Prenatal Nicotine Exposure and Glutamatergic-Cholinergic Interplay

by

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Abstract

Smoking in pregnant women is reported as a risk factor in neurobehavioral alterations such as learning and memory deficits. Prenatal exposure of nicotine (6mg/kg /day) in animal models induces memory impairment associated with cholinergic/glutamatergic dysfunction. Rodent model of prenatal nicotine exposure (PREN), receiving nicotine infusions in pregnant dams, developed memory deficits. Here, we demonstrate that basal synaptic transmission (BST), forms of synaptic plasticity, including long-term potentiation (LTP) are impaired at Schaffer collateral (SC) synapses in PREN rodents. Mean amplitudes and frequency of AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and NMDAR (N-Methyl-D-Aspartate) mediated sEPSCs (spontaneous Excitatory Post Synaptic Currents) were reduced. Concentrations of NMDAR subunits NR1/2A, postsynaptic density protein-95 (PSD-95) and CaMKII (Calmodulin Kinase II) decreased in PREN hippocampus. Moreover, deficits in LTP and BST were accompanied by altered synaptic α7/β2 nicotinic acetylcholine receptors (nAChRs) activity. Co-immunoprecipitation studies revealed α7/β2-nAChRs complexes with NR1, vesicular glutamate transporter (VGLUT), PSD-95 and synapse-associated protein (SAP102), disrupted in PREN rodents. Additionally, the nicotinic signaling pathway was impaired in them.

α7/β2-nAChRs facilitate release of other neurotransmitters including glutamate. Here, we tried to address whether nAChR subunits are responsible for deficits in synaptic plasticity in PREN rodents. First, we found that, expressions of α7and β2-nAChRs are decreased. Moreover, blockade of α7-nAChRs with Methyllycaconitine (MLA) in control animals impaired LTP.

Western blots analysis of hippocampal lysates revealed decreased expression of PSD 95/SAP102. These proteins are involved in regulating organization of postsynaptic components at nicotinic synapses. These results suggest that PREN-impaired LTP/PPF arise as consequence of dysfunctional α7/ β2 nAChRs. Hence it is logical to opine that nAChRs may be an important target to help ameliorate cognitive deficits.

Studies indicate cognitive deficits are enduring. This study was performed to investigate the effects of prenatal nicotine exposure on excitatory synaptic physiology and cellular signaling in hippocampus. Reduced nAChR expression and modified MAPK signaling, consequences of PREN are thought to be potential mechanisms for disrupted excitatory synaptic physiology in hippocampus of PREN rats. In addition, we identify alterations in synaptic plasticity, BST, decreased AMPAR synaptic currents and reduced nAChR levels and their signaling in the hippocampus of PREN rodents as mechanisms underlying long lasting cognitive impairments.

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1. Introduction:

Smoking during pregnancy has been reported as a strong risk factor for several adverse health conditions in children (Pausova et al., 2007). Such conditions range from stillbirth and sudden infant death syndrome to long term neurobehavioral alterations such as learning and memory deficits (Winzer-Serhan, 2008, Ernst et al., 2001, Linnet et al., 2003). Several human longitudinal studies conducted so far suggest a strong correlation between maternal smoking and abnormal cognitive outcomes in offspring, especially in younger children (Kafouri et al., 2009). Tobacco contains several harmful substances, but the neurobehavioral changes observed in these children are mainly due to exposure to nicotine *in utero*. Nicotine is the major psychoactive and addictive compound in tobacco and at the same time, an agonist of nicotinic acetylcholine receptors (nAChRs). Consistent with these observations, animal studies from our and other laboratories have shown that prenatal nicotine exposure results in enduring learning and memory deficits. These deficits can be used as a model of cognitive deficits observed in children born to smoking mothers (Vaglenova et al., 2008, Parameshwaran et al., 2012).

The α7- and β2-subunit containing nAChR subtypes are abundantly expressed in the hippocampus (Alkondon et al., 2004). The hippocampus is a brain region which aids the process of memory consolidation. It further helps in movement related to spatial navigation (Kogan et al., 2000). These functions of the hippocampus are likely regulated by nAChRs. During embryonic development, nAChRs play many important roles in formation of neural networks and time-dependent activation of other neurotransmitters, including the glutamate system. α7-nAChR

mRNA is expressed as early as embryonic day 13 (E13) in rat fetal brain, and receptors become functional in the prenatal brain (Leslie et al., 1997). Therefore, chronic nicotine exposure during fetal development can impair the regulatory capacity of nAChRs in neural circuitry and synapse formation. Chronic nicotine exposure during early development appears to alter hippocampal morphology, resulting in decreased gross neuronal area and increased packing densities in hippocampal and cortical neurons. This suggests that nicotine can affect neuronal morphology. These effects are a direct consequence of developmental nicotine exposure (Roy and Sabherwal, 1994; Roy et al., 2002; Huang et al., 2007). Importantly, the effects of nicotine on the developing brain are specific to certain regions. For example, the cerebellum, which develops later in the neonate, does not show altered neuronal morphology. On the other hand, nicotine has a rapid and long-lasting effect on hippocampal neuronal micro-structure with a vulnerability period that extends from prenatal to early postnatal life (Huang et al., 2007).

In the hippocampus, excitatory neurotransmission is mainly mediated by glutamate receptors. However, hippocampal glutamatergic transmission itself is modulated by other neurotransmitter systems in the brain. Specifically, presynaptic nAChRs have been shown to facilitate the release of glutamate. Indeed, presynaptic α7- and β2-nAChR-mediated increases in glutamate release in hippocampal CA1 synapses contributes to synaptic plasticity (Ji et al., 2001; Ge et al., 2005). These findings highlight the importance of α7- and β2-nAChRs in the development of glutamatergic synapses and synaptic plasticity in the hippocampus. In the case of pregnant smokers, the developing fetal brains receive chronic exposure to nicotine. Therefore, developmental disruptions of nAChRs can have deleterious impacts on glutamatergic synaptic physiology and plasticity, culminating in impaired cognitive functions. Although impairment of

hippocampus dependent memory formation in prenatal-nicotine exposed (PREN) rodents is widely reported, the underlying mechanisms have yet to be fully elucidated. Our initial findings (Parameshwaran et al., 2012) provide the first empirical evidence that hippocampal LTP is impaired in PREN rodents. In addition, our recent studies also provide evidence that basal synaptic transmission, AMPAR function, and the expression of key pre- and post -synaptic proteins are altered in the hippocampus of PREN rodents (Parameshwaran et al., 2012). The processes underlying modifications in these two-neurotransmitter systems which promote these deficits are currently not known. A comprehensive understanding of the mechanisms governing cognitive deficits in the hippocampus of PREN rodents would contribute to therapeutic strategies for increasing the quality of life for individuals affected by such conditions.

This study provides immense contributions to fill critical gaps in our previous study (Parameshwaran et al., 2012), and existing literature. ***This project tested an overarching hypothesis that alterations in nAChRs expression and function lead to modified glutamatergic transmission in the hippocampus resulting in memory deficits in PREN rodents.***

Recently published work from our laboratory demonstrated that basal synaptic transmission and LTP was impaired in PREN rat hippocampi (Parameshwaran et al., 2012). These results suggest that developmental nicotine exposure affects the synaptic transmission, mediated largely by glutamate receptors, in the postnatal brain. Previous findings established that nAChRs are modulators of synaptic transmission and plasticity in the hippocampus. Our findings suggest that protein expression of α7 and β2 nAChRs were downregulated in the PREN rat hippocampus. In the current study, evaluations of responsiveness of glutamatergic synaptic transmission to modulation of different types of nAChRs were performed. These observations raise the possibility that altered expression and function of nAChRs may have contributed to the observed deficits in

glutamatergic synaptic transmission and plasticity. This will lead to more detailed understanding of the complex mechanisms by which prenatal nicotine exposure affects synaptic transmission and plasticity. The results of this study highlight the important mechanisms by which the maternal smoking can cause cognitive deficits in offspring.

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2- Literature review

Tobacco has been documented as a profoundly used drug of abuse around the world, with an estimated 19.5% of adults in the United States identifying themselves as regular smokers (CDC, 2010). The proportion of pregnant women who smoke is believed to be 20 to 25%, resulting in approximately 800,000 babies in the United States born yearly to smoking mothers (Martin et al., 2003). In addition to the direct effects on the adult smoker, tobacco exposure exerts deleterious effects on the health of the fetus, continuing through adolescence (Kum-Nji et al., 2006; Rogers, 2008).

The use of tobacco during pregnancy exposes the developing fetal brain to the psychoactive compound nicotine. Evidence from human and animal studies have demonstrated that maternal smoking can lead to deficits appearing early in postnatal life compared to babies born to non-smokers. These symptoms include low birth weight, nicotine withdrawal syndrome, sudden infant death syndrome (SIDS), a higher risk of heart defects, cleft lip or palate, and possibly other birth defects and cognitive behavioral alterations (Hackshaw et al., 2011). The long-term effects of tobacco exposure during fetal brain development may be more profound since maturation of neural circuitry is not yet completed and the inherent plasticity of the developing brain makes it particularly susceptible to drug-induced alterations.

Although tobacco smoke contains over four thousand chemicals (US Dept. of Health and Human Services, 1989), nicotine has been reported as the main psychoactive constituent and exerts neurotoxic effects on the prenatal developing brain. Nicotine is a hygroscopic liquid alkaloid found naturally in high concentrations in the tobacco plant *Nicotiana tabacum*. Nicotine can easily penetrate the skin and cross the placenta and blood brain barrier (Luck et al., 1985). Nicotinic receptors (nAChRs) are expressed early by the first trimester in the foetal brain, which

is indicative of their crucial functional role in modulating brain development. Nicotine both activates and desensitizes neuronal nAChRs, which are a structurally diverse family of ligand-gated ion channel receptors. nAChRs are widely expressed in the fetal central nervous system and mediate the physiological effects of the neurotransmitter acetylcholine (ACh), which has shown to have a critical role in brain maturation (Dani, 2001).

Pharmacology of neuronal nicotinic receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) have caught the attention of researchers due to their involvement in several important physiological processes such as cognitive learning and memory, synaptic plasticity, neurodevelopment, and neuroprotection (Levin et al., 2002). nAChRs are also involved in many pathological changes to the brain including Alzheimer's disease, Parkinson's disease, schizophrenia, and addiction (Dani et al., 2001; Paterson and Nordberg, 2000). In addition, nAChRs are involved in other physiological functions such as arousal, cerebral blood flow and metabolism, inflammation, and presynaptic regulation of neurotransmitter release. Moreover, alterations in nAChR subunit expression in specific brain regions during development, including changes in subunit composition, can contribute to altered biochemical signaling as seen in several pathophysiological conditions (Gotti et al., 2006).

Acetylcholine receptors (AChRs) are a family of ligand-activated receptors involved in cholinergic neurotransmission in the central nervous system (CNS) (Cooper et al., 2003). Cholinergic neurotransmission involves the production of the neurotransmitter acetylcholine (ACh) in presynaptic cholinergic neurons by the synthetic activity of choline acetyltransferase (ChAT). For this mechanism, an acetyl group from mitochondrial-derived acetyl coenzyme A is transferred to dietary nutrient choline, which then uptake into the presynaptic neuron. The synthesized ACh is stored in vesicles located near the synaptic terminal and released upon

depolarization by a calcium dependent mechanism. Synaptic ACh may then bind to postsynaptic AChRs and, in some cases, regulates its own release via interactions with presynaptic autoreceptors. Unbound ACh in the synapse is hydrolyzed by acetylcholinesterase (AChE), which is present on pre- and postsynaptic neuronal membranes. Following the activation of postsynaptic receptors, ACh is released from the receptor and is degraded by AChE to acetate and choline, which is then reused in the synthetic events.

The released ACh interacts with two different cholinergic receptor subtypes: nicotinic and muscarinic (Cooper et al., 2003). Muscarinic acetylcholine receptors (mAChRs) are metabotropic receptors coupled to G-proteins (guanine nucleotide-binding regulatory proteins) and are capable of eliciting inhibitory or excitatory responses. nAChRs are ligand-gated pentameric ion channels that consist of four transmembrane-spanning subunits around a central pore (Figure 2.1). nAChRs are found in the central and the peripheral nervous systems (McKay, Placzek, & Dani, 2007). Depending on their subunit composition, nAChRs can increase either sodium or calcium permeability and hence elicit depolarization or excitation (Dani and Bertrand, 2007). To date, twelve genes encoding for neuronal nAChR subunits have been identified. A variety of receptor subtypes result from different combinations of the nine α (α2-α10) and three β (β2-β4) subunits (McGehee, 1999). nAChRs have been divided into two major classes containing either a heteropentameric (α2-α6, β2-β4) or homopentameric (α7-α10) structure. The specific subunit composition determines the pharmacological specificity, ion selectivity, and desensitization characteristics of the nAChR (Gotti et al., 2006). Accumulating evidence reveals that α7- and β2-

containing nAChRs are expressed at preterminal, axonal, somatic and dendritic locations.

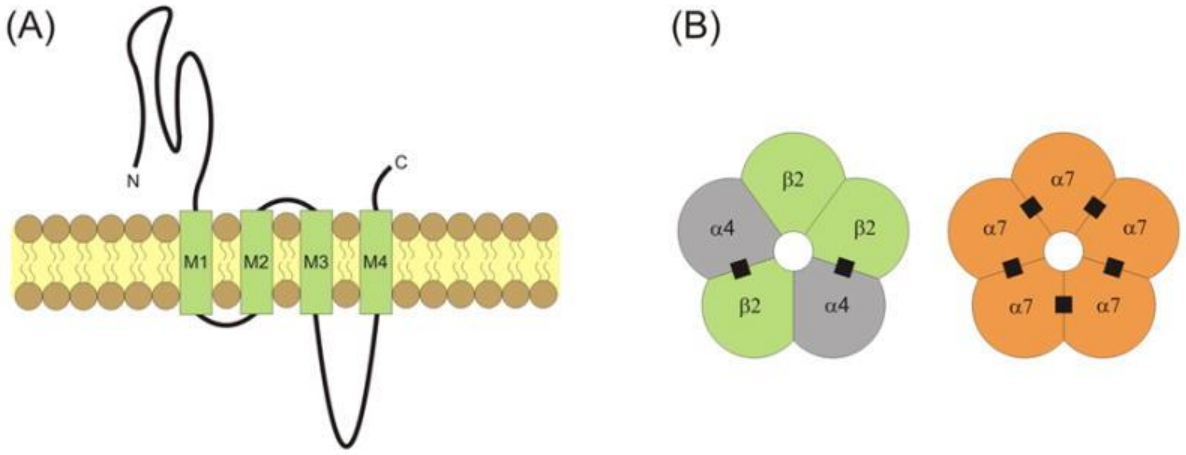

Figure 2.1: Transmembrane topology and pentameric structure of nAChRs. (A) nAChRs consist of four transmembrane domains (M1 through M4) with extracellular C- and N-termini. (B) Subunits are assembled into pentamers that include a water-filled cation-permeable pore. The most common nAChRs in the brain are hetero-oligomeric α4β2 nAChRs and homo-oligomeric α7 nAChRs. The recognized ACh binding sites are indicated by filled black squares adapted from (McKay et al., 2007).

