, Thiruchelvam et al. 2012). Here I demonstrate how various concentrations of nicotine affect synaptic plasticity associated with learning and memory by modulating ILK and subsequently impacting the expression of downstream signaling molecules in H-19 neuronal hippocampal cells. I also used acutely isolated hippocampal slices from nicotine-exposed rodents to demonstrate synaptic receptor deregulation and altered synaptic plasticity.

Synaptic plasticity and memory deficits are associated with neuronal loss. It has been reported that low doses of nicotine can enhance memory, but due to the narrow therapeutic index of nicotine it can be toxic as well (Posadas, Lopez-Hernandez et al.

2013). To determine how nicotine can affect the cell viability of H-19 cells, an MTT cytotoxicity assay was conducted. Nicotine exposure had a dose-dependent effect on hippocampal neurons. Increasing concentrations of nicotine decreased neuronal cell viability. It is interesting to note that decreased cell viability was observed even at lower concentrations (5μM) (figure 3.1). It is not in agreement with enhanced spine formation (figure 3.5), basal synaptic transmission and synaptic plasticity (figure 3.7) observed in this study with 5μM of nicotine exposure. It is reasonable to presume that decreased cell viability observed with 5μM nicotine may be due to cell differentiation. In addition, it is also important to note that the basal synaptic transmission and plasticity assessments were performed using hippocampal slices. Therefore, the differences in preparations may have contributed to the different observations noted in this study. The fact that 5μM nicotine caused decreased cell viability in H-19 could be possibly due to lack of condensed extracellular matrices in this preparation allowing easy access of nicotine to cell surface receptors and causing over excitation of neurons leading dell death.

ILK affects several downstream signaling molecules, such as Akt and Gsk3α/β (Gary, Milhavet et al. 2003, Naska, Park et al. 2006). These downstream signaling pathways were investigated in this study. Total expression of Akt and Gsk3α/β along with phosphorylated Akt and Gsk-3b was decreased when compared to the control following treatment with different concentrations of nicotine. It has been well established that Akt activation is protective against glutamate-induced apoptosis, suggesting possible roles for ILK signaling in the development of neurodegenerative conditions in which over-activation of glutamate receptors results in programmed cell death (Gary and Mattson 2001).

This Lead me to suggest that nicotine treatment affect neuroprotective signaling pathway leading to the loss of H-19 hippocampal cells. The 5μM nicotine treatment increases ILK expression, but Akt and Gsk3β are not significantly affected, suggesting another possible signaling pathway modulating synaptic transmission and plasticity. A previous study in our laboratory reported that chronic nicotine exposure is associated with reduction in the expression of Extracellular signal-regulated kinases (ERK1/2) leading to altered synaptic plasticity (Parameshwaran, K. et al., 2013). This indicate that the increase in ILK expression can possibly enhances the synaptic plasticity through other signaling cascade such as ERK, since many studies indicated the role of ERK in LTP and memory formation (Schmitt et al., 2005; Shen and Yakel, 2009). However, how nicotine, ILK and ERK signaling affect synaptic plasticity is not yet elucidated.

In neuronal cell signaling, ILK was found to be downstream of PI3K, Akt and GSK-3β during micro-spine formation, it also plays a role in determining the polarity of the axon–dendrite in hippocampal pyramidal neurons. Therefore, there is an important link in the signaling pathway involved in the formation of neuronal polarity and ILK (Guo, Jiang et al. 2007). Micro-spine formation is an important indicator for synaptic plasticity and memory formation. Immunohistochemical assessment indicates that exposing cells to low concentrations of nicotine (5μM) increases micro-spine formation, which is regulated by ILK. While higher concentration of nicotine, such as 10μM and 20μM, showed no spine formation leading me to conclude that nicotine modulates ILK signaling and is responsible for micro-spine formation and associated morphological changes. Hippocampal nicotinic cholinergic signaling plays an extensive role in attention,

learning, cognition, and memory formation (Levin, Conners et al. 1998, Kenney and Gould 2008). The most abundant form of nicotinic acetylcholine receptors (nAChRs) are the homomeric α7 subtype (α7-nAChR) with high calcium permeability and are found in presynaptic and postsynaptic regions of glutamatergic synapses in the hippocampus (Fabian-Fine, Skehel et al. 2001). The activation of the presynaptic α7-nAChRs helps in the release of glutamate, which can stimulate LTP (McGehee, Heath et al. 1995, Gray, Rajan et al. 1996). It is not clear how ILK, via the nicotinic receptor, modulates micro-spine formation, but we postulate that nicotinic receptor stimulation can induce synaptic plasticity, which is one of the important mechanisms responsible for micro-spine formation.