In CNS, the most common neuronal nAChRs are the heteromeric α4β2 subtype, which is formed in a 2α:3β stoichiometric ratio and produces two ligand binding pockets at the α/β interfaces, and the homomeric α7 nAChR consisting of five α7 subunits. α7 nAChR contains five ACh binding sites and is antagonized by α-bungarotoxin (Chen and Patrick, 1997; Orr-Urtreger et al., 1997). α4β2 nAChRs primarily gate Na^+ and have a range of effects at the neuronal level, depending on their location. The α4β2 nAChR binds nicotine and other agonists, such as epibatidine and cytisine, with high affinity (Dani and Bertrand, 2007). When activated, this receptor primarily gates Na^+, leading to membrane depolarization and facilitation of other excitatory inputs. Continued exposure to concentrations of nicotine equivalent to blood levels in a smoker will lead to desensitization of the majority of α4β2 nAChRs.

The other abundant nAChR subtype found in the brain is the homomeric α7 nAChR, which can be found in regions of the brain involved in learning and memory like the hippocampus and cerebral cortex (Breese et al., 1997a; Rubboli et al., 1994). At the cellular level, activation of α7

nAChRs is believed to modulate interneuron excitability (Frazier et al., 1998) and regulate the release of excitatory and inhibitory neurotransmitters (Alkondon et al., 2000a). The α7 nAChR displays rapid desensitization in the presence of high concentrations of agonist and conducts Na^{+} as well as being highly permeable to Ca^{++} ($1Na^{+}$:$10Ca^{++}$) (Dani and Bertrand, 2007). Presynaptic densities of α7 nAChRs play critical modulatory roles by directly inducing Ca^{++}-dependent release of hippocampal norepinephrine. Glutamate release may also be modulated by α7 nAChRs (Wonnacott et al., 2006). nAChRs shares some characteristics with N-methyl-D-aspartate receptors (NMDARs) and can modulate glutamate release: α7 nAChRs gate as much Ca^{++} as NMDARs. Thus nAChRs are implicated in synaptic plasticity at more hyperpolarized membrane potentials (McGehee and Role, 1995). Interestingly, there is some evidence that NMDARs are required for nAChR-mediated LTP (Welsby et al., 2006). Indeed, nAChRs are found at both presynaptic and postsynaptic sites in the hippocampus CA1 region, as identified by immunogold labeling and electron microscopy (Fabian-Fine et al., 2001), and additionally on sites outside the synapse where they act to regulate neuronal function. The presence of these receptors postsynaptically, where they contribute a small amount of postsynaptic membrane depolarization, presumably increases the opening probability of NMDARs via the voltage-dependent relief of Mg^{2+} blockade; in addition. nAChRs have crucial role in modulating intracellular Ca^{2+} signal in the postsynaptic cell that may stimulate plasticity-evoking Ca^{2+}-dependent signaling and gene transcription (Alkondon et al., 1998; Hu et al., 2002). However, nAChRs are more predominant in presynaptic and preterminal regions. When located presynaptically, they serve to enhance the release of many neurotransmitters in diverse regions of the brain by triggering voltage-gated Ca^{++} channels that modulate other neurochemical systems (Tredway et al., 1999).

The role of nicotinic receptor desensitization in chronic developmental nicotine exposure

Desensitization is an important characteristic of nAChRs. Acute exposure to nicotine activates a normal cholinergic response, but chronic exposure inhibits the normal biological response of ACh through desensitization. Desensitization of nAChRs is a fairly complex phenomenon. In general, nAChRs slowly desensitize in presence of concentrations of agonist much lower than that required for activation (Fenster et al., 1999). Therefore, desensitization of brain nicotinic receptors can occur due to prolonged nicotine exposure resulting from smoking (Brody et al., 2006).

Nicotine is a very potent and efficacious desensitizing agent of nAChRs. As mentioned earlier, nicotine desensitizes α4β2 nAChRs effectively in rats (Paradiso and Steinbach, 2003). The presence of increased nicotinic receptor desensitization in hypoglossal motor neurons following chronic developmental nicotine exposure has also been reported (Pilarski et al., 2012)**.** In physiological environments, neurotransmitters are typically released in amounts lower than the threshold for receptor desensitization. However, for nicotinic receptors those desensitizes quickly or have high affinities for Ach, such as the α7 nAChR desensitization occurs after prolonged or repetitive stimulation. The same is true in case of receptor sub-populations where neurotransmitters remain bound to agonists for a long time after free transmitter is hydrolyzed. Moreover, highly selective agonists for α7 nAChRs, like choline, can desensitize the receptors rapidly (Alkondon et al., 2000b; Levin and Simon, 1998; Pilarski et al., 2012).

The trend does not remain same in every species that has been studied. For example, it was reported that nAChRs are upregulated in postmortem brains of rodents and in smokers due to chronic nicotine exposure (Breese et al., 1997b;Marks et al., 1983). In addition, the

neuroprotective properties of nicotine are a result of receptor upregulation (Jonnala and Buccafusco, 2001). Many studies support the notion that inconsistent upregulation caused by chronic and repetitive stimulation of nAChRs is due to increases in the number of receptors as a result of post-translational modifications (Gentry and Lukas, 2002). This idea is supported by a study conducted in a human cell line using rat α4β2 nAChRs. The investigators demonstrated that chronic nicotine exposure stabilizes the high-affinity state of the receptor bound neurotransmitter and later shows increased binding and response (Vallejo et al., 2005).

Nicotinic modulation of synaptic plasticity and signaling

Long-term potentiation (LTP) is an activity-dependent heightening of synaptic communication induced by tetanic stimulation. It is widely accepted as a cellular model to investigate the mechanisms of learning and memory mediated through synaptic plasticity. Induction and maintenance of LTP requires release of glutamate from the neurons, leading to activation of postsynaptic NMDA receptors and Ca^{2+} entry into postsynaptic neurons (Bashir1991). Acute nicotine exposure facilitates LTP induction by decreasing the firing threshold in hippocampal CA1 regions (Fujii et al., 1999; Welsby et al., 2006). As expected, induced LTP caused by acute nicotine treatment is prevented by α7 nAChR blocker (Welsby et al., 2009). Chronic nicotine treatment however has a completely different effect on LTP induction or maintenance. This suggests that desensitized nAChRs are involved in chronic treatment (Fujii and Sumikawa, 2001). Generally, nicotine is considered to improve learning and memory in humans. A recent study reported that nicotine facilitates memory consolidation in perceptual learning (Beer et al., 2013). A different study found that acute nicotine treatment enhances working memory in rats using an active avoidance test (Whiteaker et al., 2000). It has been found that agonizing nAChRs, especially the α4β2 and α7 subtypes in the ventral hippocampus and basolateral amygdala by

nicotine, has an important role in the enhancement of cognition and memory (Levin, 2002; Pocivavsek et al., 2006).

The positive allosteric modulator PNU-120596 was found to strongly reduce the threshold for nicotinic enhancement of LTP induction and maintenance when it was blocked by the selective antagonist methyllycaconitine (MLA). This affect is believed to be mediated via the α7 nAChR (Welsby et al., 2009). Mechanisms underlying nicotinic receptor mediated enhancement of LTP, include activation of extracellular signal-regulated kinase (ERK), cAMP-dependent protein kinase (PKA), and Src molecules (Welsby et al,. 2009a). In addition, it was reported that nicotinic activation of the Ca^{2+}-permeable α7 nAChRs fills ryhonoid Ca^{+2} stores and release of Ca^{+2} by high-frequency stimulation via Ca^{+2}-induced Ca^{+2} release (CICR). It further activates mGluRs inducing an additional component of LTP which augments with controlled LTP (Welsby et al, 2006). It was later documented that Ca^{2+} influx directly through nAChR channels or indirectly via voltage-gated Ca^{2+} channels is significant for nicotinic modulation of transmitter release, synaptic plasticity, and many others biological functions such as neuronal viability, differentiation, and migration. A growing body of evidences indicates that in specific nAChR subtypes, down-stream signaling on nicotine binding leads to activation of several Ca^{2+}-dependent kinases, including Phospho-Inositol-3-Kinase (PI3K), Protein Kinase C (PKC), Protein Kinase A (PKA), calmodulin-dependent protein kinase II (CAM kinase II), and extracellular signal-regulated kinases (ERK1/2), reviewed elsewhere (Albuquerque et al., 2009). Downstream from the nicotine-stimulated kinases, a number of transcription factors are activated. Among these is the cAMP response element binding protein (CREB) (Nakayama et al., 2001).

Prenatal expression of nAChRs

The developmental and distribution pattern of the neuronal acetyl cholinergic receptor system in the growing brain of the fetus is heavily affected by exposure to nicotine from smoking mothers (Falk et al., 2002). The expression patterns of α4β2 and α7 nAChR subunits are altered in aborted fetuses of smoking women. Detection patterns vary in gradients during various trimesters. Expression of nAChR mRNA develops during the first trimester beginning in the caudal region of the CNS and further developing towards the rostral region (Hellstrom-Lindahl et al., 1998). During the end of first trimester, α4β2 nAChR subunits are expressed in the spinal cord and medulla. Henceforth, they become distinctively present in other parts of the brain such as cortex and cerebellum (Hellstrom-Lindahl et al., 1998). During the second trimester of pregnancy, the thalamus, hippocampus and basal ganglia begin to express this subunit (Agulhon et al., 1998). The pattern of expression is not the same for all nAChR subunits. For example, α7 nAChRs do not follow this trend. They appear first in the midbrain, and not in the spinal cord. Only at the end of first trimester, α7 nAChRs start expressing themselves in subcortical regions such as the forebrain and cortex (Falk et al., 2002; Hellstrom-Lindahl and Court, 2000; Hellstrom-Lindahl et al., 1998). α4 and β2 nAChR mRNAs are expressed as early as G11 in the spinal cord where they advance in a rostral fashion to reach the neocortex around G19 (Falk et al., 2002). Studies performed with radioligand binding assays exposed high-affinity binding sites appearing in a rostral to caudal fashion around G20 (Naeff et al., 1992). Around G13 and G15, α7 nAChR transcripts and binding sites are readily detected in cortical and thalamic neuro-epithelium (Broide et al., 1995). This is an example of early detection of these subunit specific protein transcripts in rodent brain. α3 and β4 transcripts rise through the mid-gestational period, but decline in the later period to reach normal levels at birth (Winzer-Serhan and Leslie, 1997). In

case of the α5 nAChR subunit, the incorporation occurs transiently in the midbrain region and frontal to neocortex in the middle part of gestational period of rodents around G13 to G15. However, the α5 subunit is minimally expressed in the neonatal brain after birth. In the late gestational period, they are found expressed in the hippocampus and cortex but not elsewhere in the brain (Zoli et al., 1995). Contrary to this, α6 and β3 nAChR subunit mRNAs, which are expressed robustly in catecholamine neurons in adult brains, are rarely expressed in the developing neonatal brain (Azam et al., 2007; O'Leary et al., 2008). This gives us a clear idea that the developmental patterns of different subunits of the nAChRs vary in different parts of the brain. There is also temporal variation, with specific subunits expressed in varying concentrations the fetus, giving way to the normal physiological receptor expression that we see in a newborn (Wang et al., 1996). Thus, it is very important to point out that any abnormal exposure to natural ligands, or other agonists, through a means such as maternal smoking can deleteriously affect the expression pattern and population of these subtypes of nAChRs. The fine balance between the subtypes, which is vital for normal brain function, cognitive growth and social behavior, can be altered as a result of such exposure. As mentioned previously, α7 nAChRs are believed to express very early in rat brain development. Gestation day 13 (G13) marks the first expression of α7 nAChR mRNA specifically in the cortical region, which is associated with memory formation. The expression levels peak during the first week of postnatal development in the rodent. This period is also related to acute changes in neurogenesis and synaptogenesis in the brain. Hence, this has direct correlations with memory formation and LTP. The β2 nAChR subtype follows a slightly different developmental plan. There is widespread expression of these receptors throughout the brain during early development. mRNA of the β2 nAChR subtype has been detected in the cerebral cortex, hippocampus, and spinal cord regions of the growing fetal brain as

early as the first trimester. Nicotine shows high affinity towards the β2 nAChR subtype. Nicotine exposure during this period of pregnancy is highly debilitating to these receptors. Overexposure to this particular ligand in the fetal brain due to maternal smoking desensitizes and downregulates expression of the nascent receptors, ultimately leading to cognitive deficits in newborns.

Prenatal functional roles of nAChRs

nAChRs are expressed early by the first trimester in many brain areas and consequently are present during critical phases of development. It is believed that nAChRs plays a fundamental role during these critical phases of brain development and the formation of neural and sensory circuits. The stimulation of α7 nAChRs by ACh promotes the retraction of neurites by Ca^{++} entry induction. Similarly, antagonism induces extension of neurites. α7 nAChRs also regulate developmental apoptosis. Recently it was reported that α7 nAChRs promote formation of glutamatergic synapses during development (Lozada et al., 2012).

The release of acetylcholine from developing motor neurons generates spontaneous bursts of activity that traverse the full length of the spinal cord. This cholinergic modulation is mediated via α4β2 nAChR activation and inhibitory glycinergic input. Similar bursts can be confined to local circuits through non-α4β2 nAChRs and GABA excitation. This coordinated mechanism is critical to the development of neuronal paths linking motor neurons to their appropriate targets. It is also vital for the initiation of sensory circuitry.

During the early prenatal period, α4 and β4 nAChR subunit mRNAs have correlating distributions in developing sensory structures. α3β4 nAChR channels are capable of generating burst firing patterns.

Effects of prenatal nicotine exposure

The early expression of nAChRs in the fetal brain suggests an important role for this receptor during development of the human nervous system (Candy et al., 1985). This receptor is believed to be involved in the regulation of neuronal growth, differentiation, and synapse formation during the development of the human brain.

Many studies have shown that acute nicotine treatment stimulates dopamine release in the foetal rat forebrain through activation of α4 nAChRs (Azam et al., 2007; Meyer et al., 2008). On the other hand, α7 controls glutamate release (McGehee and Role, 1995). The β2 nAChR was reported to modulate GABA release (Lu et al., 1998), and α3 and β4 nAChRs modulate noradrenaline release (Wonnacott, 1997). Chronic nicotine infusion alters the brain levels of dopamine, norepinephrine and their metabolites during the late prenatal period (Onal et al., 2004; Ribary and Lichtensteiger, 1989). In the early postnatal period, prenatal nicotine exposure elevates the levels of both dopamine and norepinephrine in the forebrain of rats. The neurotransmitter levels return to normal by adulthood (Ribary and Lichtensteiger, 1989). During prenatal development, nAChRs regulates catecholamine neurons. This is when dopaminergic neurons start to innervate their targets, and they are necessary for cholinergic regulation (Azam et al., 2007; Jung and Bennett, 1996). The expression of α4β2 nAChRs on dopaminergic terminals takes place during the prenatal period (Azam et al., 2007), and serves to control the timing of neurotransmitter release (Labarca et al., 2001). Mice lacking α4 or β2 nAChR subunits show maturational deficiencies, resulting in alteration of both presynaptic signaling and transmitter release (Parish et al., 2005). Many studies indicate that high levels of nAChRs are expressed in adrenergic neurons during development (Leslie et al., 2002; O'Leary et al., 2008), leading to further confirm the role of nAChR in regulating transmitter release in an embryo (O'Leary and

Leslie, 2006). Such findings indicate that nAChRs may also regulate the early development of these neuronal pathways which play significant roles in regulating arousal, maternal bonding, and autonomic function during the prenatal period (Nelson and Panksepp, 1998). Thus, it is expected that prenatal nicotine exposure affects these circuits in offspring.

Prenatal nicotine exposure has been shown to decrease the total number of cells in the brain during the fetal and early neonatal period, as well as increasing the expression of c-fos. This suggests a role of nAChRs in promoting apoptosis (Onal et al., 2004; Slotkin et al., 1987). The enzyme activity of ornithine decarboxylase was increased by prenatal nicotine exposure, indicating an abnormal switch from cellular proliferation to differentiation in the neonatal rat brain (Navarro et al., 1989; Slotkin et al., 1987). Prenatal nicotine exposure appears to have long-term significances in male rats. Prenatal nicotine exposure influenced the endocrine systems in males by increasing corticosterone levels during G18, and inhibits testosterone production during the prenatal hormonal surge (von Ziegler et al., 1991; Sarasin et al., 2003). Nicotine is also reported to affect neural sexual differentiation by reducing aromatase activity in the male foetal brain during development (Barbieri et al., 1986). In humans, the onset of puberty in males is influenced by prenatal nicotine exposure, suggesting that prenatal nicotine exposure results in endocrine dysfunction (Fried et al., 2001).

Behavioral studies of prenatal nicotine-treated rats reveal phenotypes consistent with alterations in the dopamine system, with changes in both motor function and reward. Prenatal nicotine exposure leads to changes in both spontaneous and ligand mediated motor responses (LeSage et al., 2006; Paz et al., 2007). In addition, levels of anxiety and depression are elevated, and memory is impaired in rats exposed to prenatal nicotine (Parameshwaran et al., 2012).

In order to cover the entire prenatal period, it is required to expose pregnant rodents with nicotine for 28 days. Hence, an osmotic mini-pump was used for this study to deliver nicotine to the pregnant dams. This method produces nearly constant blood levels of drug which decline gradually as pregnancy progresses and the dam gains weight. An initial infusion of 6 mg nicotine base/kg/day produces maternal blood levels analogous to that resulting from moderate to heavy smoking (Fewell et al., 2001; Murrin et al., 1987). This concentration is similar to that found in adults who smoke approximately 30 cigarettes per day. (Benowitz et al., 1982). In addition, this delivery method minimizes stress to the dam from repeated drug administration and prevents hypoxia which could result from spikes in blood nicotine concentration.

To summarize, there is strong evidence from published literature demonstrating extensive deleterious effects of prenatal nicotine exposure, consistent with an important developmental role played by nAChRs. There is also considerable overlap between the findings of animal studies and those of the clinical literature, indicating that nicotine may be an important neuroteratogen. Thus, the results of these animal studies provide an important conceptual background for understanding the mechanisms underlying clinical disorders in children born to mothers who smoke during pregnancy.

The hippocampus

The hippocampus located in the medial temporal lobe brain region is one of the most profoundly studied regions of the brain. The reason behind this observation is that scientists and researchers through decades of work have found this brain formation to be important in storage of early memory (Kerchner and Nicoll, 2008). Scientists have proven that damage to the hippocampus in human subjects results in anterograde amnesia. Another exciting characteristics of the

hippocampus is its comparatively simple cellular stratification and highly arranged leaf-like networking of its inputs, capable of quickly processing and storing a variety of unrelated events and facts (Neves et al., 2008).

The four sub-regions of the hippocampus are called the Cornus Ammonis or Horn of Ammonis (CA): CA4, CA3, CA2 and CA1. The main sub-regions with excitatory neurons in the hippocampus are as follows:

A .Dentate gyrus- Granule cells project to the nearby dendrites of CA3 pyramidal cells via axons. B. mossy fibers- The CA3 neurons in this region project on ipsilateral CA1 pyramidal cells passing within Schaffer Collateral pathways. The neurons in CA3 region further receives input the contralateral hippocampus, passed the commissural pathway from (Morris et al., 2006; Neves et al., 2008) (Figure 2). The hippocampus interacts functionally with the neocortex in order to transfer older memories overtime out of the hippocampus and into the cortex (Morris et al., 2006). Neocortical input enters the hippocampus via the perforant pathway from layer II of the entorhinal cortex to the dentate gyrus. CA3 pyramidal cells receive a direct input from layer II entorhinal cortex. Moreover, a direct input from layer III cells of the entorhinal cortex innervates the CA1 pyramidal neurons (Morris et al., 2006; Neves et al., 2008).

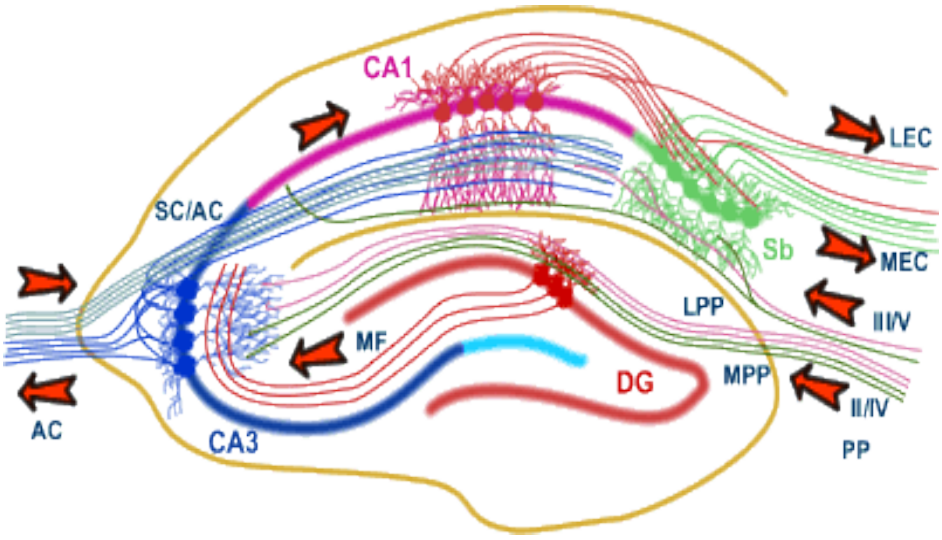

Figure 2.2: The hippocampal network: The hippocampus forms a principally uni-directional network, with input from the entorhinal cortex (EC) that forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the perforant pathway (PP - split into lateral and medial). CA3 neurons also receive input from the DG via the mossy fibres (MF). They send axons to CA1 pyramidal cells via the Schaffer collateral pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the associational commisural (AC) pathway. CA1 neurons also receive inputs direct from the perforant pathway and send axons to the subiculum (Sb). These neurons, in turn, send the main hippocampal output back to the EC, forming a loop. Adapted from MRC center for synaptic plasticity. Adapted from Department of Anatomy, School of Medical Sciences, University Walk Bristolhttp://www.bristol.ac.uk/synaptic/receptors/.

The glutamatergic system

The major excitatory neurotransmitter in the hippocampus, as well as the entire CNS, is glutamate. Glutamate passes through the synaptic cleft after being released from the presynaptic boutons of a neuron. It can activate both ligand-gated ion channels (ionotropic receptor) and G-protein coupled (metabotropic) receptors. The two major types of ionotropic glutamate receptors are NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (AMPARs). These receptors play important roles in synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) which represent the molecular and cellular mechanisms by which memories are generated (Citri and Malenka, 2008; Lynch, 2004; Malenka, 1994).

AMPA Receptors

Activation of AMPARs provides the majority of the inward current that causes the fast excitatory synaptic responses. The ionotropic glutamate receptors composed of four to five subunits, each containing four hydrophobic transmembrane regions within the central region of the amino acid sequence. The second transmembrane region forms a reentrant loop resulting in an extracellular N-terminus and an intracellular C-terminus, making them unique compared to the subunits of most ionotropic receptors. AMPARs are expressed as tetramers composed of four subunits: GluR1-4 (or GluRA-D) (Braithwaite et al., 2000; Citri and Malenka, 2008; Jiang et al., 2006). Kinetics of the ion channel itself and the localization of the receptors are directly influenced by the subunit composition. AMPA subunits GluR1, GluR3 and GluR4 are homomeric channels; they are calcium permeable and inwardly rectifying. In contrast, homomeric GluR2 channels are impermeable to calcium and have outwardly rectifying properties because they undergo RNA editing such that the arginine (R) codon replaces the glutamine (Q) codon on residue 607 (Burnashev et al., 1992b; Hollmann et al., 1991). Alternate splicing of the second extracellular region is another structural basis of AMPAR properties and is designated as “flip” and “flop” (Sommer et al., 1990). In GluR2 and GluR3, an arginine codon can be replaced by a glycine codon to produce “flip” and “flop” isoforms of AMPARs which possess differing desensitization and resensitization properties (Figure.3.2) (Braithwaite et al., 2000; Jiang et al., 2006). In addition, it has been suggested that the synaptic delivery of AMPARs is also governed by subunit-specific rules (Citri and Malenka, 2008; Derkach et al., 2007). GluR1-containing AMPARs are slowly incorporated into synapses under basal conditions. However, the insertion of GluR1-containing AMPAR subunits into the postsynaptic density is strongly stimulated by NMDAR activation. The incorporation of GluR2/3 heteromeric AMPARs occurs constitutively

on a rapid time scale (Braithwaite et al., 2007; Citri and Malenka, 2008). In addition, it has since been found that AMPARs associate with a four-pass transmembrane protein termed stargazin that in turn directly interacts with postsynaptic density-95 (PSD-95) via a C-terminal PDZ-binding domain (Bats et al., 2007; Chen et al., 2000; Schnell et al., 2002). This transmembrane AMPAR regulatory protein (TARP) plays a crucial role in the trafficking of AMPARs to the surface of the postsynaptic membrane (Hashimoto et al., 1999; Vandenberghe et al., 2005). Moreover, recent studies reported that stargazin influences the functional properties of AMPARs by slowing the rate of AMPAR deactivation and desensitization (Milstein et al., 2007; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). AMPARs are phosphorylated by calcium-calmodulin-dependent kinase II (CaMKII), protein kinase C (PKC) and protein kinase A (PKA) at several sites along the C-terminal domain (Roche et al., 1996). Phosphorylation on the PKC/CaMKII site (serine 831), results in an increase in their single-channel conductance, whereas phosphorylation on the PKA-sensitive site (serine 845) increases the single-channel open probability of GluR1-containing AMPARs (Boehm and Malinow, 2005; Wang et al., 2005).

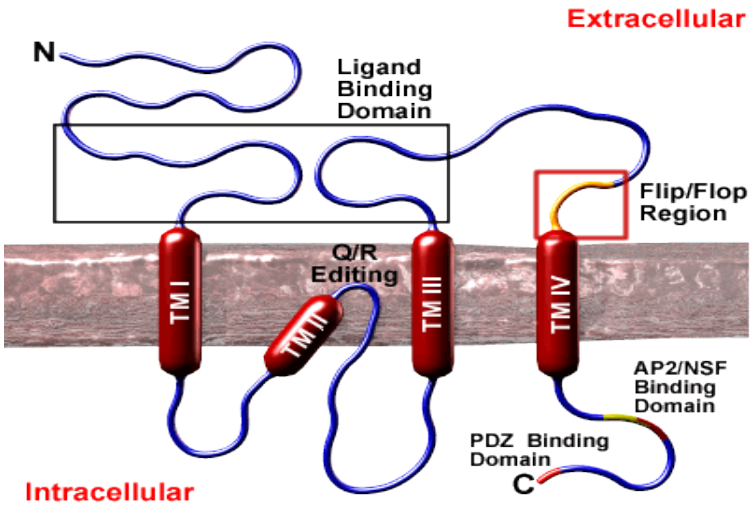

Figure.3.2: Structure of the AMPA. The subunit is used to illustrate the structural properties of the AMPA receptor subunits. The N-terminus is extracellular and C-terminus is intracellular. Splice variation occurs in the 'Flip/Flop' region, giving two variants for each gene sequence. The C-terminus contains binding regions for AP2, NSF and PDZ

proteins such as PICK1 and GRIP. Department of Anatomy, School of Medical Sciences, University Walk Bristol http://www.bristol.ac.uk/synaptic/receptors/.

NMDA Receptors

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated voltage-dependent glutamate receptors. In contrast to AMPARs, NMDARs require both presynaptic release of glutamate and postsynaptic depolarization in order to produce a postsynaptic response. At negative membrane potentials, NMDAR channels are blocked by extracellular magnesium. However, magnesium dissociates from its binding site on the NMDAR channels upon depolarization of the cell, resulting in the influx of Na^{+}, K^{+} and Ca^{2+} (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008). NMDARs are formed from heteromeric assemblies of NR1 (GluN1, with 8 different splice variants), NR2 (GluN2A, GluN2B, GluN2C and GluN2D) and NR3 (GluN3A and GluN3B) subunits (Figure 4.2) (Cull-Candy and Leszkiewicz, 2004; Rebola et al., 2010). NR1 is the essential subunit in heteromeric complexes (Forrest et al., 1994; Rebola et al., 2010) and contains the binding domain for the co-agonist glycine (Banke and Traynelis, 2003; Burnashev et al., 1992a; Monyer et al., 1992). NR2A and NR2B NMDARs subunits form either di-heteromers (NR1/NR2A or NR1/NR2B) or tri-heteromers (NR1/NR2A/NR2B). The structural and pharmacological properties of NR2 subunits differ and are important determinants of NMDAR function (Lau and Zukin, 2007; Rebola et al., 2010).