The stimulation and strength of the glutamatergic synapses are crucial for memory encoding. It was proven that nicotinic signaling plays a major role in stimulating the fast glutamatergic transmission (Halff, Gomez-Varela et al. 2014). I performed in vivo studies to further investigate the role of the nicotine and ILK in glutamatergic transmission. The electrophysiology results revealed that chronic nicotine exposure in resulted in decreased LTP as reported earlier (Kenney and Gould 2008). In addition, western blot analyses showed a decrease in the ILK expression, suggesting a possible molecular mechanism involved in nicotinic modulation of synaptic mechanisms associated with memory formation.

In this study I evaluated the changes in fEPSPs and fiber volley amplitude, which care considered as synaptic plasticity markers, in response to various nicotine concentrations. First, I measured the fiber volley amplitude in response to varying

stimulus intensities. With 5μM nicotine application to the acutely isolated hippocampal slices, there was gradual increase in fiber volley amplitude while 10μM and 15μM showed a decrease, compared to control (figure 3.7G). The fiber volley amplitude represents presynaptic action potential. An increase in action potential in response to various stimulus intensities indicates that 5μM nicotine increased the efficiency of conversion of electrical stimuli into action potentials. Increase in presynaptic action potential will enhanced basal synaptic transmission and thus plasticity. Next, I measured the postsynaptic response to various stimulus intensities. With 5μM nicotine application, there was an increase in the gradual increase in fEPSP amplitude while 10μM and 15μM showed a decrease compared to control (figure 3.7G). This indicates that low concentration of nicotine can enhance the basal synaptic transmission in the hippocampus, which is important for learning and memory. To further validate these results, I studied the paired pulse facilitation (PPF) that is related to the glutamate release and conceder as short-term synaptic plasticity. My results showed with 5μM of nicotine, increased paired pulse ratio, but with 10μM and 15μM it was decreased compared to the control. Therefore, it was concluded that 5μM nicotine can enhances the release of neurotransmitters related to synaptic plasticity. The measure of synaptic plasticity in the form of LTP indicated that acute exposure of lower concentrations (5μM) of nicotine increased LTP whereas, 10μM and 15μM of nicotine decreased LTP compared to controls.

Lower concentrations of nicotine enhanced synaptic transmission and LTP whereas, similar concentrations showed increased expression of ILK and decreased

expression of p-Gsk3β. Decreased expression of p-Gsk3β should lead to decrease in LTP but this is not what was observed in my study. Therefore, it appears that ILK modulated LTP through another mechanism not involving p-Gsk3β. A recent study from our laboratory reported the role of nicotine in modulating ERK1/2 and LTP. Therefore, I measured the expression of ERK in the presence of nicotine and found that 5μM nicotine increased the expression of ERK, whereas, 10 and μM 15μM nicotine resulted decreased expression (Figure 3.8).

Certain subtypes of nicotinic receptors are permeable to calcium and calcium permeability can influence synaptic plasticity. Therefore, I also carried out studies to determine the differential modulation nicotinic receptors by varying concentrations of nicotine. Results indicate that the open channel probability of nicotinic receptors are increased by 5μM nicotine and decreased by10-15μM nicotine. Increased channel open probability can increase calcium influx into neurons and thus increase plasticity. In addition, increased calcium permeability can increase transmitter release. The cholinergic projections from medial septum modulate the activity of glutamate release in the hippocampus. Therefore, an increase in carefully controlled glutamate release can also increase LTP. This notion is further supported by my findings that lower concentrations of nicotine increased paired pulse ratio leading to increased transmitter release. Taken together, my data demonstrate that nicotine by modulating ILK signaling alters synaptic transmission and plasticity required for learning and memory-mechanisms.

CHAPTER IV

Conclusion and Prospective

The molecular and cellular mechanisms of memory encoding have been a crucial factor regarding learning and memory along with neurodegenerative diseases. Low concentrations of nicotine enhance cognition, memory and attention in humans and animals. In Alzheimer's disease and schizophrenia, nicotinic receptor deficits are observed, suggesting nicotinic receptors are fundamental in memory and cognition. On the other hand, studies indicate that chronic nicotine exposure can lead to impairments in memory and cognition. We demonstrate a link between nicotinic receptors and other biochemical signaling molecules that are affected during memory formation. The molecule I focused on was ILK and its down stream signaling. ILK signaling is essential in cell survival and proliferation. Recent studies indicate a relation between ILK and synaptic plasticity, a decrease in expression of ILK occurs with diminished LTP.

In conclusion, our results suggest a potential biochemical link between the nicotinic receptor and ILK. Our data shows that varying concentrations of nicotine differentially modulates ILK and its downstream signaling. Further investigations should be directed at identifying the molecular and cellular mechanisms using pharmacological modulations, gene knockdown and overexpression to gain a clear understanding of the role of ILK in learning memory encoding.

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