The NR2A subunits are deactivated more quickly but have greater channel open probability than the NR2B subunits (Lau and Zukin, 2007; Yashiro and Philpot, 2008). However, the low open probability of NR2B subunits is compensated by the slow deactivation of NR2B subunits; as a result, more charge and more calcium per unit current are carried by NR2B subunits (Yashiro and Philpot, 2008).

The findings of several reports suggest that mutation-induced truncation of the PDZ binding site where NR2B-containing receptors interact with SAP-102 and PSD-95 subunits causing complete loss of NR2B receptors in the synaptic pool (Barria and Malinow, 2002; Mohrmann et al., 2002; Prybylowski et al., 2005).

NR2A and NR2B subunits possess PDZ-binding motifs in their C-terminal domain by which they can interact with a family of synaptic scaffolding proteins known as membrane-associated guanylate kinase (MAGUK). NMDA receptors interact with PDZ-binding domain proteins, whose synaptic localization is regulated by PSD-95 (Li et al., 2003; Lim et al., 2003). NR2A-containing receptors were predominately present in the synaptic pool. Whereas, NR2B was primarily located in the extrasynaptic pool (Carmignoto and Vicini, 1992; Kew et al., 1998; Khazipov et al., 1995; Liu et al., 2004; Shi et al., 1997; Tovar and Westbrook, 1999). The reason behind this was assumed to be that NR2A preferentially binds to PSD-95 while NR2B bind to SAP102 (Barria and Malinow, 2002; Mohrmann et al., 2002; Prybylowski et al., 2005). However, a study found that PSD-95 and SAP-102 interact with NR2A and NR2B subunits at comparable level (Yashiro and Philpot, 2008).

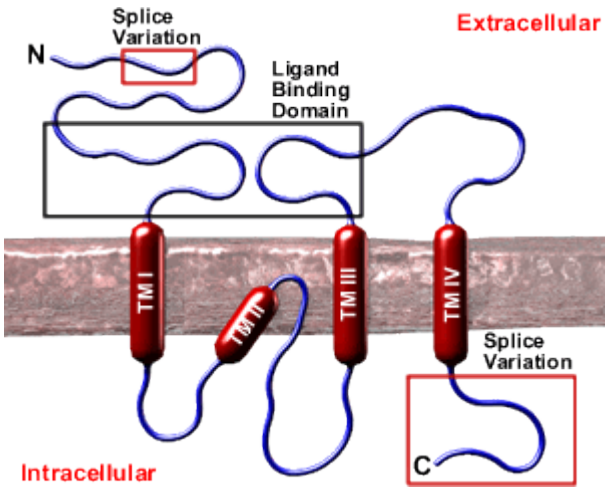

Figure 4.2: Structure of the NMDA receptor subunit, showing regions of splice variations. Adapted from Department of Anatomy, School of Medical Sciences, University Walk Bristol http://www.bristol.ac.uk/synaptic/receptors/.

Long-term potentiation

In 1949, Donald Hebb hypothesized that the process of information storage or memory requires organization and activity of neurons (Hebb, 1949). Bliss and Lomo in 1973 conducted the first experiment that proved the Hebbian model term (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). They reported strengthening of synaptic connections lasting for hours or even days caused by repetitive activation of excitatory synapses; this phenomenon was termed 'long-term potentiation' (LTP). LTP is a particular form of synaptic plasticity, activity-dependent changes in synaptic efficacy that is an attractive cellular mechanism for learning and memory (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008).

Like memories, LTP is composed of two phases: induction or early LTP (E-LTP) and maintenance or late LTP (L-LTP), corresponding to short-term and long-term memories. The increase in postsynaptic calcium, following postsynaptic depolarization and NMDAR activation, results in induction of NMDA-dependent LTP in the CA1 region of the hippocampus. Calcium-calmodulin-dependent protein kinase II (CaMKII) is activated by the increased Ca^{2+} concentration

in the dendritic spine (Malenka, 1994). Other signaling molecules are also implicated in triggering LTP, including cAMP-dependent protein kinase (PKA) (Esteban et al., 2003), protein kinase C (PKC) (Boehm and Malinow, 2005), extracellular signal-regulated kinase 1/2 (ERK 1/2) in the mitogen-activated protein kinase (MAPK) cascade (Blum et al., 1999), phosphatidylinositol 3-kinase (PI3-kinase) (Opazo et al., 2003) and the tyrosine kinase Src (Hayashi and Huganir, 2004). The expression of LTP at hippocampal CA1 synapses is mediated by an activity-dependent increase in the number of AMPARs within the postsynaptic density (Bredt and Nicoll, 2003; Derkach et al., 2007; Malinow and Malenka, 2002; Song and Huganir, 2002). During LTP, AMPARs are exocytosed at extrasynaptic sites, and then AMPARs laterally diffuse in the plasma membrane and are inserted within the postsynaptic density (Derkach et al., 2007; Citri and Malenka, 2008). Extrasynaptic trafficking of AMPARs is mediated by protein kinase A (PKA)-induced phosphorylation of ser 845 in the GluR1 AMPAR subunit. The subsequent insertion of GluR1-containing AMPARs into the synaptic sites during LTP requires phosphorylation of ser 818 in GluR1 AMPARs subunits by protein kinase C (PKC), a process mediated by NMDARs/calcium dependent mechanisms (Citri and Malenka, 2008; Derkach et al., 2007; Jiang et al., 2006; Wang et al., 2005). The maintenance phase of LTP is also termed as the protein synthesis-dependent phase. During the maintenance of LTP, a variety of proteins are synthesized, including AMPAR subunits, transcriptional factors and cytoskeletal components involved in morphological and cytoskeletal reorganization of dendritic spines (Fukazawa et al., 2003; Matus, 2000).

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3-Prenatal nicotine exposure and mechanism of memory deficits in offspring

Abstract

Maternal smoking during pregnancy has been reported as a strong risk factor for neurobehavioral alteration in offspring. A rodent model of prenatal nicotine exposure (PREN), where nicotine is infused (6 mg/kg/day) via mini-pumps to pregnant dams, resulted in cognitive deficits. Here, we demonstrate that basal synaptic transmission and long-term potentiation (LTP) are decreased in the Schaffer collateral-CA1 synapses of PREN animals. Moreover, the amplitude and frequency of AMPAR-mediated spontaneous excitatory postsynaptic currents (sEPSCs) recorded from PREN hippocampal slices were significantly reduced. The deficits in LTP and basal synaptic transmission were accompanied by alterations in the function and expression of synaptic α7/β2 nicotinic acetylcholine receptors (nAChRs). In addition, co-immunoprecipitation studies in rat hippocampi revealed that α7/β2 nAChRs complex with vesicular glutamate transporter (VGLUT), postsynaptic density protein-95 (PSD-95) and synapse-associated protein (SAP102). This interaction was disrupted in PREN rats. Interestingly, the nicotinic receptor signaling CAMKII-ERK-CREB pathway is altered in PREN. Also, we found α7 but not β2 nAChRs co-immunoprecipitated with ERK1/2. The blockade of α7 nAChRs with 100 nM methyllycaconitine (MLA) in control animals impaired LTP. Furthermore, Western blots studies revealed that expression of PSD-95 and SAP102, which are involved in regulating the organization of pre and postsynaptic components of nicotinic and glutamatergic synapses was significantly decreased in PREN rodent hippocampi. Taken together, our study suggests that alterations in nAChRs expression and function lead to modified glutamatergic transmission in the hippocampus resulting in memory deficits in PREN rodents.

1. Introduction

The use of tobacco during pregnancy exposes the developing fetal brain to the psychoactive compound nicotine. Accumulating evidence has demonstrated the deleterious effects of nicotine in the offspring including cognitive behavioral alterations. Recent studies from our laboratory have demonstrated that prenatal nicotine exposure results in increased anxiety, depression and cognitive deficits in rodent offspring (Parameshwaran et al., 2012).

Nicotine is a natural agonist of nicotinic acetylcholine receptors (nAChRs), which play a crucial role during development and plasticity. Several lines of evidence have pointed to a vital role of brain nAChRs in cognitive functions; however, the underlying mechanism is not fully understood. In the hippocampus, excitatory neurotransmission is mainly mediated by glutamate receptors. However, hippocampal glutamatergic transmission itself is modulated by other neurotransmitter systems. Specifically, presynaptic nAChRs have been shown to facilitate release of glutamate. Indeed, a presynaptic α7 and β2 nAChR-mediated increase in glutamate release in hippocampal CA1 synapses is known to contribute to synaptic plasticity (Ge and Dani, 2005; Ji et al., 2001). Activation of nAChRs promotes synaptic connections and contacts in the hippocampus during critical periods of development (Maggi et al., 2003); cholinergic innervation commences prenatally in the mammalian brain (Berger-Sweeney and Hohmann, 1997). Prenatal nicotine exposure (PREN) would interrupt the normal developmental onset of the nicotinic cholinergic system and its regulation of other neurotransmitter systems. In support, studies have reported chronic administration of nicotine during development results in changes in nicotine binding in the brain (Narayanan et al., 2002; Schwartz and Kellar, 1983; van de Kamp and Collins, 1994). Apart from these changes, prenatal nicotine is believed to cause neuronal death and morphological changes in specific brain regions including the hippocampus (Roy et al., 1998;

Roy and Sabherwal, 1994; Roy and Sabherwal, 1998; Roy et al., 2002), which plays a critical role in learning and memory. Our work has provided valuable insights into the mechanistic basis of cognitive decline in cases of prenatal nicotine exposure. Our laboratory has previously shown that PREN exposure leads to decreased AMPA receptor-mediated synaptic transmission in the CA1 pyramidal neurons of the hippocampus (Vaglenova et al., 2008). Moreover, long-term potentiation (LTP), a well-established model of synaptic plasticity, decreased with concomitant deficits in basal synaptic transmission (Parameshwaran et al., 2011). In contrast, nicotine administration in adult rodents has been shown to promote LTP and memory. Therefore, nicotine exposure during development has a long-lasting effect on excitatory synaptic transmission and synaptic plasticity in the hippocampus. Specific molecular mechanisms by which nicotine exerts its developmental effects warrants further investigation in order to develop potential therapeutic interventions for cognitive deficits. Epidemiological studies reveal about 25% of pregnant mothers are smokers in the United Stated, leading to thousands of babies born annually to smoking mothers. Currently, there are no therapies available to treat the deficits caused by PREN exposure.

Desensitization is an important characteristic of nAChRs; acute exposure to nicotine may activate a normal cholinergic response, but chronic exposure may inhibit the normal biological response of ACh through desensitization. In general, nAChRs are slow to desensitize in the presence of concentrations of agonist much lower than that required for activation (Fenster et al., 1999). Thus, desensitization of brain nicotinic receptors can occur as a result of prolonged nicotine exposure during smoking (Brody et al., 2006). Consistent with this, we hypothesize that ***alterations in nAChRs expression and function lead to modified glutamatergic transmission in the hippocampus resulting in memory deficits in PREN rodents.***

2. Materials and methods

Animals and chemicals: Time-pregnant Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). On approximately third day of pregnancy, osmotic minipumps (Alzet, Model 2004, Cupertino, CA) were implanted aided by isoflurane anesthesia underneath the skin to deliver subcutaneous dose of nicotine at a rate of 6 mg/kg/day. Pumps were removed after the pups were delivered in order to restrict postnatal exposure. Animals were housed in a temperature-controlled room with access to food and water *ad libatum*. Animal care and all experimental procedures were approved by the Institutional Animal Care Committee of Auburn University (IACUC protocol no.1761). All chemicals were purchased from Tocris (Ellisville, MO), 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL), - (-) nicotine base free from Sigma (St. Louis, MO).

Preparation of hippocampal slices and synaptosomes: Transverse hippocampal slices (400 μm) were prepared as described earlier (Parameshwaran et al., 2007) with some modifications. briefly, hippocampal slices were cross-sectioned while in ice cold cutting buffer containing (in mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5$CaCl_2$, 1.25NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 0.5 ascorbate, and 2 kynurenic acid. The solution was bubbled with 95%CO_2/5%O_2, and the pH was made upto to 7.4. Slices were incubated for one hour in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5$CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$ and 11 dextrose. The solution was bubbled with 95%CO_2/5%O_2. Synaptosomes were prepared as previously described (Johnson et al., 1997; Suppiramaniam et al., 2006), in which the hippocampi were taken out and homogenized in homogenization buffer (modified KRBS) using 10 strokes in a Potter homogenizer. The m-Krebs buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose and 10 mg/ml

adenosine deaminase. The homogenate was filtered through a 13 mm diameter Millipore syringe filter holder attached to a 1 cc tuberculin syringe, followed by filtration through three layers of nylon filters (Tetko, 100 μm pore size) and finally collected in a 1.5 ml Eppendorf tube. Earlier, pH was adjusted to 7.4 by bubbling with 95%O_2/5%$CO_2$and was supplemented with 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM benzamide to minimize proteolysis. The filtrate was then loaded into another 1 cc tuberculin syringe and forced through a pre-wetted 5 μm Millipore nitrocellulose filter, then centrifuged at 1000 x g for 15 min in a microcentrifuge at 4°C. The supernatant was removed, and the synaptosome-containing pellet was resuspended in 20 μl of mKRBS buffer.

Slice electrophysiology: Brain slices are incubated for two hours; electrophysiological recordings were performed in recordings chambers continuous perfused in ACSF bubbled with 95%CO_2/5%O_2. Field excitatory postsynaptic potentials (fEPSP) from Schaffer collateral/commissural CA1 synapses were obtained by stimulating CA1 stratum radiatum with bipolar electrodes and the recording glass electrode was filled with ACSF (2-4 MΩ), and placed approximately 200 μm from the stimulating electrode. The frequency of the test stimulation was every 20 s. For stimulus response curves, current intensity was changed from 0 to 200 μA in steps of 25 μA. For paired pulse ratio (PPR) and LTP experiments, current intensity was set at 50% of maximal fEPSP and interpulse intervals were set to 25, 50, 75, 100, 150 and 200 ms in PPR experiments. In LTP experiments, after 15 min of stable baseline recording, theta burst stimulation were induced. The TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ, with an interburst interval of 200 msec. LTP was measured 60 min post TBS.

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% O_2/5% CO_2at a rate of

2 ml/min. Individual hippocampal slices were visualized by Olympus BX51WI microscope (Olympus,USA) . Recordings from CA1 pyramidal neurons were made with patch pipettes (5–7 MΩ), 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. The pH was adjusted 7.3 using KOH and the osmolarity was set to 280–290 m-Osmoles. To conserve G-protein-mediated responses, GTP was added to the pipette solution and ATP was included to supply energy for other intracellular phosphorylation reactions and to prevent rundown of calcium channels. Cs^+ was added to eliminate K^+ currents. We obtained tight seal on pipettes by applying negative pressure (≥ 2 GΩ before breaking into whole-cell mode). Using the whole-cell voltage-clamp technique, AMPA receptor-mediated quantal events (sEPSCs) were isolated from CA1 pyramidal neurons in the presence of 50 μM APV and 30 μM bicuculline methiodide (BMI). An 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 a Digidata 1200 digitizer (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 a student *t*-test. Results are presented as mean ± SEM.

Single-channel electrophysiology: Incorporation of nAChRs from synaptosomal fractions in artificial lipid bilayers was carried out using 'tip-dip' method. In brief, a phospholipid bilayer was formed at the tip of a polished glass pipette (100 MΩ). The artificial phospholipids were prepared by dissolving 1,2-diphytanoyl-sn-glycero-3-phosphocholine in hexane (Aldrich Chemical Co., Milwaukee, WI) to get a concentration of 1 mg/ml. Approximately 3-5 μl of synthetic phospholipids were delivered into 300 μl of bath solution containing 125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, and 5 mM Tris HCl. The pipette solution consisted of 110 mM KCl, 4 mM NaCl, 2 mM NaHCO3, 1 mM MgCl2, 0.1 mM CaCl2, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS) (pH adjusted to 7.4). Bilayer formation was initiated by consecutive transfer of monolayers onto the tip of pipette in an asymmetric saline condition with an 'outside-out' fashion. After forming a stable membrane, a 3-5 μl suspension of the synaptosomes was delivered to the ECF. After addition of low concentration of nicotine (500 nM) and/or PNU-120596(1 μM), voltage was applied to evoke single-channel activity. Single-channel currents were digitized at 2 kHz and digitized at 5 kHz (Mini-digi, Molecular Devices) with pClamp 9 software (Molecular Devices) and saved to a computer hard disk. Only the data exhibiting long stretches of single-channel current transition without base line drifts was chosen for quantitative analysis. The current amplitude histograms were constructed. The single-channel open probability was calculated from the area under the current-amplitude histogram. Log-transformed dwell time count histograms were constructed and fitted with variable metric fitting method to identify distinct open and close times.

Western blot analysis: Hippocampal cell lysates were separated by 10% SDS-PAGE and blotted to PVDF membranes (Immobilon-P; Millipore). Membranes were blocked with 5% non-fat dry milk in TBS (0.01% Tween 20) for 1 h and then incubated with anti-α7 nAChR (1:1000; Cell

Signaling Technology), anti-β2nACh (1:1000; Cell Signaling Technology), p-CAMKII, p-CREB, pERK1/2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), PSD-95, synaptophysin, SAP102 and anti-β-actin (1:1000; Cell Signaling Technology) antibodies overnight at 4 °C. Membranes were then probed with corresponding anti-rabbit (1:5000; Cell Signaling Technology) or anti-mouse (1:5000; Cell Signaling Technology) horseradish peroxidase-conjugated second antibodies for 1 h. It was then developed using enhanced chemiluminescence (Super Signal West Femto ECL reagent, Pierce Biochem). All immunoreactive bands were subsequently quantified by densitometric analyses using Quantity 1 analysis software (Bio-Rad). The densities of each band representing individual animals were normalized to β-actin and then compared with control levels for both control and treated groups. Data is represented as mean ± SEM. Significance was determined using a two-tailed Student's *t*-test.

Co-immunoprecipitation studies: Tissues were homogenized as described previously (Shonsey et al., 2010) 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 *g* and 4 °C to remove cellular debris, and the protein content of the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL). α7nAChRs and β2nAChRs were immunoprecipitated using anti-α7 nAChR and anti-β2 nAChR antibodies or mouse IgG (Invitrogen)as a negative control, preconjugated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described above. To examine interactions between nAchRs and differently proteins in the hippocampi homogenates, Western blots were then performed using the following antibodies: anti-VGLUT (1:1000; Millipore), anti-ERK1/2 (1:1000; Cell Signaling Technology), anti-SAP102 (Cell Signaling Technology 1:1000), anti-PSD-95 (1:1000; Millipore) and anti-PSD-95 (Cell Signaling Technology 1:1000). The blots were stripped and reprobed with anti-α7and anti-

β2 antibodies (Cell Signaling Technology 1:1000). To further examine these interactions,VGLUT, ERK1/2, SAP102 and PSD-95 proteins were immunoprecipitated using anti-VGLUT, anti-ERK1/2, anti-SAP102 and anti-PSD-95 antibodies or mouse-IgG (Invitrogen) as a 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 anti-α7and anti-β2 antibodies.

Drug application and AMPARs sEPSC recording parameters: sEPSCs were recorded continuously, without utilizing a sodium channel blocker, during and immediately following drug applications so that the one-minute peak period of sEPSCs could be detected. Nicotine- and PNU120596-induced sEPSCs started within 5–10 s of application and reached a peak between 30 and 60 s, depending on the drug used. To avoid desensitization, nicotine was applied for no more than 45 s at a time. If nicotine applications were to be repeated, this desensitized compound was applied at a low concentration at short period of time for no more than 45 s followed by a prolonged washout time with ACSF (minimum 30 min) determined to allow a reproducible response. Once the peak period was noted for a specific flow rate, the same measurement period was used for all neuronal cells. For analysis of sEPSC kinetics, time constants of decay were estimated by single exponential curve fitting. Drugs were applied in the fast-flowing bath. Picrotoxin (0.5 μM) was applied for 5 min to block action-potential-dependent transmitter release before applying nicotine (5μM), PNU120596(1μM) and MLA (100 nM).

Electrochemical detection of hippocampal glutamate and gamma-aminobutyric acid (GABA) levels: Animals were sacrificed by cervical dislocation in the morning before noon in order to prevent any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, the hippocampi were dissected out from the

treated coronal slices, rinsed in ice-cold normal saline, and blotted dry on ash-free filter paper. The hippocampal sections were weighed and homogenized by sonication in 1ml of chilled homogenization buffer (0.1M citric acid, 0.1M sodium dihydrogen phosphate monohydrate, 5.6mM octane sulfonic acid, 10 µm EDTA in 10% (v/v) methanol solution, pH 2.8 with 4M NAOH). After homogenization, samples were centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant stored at −80 °C until final HPLC analysis.

*Measurement of glutamate and GABA:*Amino acids were estimated by employing HPLC-electrochemistry detection as described by Clarke et al. (Clarke et al., 2007). Briefly, the method utilizes the chemical properties of primary amines to react with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide to produce 1-cyanobenz-isoindole (CBI) derivatives that can be detected by either electrochemical or fluorescent detectors. After derivitization of both standards and tissue samples, 20 uL of injection of each was injected in the HPLC system.

The derivitization of GABA and glutamate was done according to the method published by Clark et al. (2007) with some modifications. Briefly, 10µl of either standard mix or sample supernatant, 90 µl of borate buffer (0.1 M, pH 9.5), 10 µl of potassium cyanide (10 mM) and 10 µl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. 20 µl of the derivative was injected into the appropriate HPLC system. The composition of the mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). The mobile phase was filtered through 0.45 µm filters and vacuum degassed prior to use. The samples were

eluted isocratically over a 30 min runtime at a flow rate of 0.65 ml/min after a 20µl injection. A standard solution containing glutamate and GABA was run immediately prior to, and following, sample injections. Results are presented as pmol/mg tissue.

Statistical analysis: Statistics was performed using the Prism-V software and results were expressed as mean ± SE. Column statistics were run along with the Kolmogorov–Smirnov test to check for the normal distribution of the data. Experimental data that pass the normal distribution test was subjected to one-way ANOVA, followed by Tukey test for the comparison between different treatment groups. Failing the normality test, a non-parametric Kruskal-Wallis test was performed on the experimental data set, which was followed by Dunn's multiple comparison post-hoc analysis to test for statistical significance among different treatment groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1 Prenatal nicotine exposure alters the nicotinic receptors expression

We examined the effect of prenatal nicotine exposure on protein levels of nicotinic receptors using Western blot analysis. A reduction in nicotinic receptor expressions was found in prenatal hippocampal slices exposed to nicotine (6 mg/Kg) as compared to an equivalent volume of the vehicle. As shown in (Fig.1 (102.0 ± 1.518, for controls and 58.11 ± 4.545, 75.46 ± 2.804 for α7 and β2-nAChRs respectively in PREN, *** $p< 0.0001$, ** $p< 0.001$) suggesting ~40% decreased expression of α7 and ~25% reduction in β2-nAChRs in PREN rat hippocampi (Fig. 1).

3.2 Prenatal nicotine exposure results in diminished LTP and basal synaptic transmission in rodent hippocampus

3.2.1 Failure to rescue basal synaptic transmission and LTP in PREN acute hippocampal slices after nicotinic receptor modulation:

3.2.1.1 LTP

Long-term potentiation (LTP) of hippocampal synaptic efficacy has been considered as a cellular correlate of synaptic model of learning and memory. We studied LTP in SC synapses in saline- and nicotine-exposed rodent hippocampal acute slices by inducing LTP with theta-burst stimulation (TBS). The TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ, with an interburst interval of 200 msec. TBS has been found to be the most effective and the most physiologically relevant method as it induces the normal discharge characteristic of

hippocampal neurons (Albensi et al., 2007). A one-way repeated-measures ANOVA showed that LTP was significantly impaired in six of six slices that were prenatal nicotine-exposed (Fig. 3 A and B, ; $p< 0.001$; n= 6 slices from 6 animals) with an average of 104.5% ± 1.197%, compared with control slices (prenatal saline exposed) in which LTP was induced and stably maintained in six of six slices with an average of 147.24% ± 2.63% in saline-control slices. LTP increased with the addition of 5μM nicotine in saline-exposed slices to an average of 161.8% ± 4.437%, and further increased with the addition of 1 μM PNU120596, a α7 agonist, in the presence of nicotine to an average of 197.34% ± 6.643%. The increase was less prevalent in the presence of 5-iodo-A, a β2 agonist (average of 177.64% ± 5.388%). These increases were further diminished by adding MLA (100nM) to an average of 118.6% ± 1.583%. These findings indicate that activation of α7 nicotinic acetylcholine receptors is crucial to persistently enhance hippocampal synaptic transmission. LTP impairment among PREN groups was irreversible, as fEPSPs slope was not recovered to a potentiated level after nicotine and/or PNU120596 addition (5 minutes before TBS delivery) as compared to saline-control slices (Fig. 3 B; 125.2% ± 2.368%; 120.31% ± 2.373% respectively n= 6 slices from 6 animals). These data suggested that prenatal nicotine exposure is associated with impaired synaptic plasticity due to alterations in nicotinic receptors.

3.2.1.2 Input/output curves

To test the deficits in synaptic function in prenatal nicotine exposed rodents, we studied the input-output relationship of Schaffer collateral-CA1 synapses in response to single electrical stimuli. After placement of stimulating and recording electrodes in the CA1 region of a stratum radiatum hippocampal slice, the stimulus strength was reduced until no field

excitatory post-synaptic potential (fEPSP) was induced. The stimulus was then increased in increments, with six responses collected and averaged at each increment. Data were averaged across all rodents in each group (saline- and nicotine-exposed) to construct input-output curves (Fig. 4A). There were no significant differences among PREN group hippocampal slices compared to saline-exposed groups in this measure. However, there were significant differences observed among the saline exposed rodents ($P< 0.05$). The reduction observed in input-output curves for PREN fEPSPs could result from a number of possible reasons, such as differences in synaptic density of nicotinic receptors or altered strength of individual synapses within the population. A lack of difference among PREN group slices indicates that PREN have reduced densities of nicotinic receptors in the synapses within the stratum radiatum of CA1 and that, as a population, these synapses are equally functional in response to single stimuli. Thus, there appears to be no differences in synaptic organization or baseline function among the PREN groups.

To investigate the mechanisms underlying the excitatory effect and the strength of synaptic transmission seen in the saline-exposed rodents, we measured the size of the presynaptic fiber volley, a small deflection of the field response that precedes the postsynaptic potential and correlates with the number of presynaptic afferents activated by the stimulation pulse at different input current intensities. Figure. 4B shows that perfusion of nicotine (5 μM) in saline exposed rats resulted in an increase in the EPSP slope of the presynaptic fiber volley across different stimulation intensities compared with saline-control (n = 6 slices, $p < 0.001$; Fig.4B). Application of 1 μM PNU120596 in the presence of nicotine further enhances the presynaptic fiber volley slope (n = 6 slices, $p < 0.001$, Fig.4B). Application of 100 nM MLA reduced the presynaptic fiber volley slope, indicating that the conversion of the presynaptic

stimulus into axonal depolarization was affected by nicotine and/or PNU120596 treatment. No changes were shown in the EPSP slope of the presynaptic fiber volley from the hippocampi of prenatally nicotine-exposed group in presence of nicotine and/or PNU120596 ($p > 0.05$), suggesting that the numbers of active afferent axons are reduced by nicotine exposure during prenatal development (Figure 4B).

3.2.1.3 Paired pulse facilitation (PPF)

Changes in the ratio of amplitudes of first and second potentials are generally accepted as representing a modification to the presynaptic component of the synapse (Chen et al., 1996; Commins et al., 1998; Gottschalk et al., 1998). To investigate whether the decrease in fEPSP slope in PREN slices was due to altered probability of glutamate release, we measured PPF across a range of inter-stimulus intervals. PPF is a presynaptic, short-lasting form of synaptic plasticity where two stimuli are delivered to synapses in rapid succession, resulting in augmentation in synaptic response to the second stimulus relative to the first stimulus. This phenomenon is likely mediated by additional neurotransmitter release during the second stimulation caused by residual calcium left over after the first action potential (Wu and Saggau, 1994; Zucker and Regehr, 2002). The probability of release is inversely related to paired-pulse ratio, if PREN decreases EPSC amplitude through presynaptic alteration of nicotinic receptors expression, then an increased paired-pulse ratio is expected in the PREN slices. Alteration of EPSC amplitude in prenatal nicotine-exposed slices was accompanied by an increased paired-pulse ratio (EPSC2/EPSC1 from 1.01±0.05 to 1.25±0.20; n=6; p=0.019; paired t-test) compared to saline-exposed slices (Fig.4C), suggesting that PREN reduced probability of glutamate release. In saline-exposed slices, paired-pulse facilitation of fEPSCs

monitored in CA1 pyramidal neurons was significantly depressed after treatment with nicotine and/or PNU120596, 5-iodo-A, indicating that the nicotinic modulator effect on synaptic transmission is actually due to an increase in glutamate release from presynaptic terminals. The fEPSP slope 2/slope 1 ratio observed in control-PREN slices did not differ significantly from the PREN-nicotine treated slices at any inter-stimulus interval (ISI) examined (Fig. 4.C; $p > 0.05$; n= 6 slices from 6 animals), indicating that PREN altered the presynaptic release due to alteration of presynaptic nicotinic receptors. As PNU120596 induced higher enhancement of EPSC amplitude than 5-iodo-A, a β2-nACh agonist, in saline-exposed control slices are associated with more decrease in the paired-pulse ratio (EPSC2/EPSC1 from 1.03±0.05 to 0.91±0.05; n=6;; p=0.03; paired t-test; Fig.4C). Altogether, these data suggest that α7 subunits are the main components of nAChRs located on glutamatergic presynaptic neuron in hippocampal Schaffer collateral-CA1 synapses. In support, it was reported that presynaptic α7 nACh receptors on the glutamatergic terminals are involved in excitatory synaptic transmission through glutamate release (Gray et al., 1996; Wonnacott, 1997; Wonnacott et al., 2006). These observations raise the possibility that altered expression and function of nAChRs may have contributed to the observed deficits in glutamatergic synaptic transmission and plasticity.

3.3 Impairments in AMPAR-sEPSCs in prenatal nicotine exposed rodents

Most of the excitatory neurotransmission in the hippocampus is mediated by AMPARs. As per our previous report, the basic properties of AMPAR-mediated EPSCs of the CA1 pyramidal neurons of the hippocampus are altered in PREN (Parameshwaran et al., 2012). It was reported that activation of nAChRs regulates the downstream turnover of the AMPAR GluR1 subunit (Rezvani et al., 2007). Therefore, in order to test whether alterations in AMPAR-mediated

currents contribute to the LTP deficits as a result of decreased synaptic α7-nAChRs expression and/or function contributed to the postsynaptic deficits observed in PREN rodents, we recorded sEPSCs that occur without inhibition of the Na^+ channels. The AMPA-mediated sEPSCs were recorded in the presence of NMDAR blocker (APV 50 µm) and GABA blocker (picrotoxin 0.5µM). We analyzed the action potential-independent, spontaneous neurotransmitter release-elicited AMPA receptor-mediated sEPSCs in hippocampal Schaffer collateral-CA1 synapses. Nicotinic receptor stimulation, either with nicotine alone or with PNU120596 in saline-exposed control animals, was found to induce a characteristic increase in AMPA receptor-mediated sEPSCs, as shown in Figure 5A. Spontaneous EPSC frequency and current amplitude was significantly increased by nicotine (5 µM) (5.2 ±0.439, 54.587± 2.9 pA), with further larger increases in the presence of PNU120596 (1 µM) (7.17167±0.25, 78.548±4.29 pA) and was reduced by MLA (100 nM) (1.60±0.594, 17.278±2.66) compared with saline-control (4.697±0.2075, 48.268±1.4 pA) respectively. The decay phase of the sEPSCs was best fitted with two time constants and the decay times were enhanced in saline-control slices perfused with nicotine and/or PNU120596 (τ_1=12.697 ± 0.29, 13.3637± 0.4946 ms, τ_2= 12.89 ± 1.132, 14.7143±0.4493 ms) respectively, and reduced in the presence of MLA (τ_1=9.135 ± 0.33ms, τ_2= 6.45 ± 1.647) compared to control slices τ_1= 10.515 ± 0.73 ms, τ_2= 12.763 ± 1.352 ms (Figure 5 F; $p < 0.05$; n = 6). A rapid desensitization was observed during the application of higher concentrations of nicotine (30µM). Levels of nicotine (5 µM) and PNU120596 (1 µM) that gave consistently large increases in sEPSCs and manageable washout times were chosen for further experiments to characterize the mechanism and nAChR subunits involved. These results suggest hippocampal glutamatergic transmission itself is modulated pre- and postsynapticaly by cholinergic nicotinic receptors.

In contrast, our results indicate that the mean current amplitude of AMPAR-sEPSCs was significantly reduced from 48.26 ± 1.38 pA in prenatal saline-exposed rodents (control) to 33.330 ± 0.1837 pA in PREN rodents (Fig. 5; $p < 0.01$; n = 5). Next, we examined the effect of nicotine and/or PNU120596 on altered AMPAR-sEPSCs in PREN hippocampal slices. Our data showed that no significant changes in mean current amplitude of AMPAR-sEPSCs existed among the PREN groups in control PREN slices (33.330 ± 0.1837 pA), PREN slices with nicotine-perfused (35.342 ± 0.342 pA), or in the presence of PNU120596 (34.654 ± 0.5554 pA) (Fig. 5; $p > 0.05$; n = 5). In addition, the frequency of AMPAR-sEPSCs in PREN was significantly different from that in saline-exposed rodents (control). The frequency of AMPAR-sEPSCs was not changed among the PREN groups perfused with nicotine and/or PNU120596, (3.820 ± 0.1281, 3.93±0.124 respectively), compared with control (3.6993 ± 0.1246) (Fig. 5; $p>0.05$; n=5). These sEPSCs for PREN in the presence of nicotine and/or PNU120596 showed respective decay times of τ_1 = 6.364 ± 1.24, τ_2 = 12.77 ± 1.326, and τ_1 = 7.364 ± 0.585, τ_2 = 13.05 ± 0.91 ms, which did not differ significantly from the decay times of PREN baseline sEPSCs (τ_1 = 6.515 ± 0.7334 and τ_2 = 11.77 ± 0.7361 ms, respectively). This could be due to changes in the kinetics of the nicotinic channel closure or increased receptor desensitization.

Our data showed that in PREN, neither nicotine nor PNU120596 affected the deficits in frequency of AMPAR-sEPSCs to any significant extent. These results suggest that postsynaptic expression/function of nicotinic receptors as well as the presynaptic glutamate release were both impaired in PREN rats.

The reduction of EPSC amplitude observed in PREN could be interpret by a presynaptic effect on glutamate release, a postsynaptic alteration of the AMPA receptors, change in the properties of nicotinic receptors, or a combination of these. Therefore, we measured the paired-pulse ratio,

whose alterations are considered to reflect changes in presynaptic transmitter release (see previous section).

3.4. Effects of PREN on single-channel properties of synaptosomal nicotinic receptor

Alterations in single-channel activity could result in alterations in overall synaptic currents and LTP (Ambros-Ingerson and Lynch, 1993; Benke et al., 1998; Parameshwaran et al., 2007; Wijayawardhane et al., 2008). Our previous study results showed that channel open probability (Po) of synaptic AMPA receptors in nicotine-exposed rats was reduced compared to controls (Parameshwaran et al., 2012). These experiments were designed to determine whether the decrease in amplitude and frequency of AMPAR-sEPSCs in PREN slices is due to modified single-channel properties of synaptic nicotinic receptors. We analyzed synaptic nicotinic receptor single-channel currents from synaptosomes reconstituted in lipid bilayers. Results show that the nicotinic channel open probability (Po) in nicotine-exposed rats was reduced in presence of nicotine (Po = 5.23% ± 0.6%) compared to controls (Po = 22.15% ± 1.4%; Figure 6, n=5, $p^{***} < 0.001$). The conductance of nicotinic channels was drastically reduced from 60 Ps in control to 10.83 Ps in PREN synaptosomes. Furthermore, analyses of dwell times revealed open times (τ_o) were reduced, and close times were increased (τ_c) in nicotine-exposed rats. The histograms of channel open, and close dwell times were fitted with 2 exponentials by the Marquart least squares methods (Fig.6). The open dwell time data showed shorter open times in PREN (Fig. 6; PRENl: τ_1 0.364±0.0723ms; τ_2 2.871±0.22ms control: τ_1 0.7186±0.0367ms; τ_2 8.204 ± 0.636ms; $^{*}p<0.05$; n=10), and longer close times (control: τ_1 1.302±0.17ms; $\tau_2$20.45±0.28ms, PREN: τ_1 2.209±0.47ms; τ_2 49.187±0.17ms; $^{**}p<0.001$; n=10). PNU-120596 induces the opening probability of α7 nAChR ion channels in control without producing significant changes in ion

channel properties, single channel conductance (Fig. 6). These results suggest that alterations in single-channel properties may play a role in deficient mEPSCs and LTP in nicotine-exposed rats.

3.5 Effects of nicotinic receptor activation on expression of AMPARs

Alteration in the expression of synaptic Glur1 AMPAR subunits could also contribute to the impairment in synaptic transmission and plasticity, as a result of alteration in nAChR expression (Rezvani et al., 2007). Therefore, we performed Western blot experiments to quantify the protein expression levels of the most abundantly expressed AMPARs subunit, GluR1. We found that the protein level of GluR1, was decreased in PREN hippocampal slices and enhanced after nicotinic receptor activation compared to saline-control (Figure 5A, 100.0 ± 3.208 for controls, 48.11 ± 3.604 for PREN, and 120.0 ± 2.208 after nicotinic receptor activation; $^{**}p < 0.001$ and $^{*}p < 0.05$ respectively, n = 6; fig.5G).

3.6 Effect of PREN on the expression of synaptic proteins and nicotinic receptor signalling in whole hippocampal lysate

Certain synaptic proteins regulate presynaptic glutamate release, including synaptophysin, and thus can a have a significant role on regulation of synaptic transmission and plasticity (Mullany and Lynch, 1998). PSD-95 and SAP102 together support postsynaptic receptor expression and nicotinic synapse formation. Interestingly, PSD-95 and SAP102 at least partially compensate for each other in supporting nicotinic input. We found that levels of synaptophysin (Figure 7.; $p^{*} < 0.05$, n = 5), SAP102 (Figure 7; $p^{**} < 0.01$, n = 5), and PSD-95 (Figure. 7; $p^{**} < 0.01$, n = 5) were decreased in the hippocampus of the prenatal nicotine group. These results suggest that prenatal nicotine exposure alters the levels of pre- and postsynaptic proteins in the hippocampus, which may have caused the alteration in synaptic function and receptor expression. In addition, we

found that levels of CAMKII (Fig. 7; $p^{**}<0.01$, n = 5), pERK (Fig. 7; **p <0.01, n = 5), and pCREB (Fig. 7; **p <0.01, n = 5) were decreased in the hippocampus of the prenatal nicotine group. These results suggest that prenatal nicotine exposure results in reduced expression of nicotinic receptors signaling proteins in the hippocampus, which may have caused the deficiencies in synaptic plasticity and transmission.

3.7 Nicotinic receptors interact with PSD-95 and SAP102 scaffolding proteins

Since PREN impaired the expression of PSD-95 and SAP102 proteins, we hypothesized that nicotinic receptors may physically interact with these proteins and alter their cellular trafficking. To determine if nicotinic receptors complex with these scaffolding proteins, we conducted co-immunoprecipitation experiments using homogenates from the hippocampus. α7- and β2-nAChRs antibodies were sufficient to immunoprecipitate PSD-95 and SAP 102 proteins (Figure 8, n = 6). To further examine these interactions, co-IP studies were performed using PSD-95 and SAP102 antibodies. These antibodies were sufficient to immunoprecipitate α7- and β2-nAChRs (Figure 8, n = 6).

3.8 Effects of PREN on nicotinic interactions with PSD-95 and SAP102 scaffolding proteins

In order to determine whether PREN could impair the interaction of nicotinic receptors with PSD-95 and SAP 102 and thereby impair their membrane localization and trafficking (Neff et al., 2009), we performed co-IP experiments in control and in PREN hippocampi by using α7-antibody. We found a significant decrease in the co-immunoprecipitated protein levels of SAP 102 and PSD-95 proteins from PREN slices compared to control (Fig. 9, 100.0 ± 6.437 and

100.0 ± 4.783 for controls; 67.123 ± 6.521 and 65.432 ± 4.418 for PREN hippocampal homogenates; $p < 0.05$ and $p < 0.05$, respectively, n = 6).

3.9 PREN alters the neurochemical levels of glutamate and GABA

Glutamate and GABA are two predominant neurotransmitters that regulate excitatory and inhibitory neurotransmission in the brain, respectively. Nicotinic α4β2 and α7 nACh receptors are also abundantly expressed on GABAergic interneurons in the hippocampus and mediate inhibition and disinhibition of hippocampal neuronal networks (Alkondon and Albuquerque, 2002). The used concentrations of (5μM) nicotine and/or (1μM) PNU120596 in this study, therefore, might enhance excitation in pyramidal neurons due to disinhibition in association with a decrease in GABA release. To confirm that the used concentrations do not produce disinhibition of hippocampal neurons, glutamate and GABA levels were determined in the homogenized lysate from the hippocampal region of the brain tissue. In nicotine and/or PNU120596-treated slices (saline-exposed) group, there were significant increase in the glutamate levels compared to control slices (saline- exposed) group (Fig. 10, Control (18.13 ± 1.612) ; nicotine treated slices (24.53 ± 1.166); and PNU120596 in presence of nicotine treated slices (28.79 ± 0.151 $p<0.05$, n=5). On the other hand, there was no significant change in GABA levels in nicotine and/or PNU120596 treated slices compared to control group (figure 2b, Control (4.302±0.2746); nicotine treated slices (3.969 ± 0.4585); and PNU120596 in the presence of nicotine treated slices (4.546 ± 0.5117 $p >0.05$, n=5). These data confirm that these concentrations did not induce excitation of hippocampal interneurons. The levels of glutamate and GABA were changed in PREN compared to control saline-exposed group (figure 2B, Control (18.13± 1.612 for glutamate; 4.302±0.2746 for GABA) and PREN (10.09 ± 0.1435 for

glutamate; 2.592±0.3217 for GABA n=5). The neurotransmitter levels were measured as pmol/mg of protein of hippocampal tissue.

4. Figures and Figure Legends:

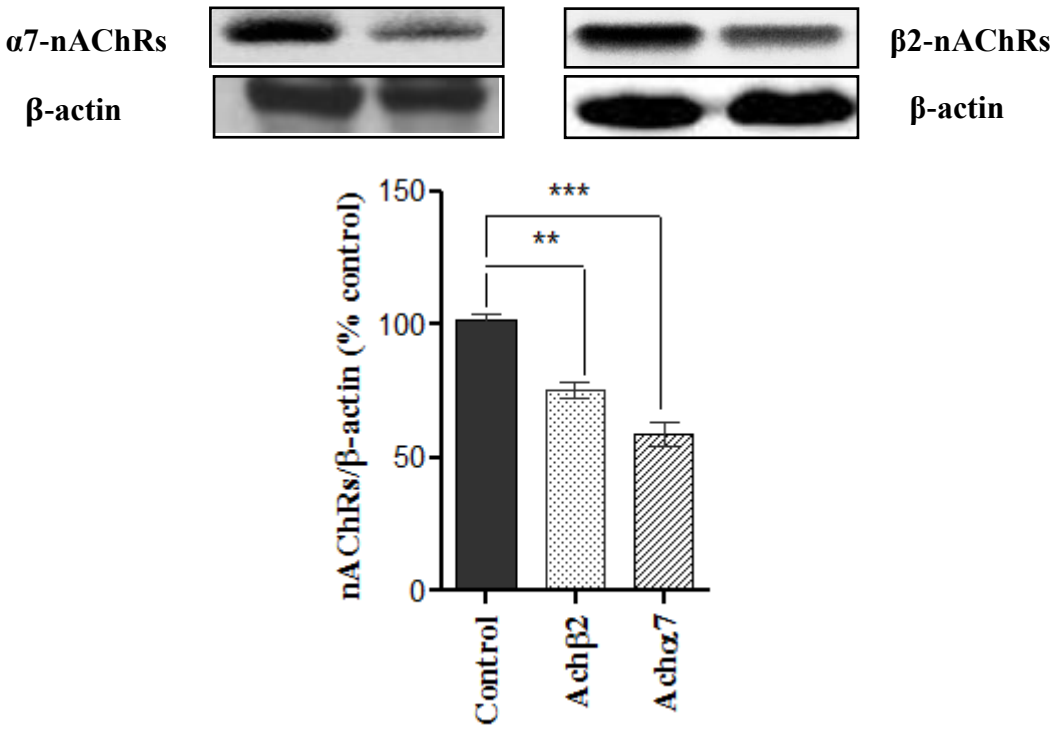


Figure 3.1 Effect of prenatal nicotine exposure on protein levels of α7-nAChR and β2-nAChR in the whole hippocampus in PREN offspring rodents. Western blot analysis shows α7-nAChR expression was significantly down regulated by 40% following prenatal nicotine exposure (***P<0.001, n=8 CTL, 8 PREN). Whereas, the level of β2-nAChR expression in the nicotine group was significantly decreased by 25% (**P<0.01; n=8 CTL, 8 PREN). Values are the mean±SEM relative to saline-exposed controls. Protein levels in the control and treated whole hippocampus lysate. The density of α7-nAChR and β2-nAChR protein levels are normalized to β-actin and expressed as a percentage of the control group which was set as 100%. Typical Western blots for each treatment are shown in band pairs above the bars (left band: control; right band: PREN). Abbreviation CTL=control, PREN= Prenatal nicotine-exposed, n=the number of pups used for each bar, two-tailed, unpaired Student's t-test.

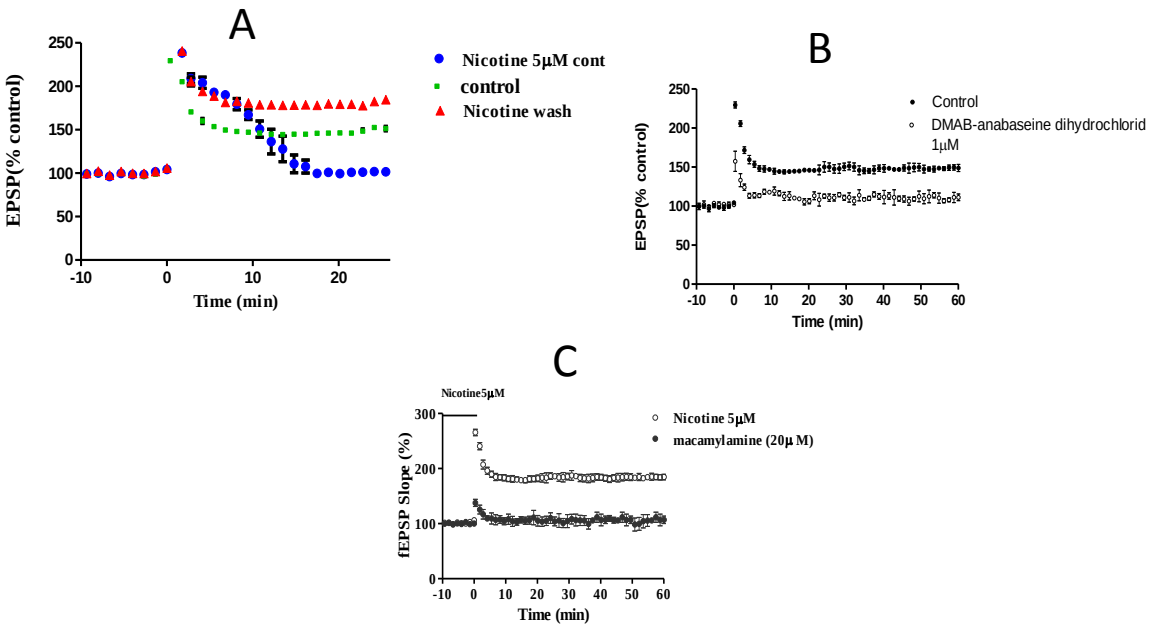


Figure 3.2 Nicotinic-enhanced TBS-LTP in control rat Shaffer collateral. (A) The graph shows the induction of control/saline LTP (filled red triangles), the enhancement of LTP induction by pre-application of nicotine (5 µM; green squares), and impair maintenance of LTP due to continuous application of nicotine (5 µM; filled blue circles), (B) Acute application of Anabasein dihydrochlorid (1 µM) prevents nicotine enhancement of TBS-LTP (open circles), (C) LTP impairment due to inhibition of nicotinic receptors by macamylamine.

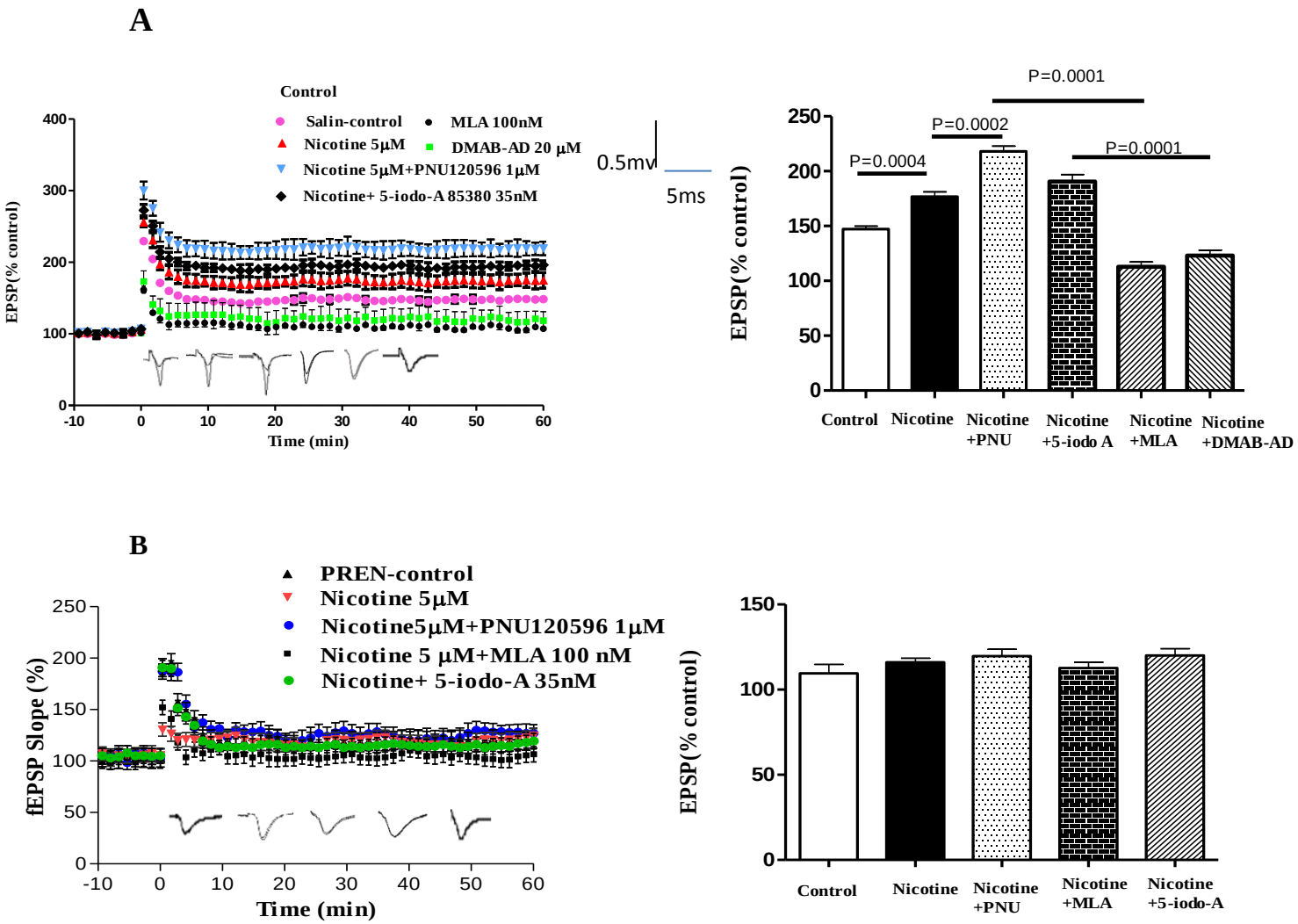


Figure 3.3 Effect of nicotinic receptor stimulation on hippocampal LTP. LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS; the normalized fEPSPs slopes show significantly differences between PREN and saline exposed groups. (A) In saline exposed animals acute slices activation of nAChRs by addition of nicotine and/or PNU120596, 5-iodo-A in ACSF enhances LTP, compared to control-saline, blocking α7- nAChRs drastically reduced LTP (B) LTP in PREN acute hippocampal slices were impaired in all four groups compared to saline exposed slices.

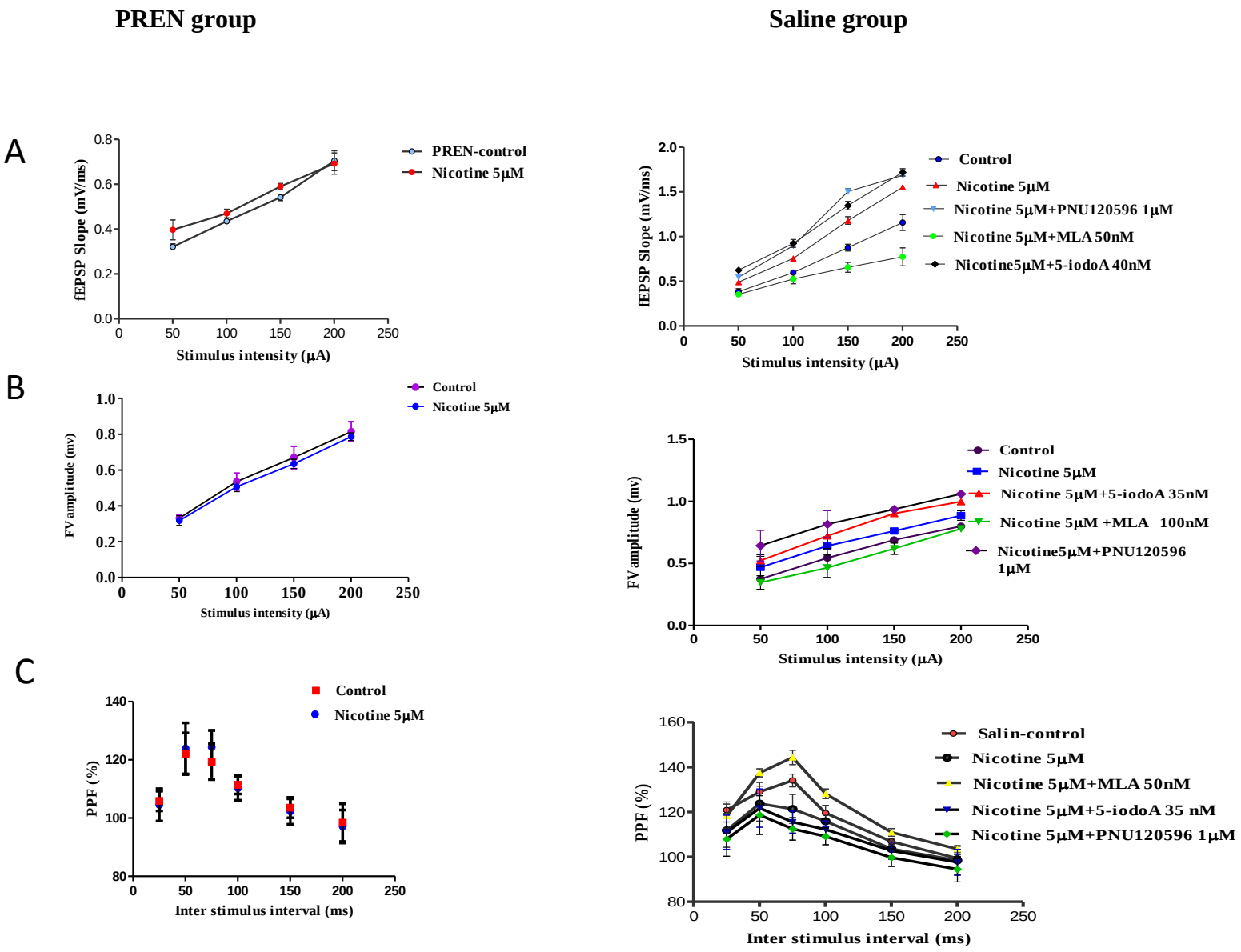
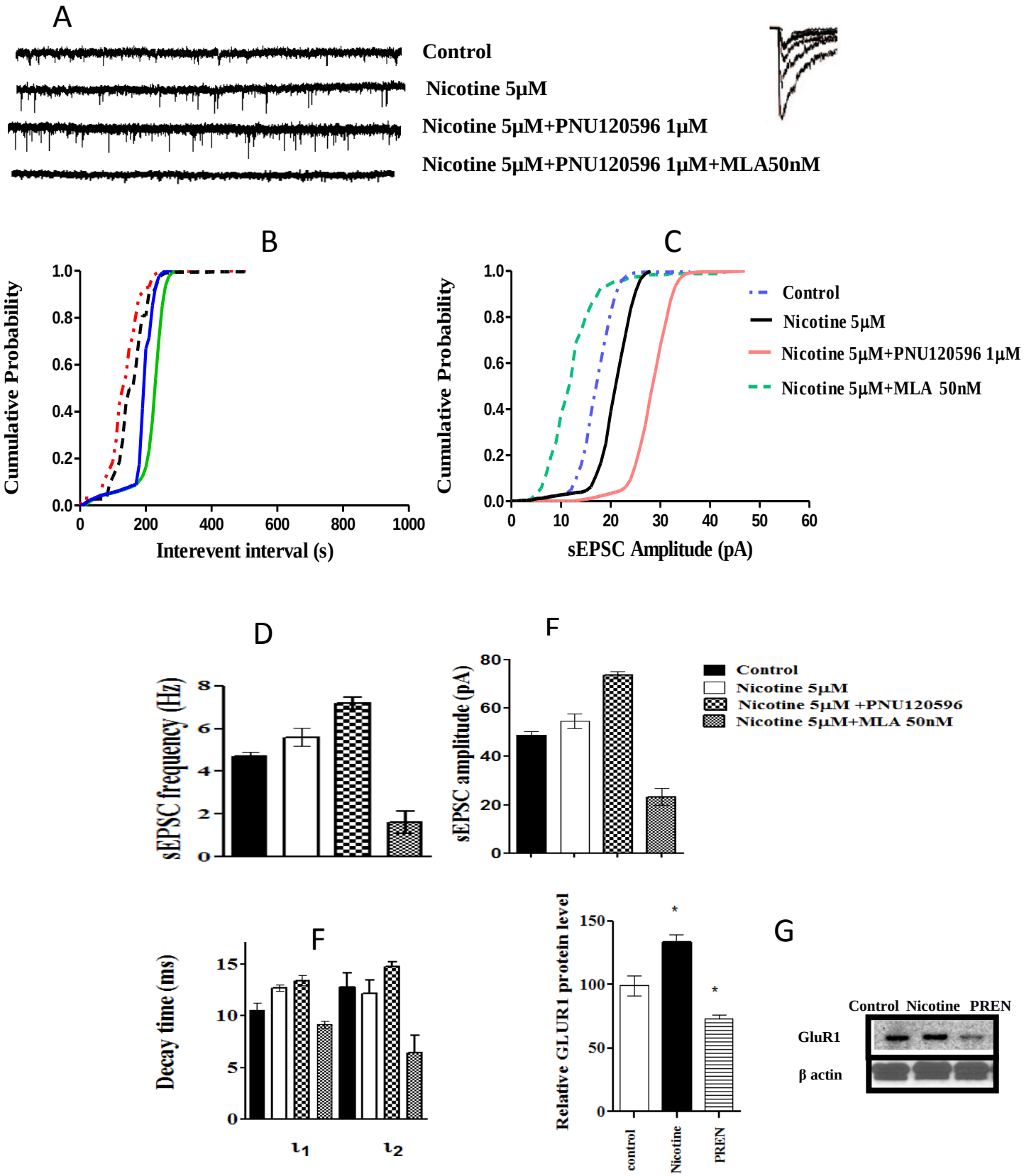


Figure 3.4 Basal synaptic transmission is enhanced by stimulation of nicotinic receptor. (A) input/output plot of fEPSP slope versus stimulus intensity, which depict difference in I/O response of PREN and saline exposed groups. At all stimulus levels, the average fEPSP slope for PREN-slices after addition of nicotine was no statistical difference compared with PREN-control (p> 0.05; n= 6). There was significant difference between saline-control and saline-nicotine and/or PNU120596 fEPSPs slopes; MLA fEPSPs slope was reduced compared with PNU120596 fEPSPs slope. (B) Plot of presynaptic fiber volley amplitude (FV) versus different stimulus intensity. (FV)

of PREN group was significantly reduced compared with saline group, (FV) of saline –nicotine and /or PNU120596 plotted at different stimulus intensities was enhanced compared with saline – control slices, (FV) for PREN-slices after addition of nicotine was no statistical difference compared with PREN-control (C) Comparison of PPF (higher PPR indicates reduced probability of release) in saline and PREN-slices. At different interpulse intervals, there was no statistical difference in the PPF ratio (slope2/slope1) among PREN group, there was significant difference between saline-control and saline-nicotine and/or PNU120596, and high PPR in MLA treated slices.

Saline exposed group



Prenatal nicotine exposed group

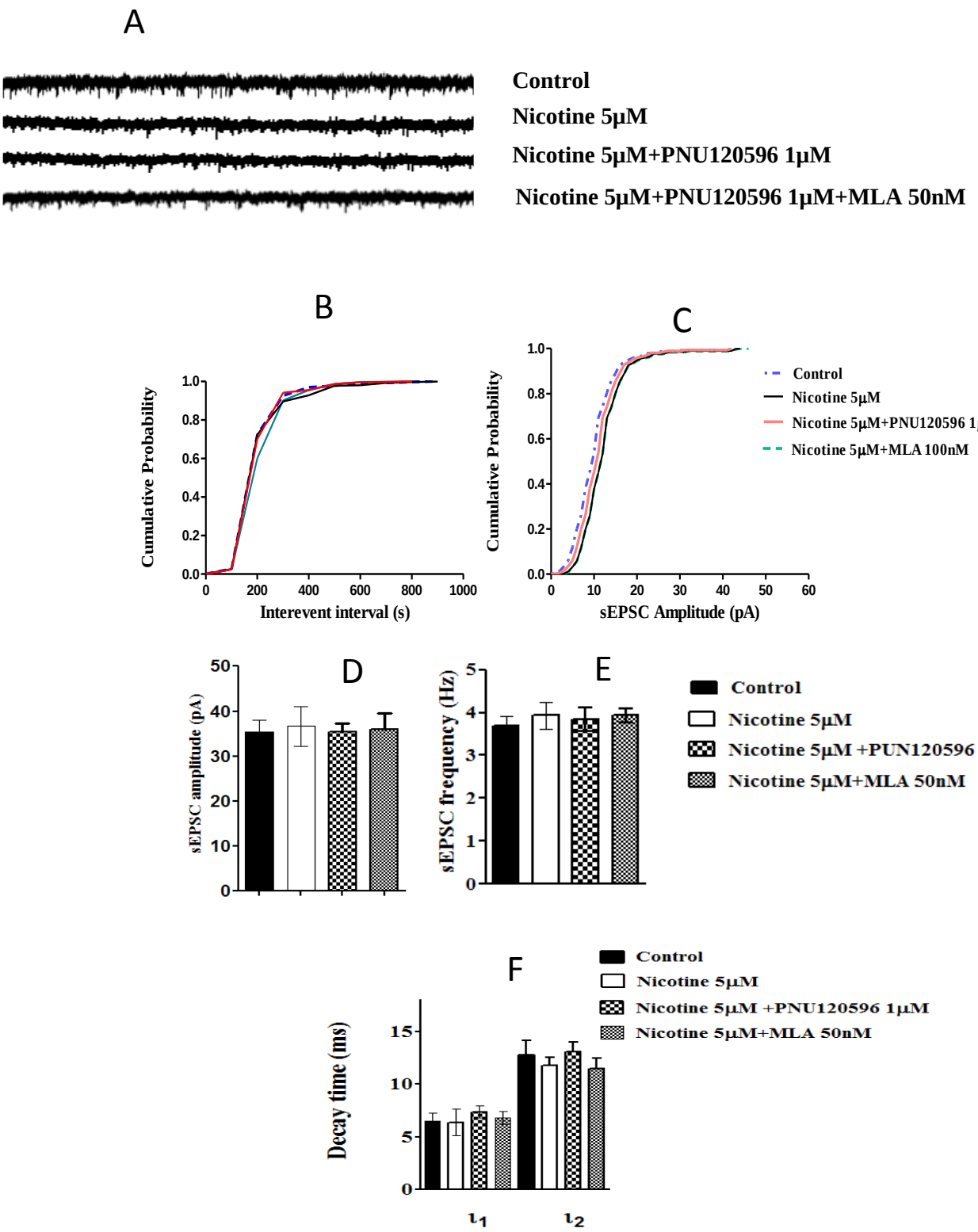
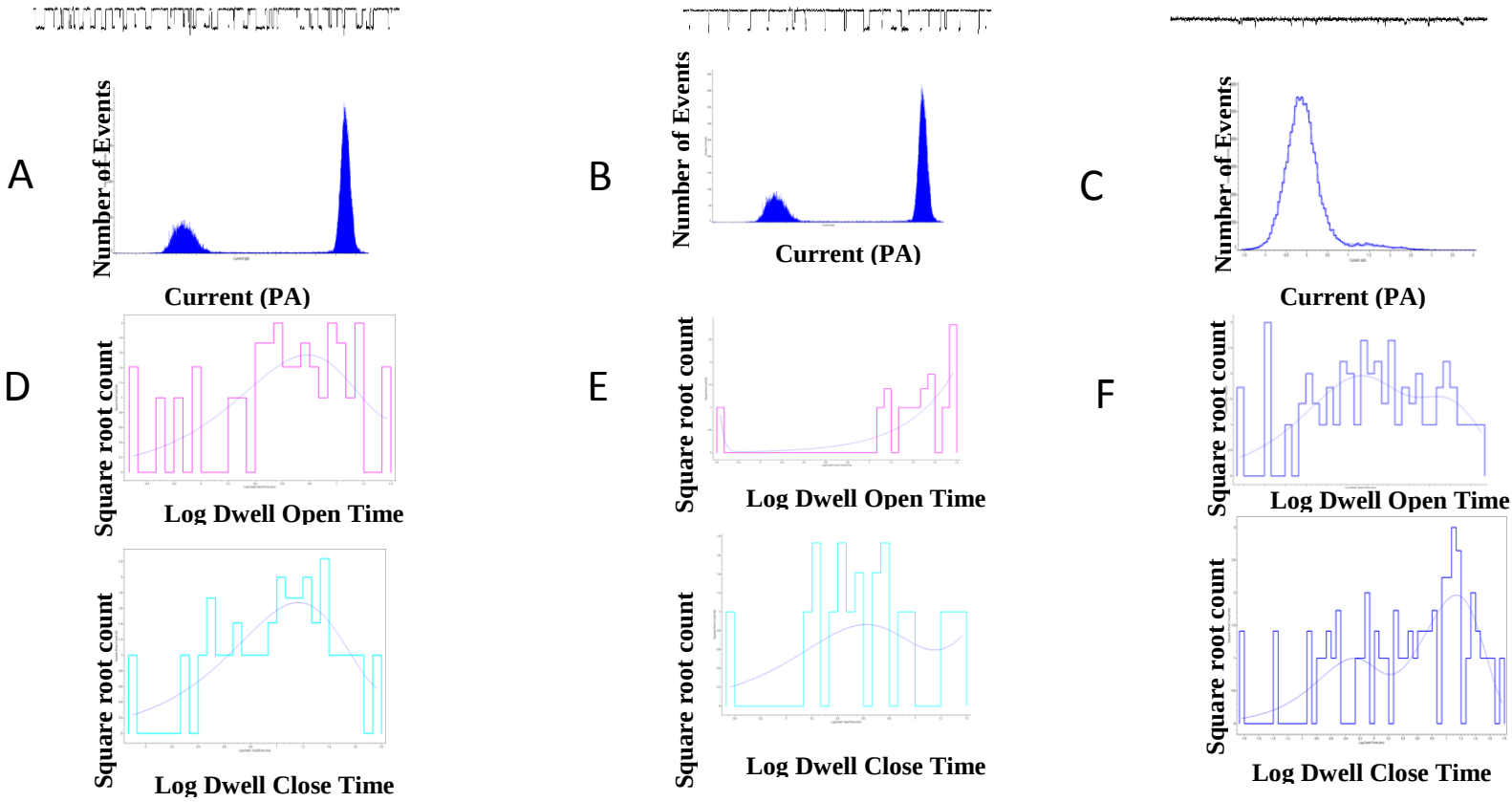


Figure 3.5 Effect of nicotinic receptor activation on AMPARs-mediated whole-cell synaptic currents. (A) Sample sweeps show AMPARs-mediated sEPSC recorded at -70 mv membrane potential from a representative neuron showing increase in amplitude and frequency, in saline-nicotine treated slices and further increase with PNU120596 compared to control- saline slices and with no change in PREN among the four groups. (B) Cumulative probability of distributions of amplitude shows a significant shift of nicotine treated curve to the right from the controls, suggesting increase in amplitude. (C) Cumulative probability of distributions of interevent interval shows a significant shift of nicotine and /or PNU120596-saline treated curve to the left from the control-saline treated, implying increase in frequency of sEPSCs in the saline treated group curve compared to PREN group which shows no change in interevent interval among the four PREN group. (D) Bar chart shows the significant increase in mean amplitude of AMPARs-mediated sEPSCs in nicotine and/or PNU120596 –saline treated slices than that of saline-control ($p< 0.01$; n= 6). (F)The decay times of AMPARs-mediated sEPSCs were fitted with two terms. And decay times were depict a significant increase in AMPARs-mediated sEPSCs decay times in nicotine and /or PNU120596 saline-treated slices than that of control (Student's *t*-test, $p<0.05$; n=6) compared to PREN group which shows no change in decay time among the four PREN group ($p< 0.01$; n= 6). (G) Enhancement of AMPARs expression in nicotine treated slices relative to untreated control slices in comparison to PREN.


A
Number of Events
Current (PA)
B
Number of Events
Current (PA)
C
Number of Events
Current (PA)
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Square root count
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Log Dwell Close Time
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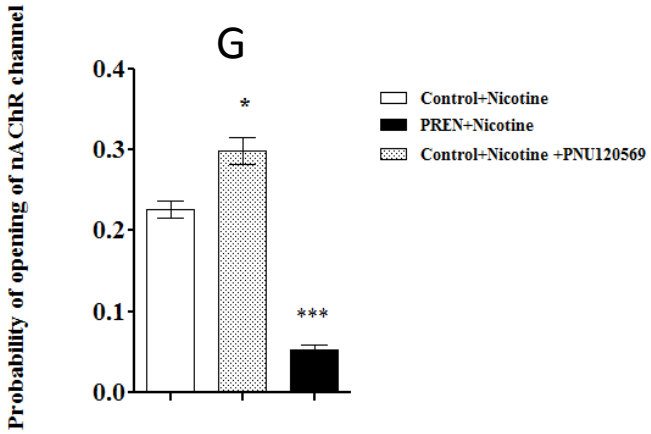
G
Probability of opening of nAChR channel
0.4
0.3
0.2
0.1
0.0
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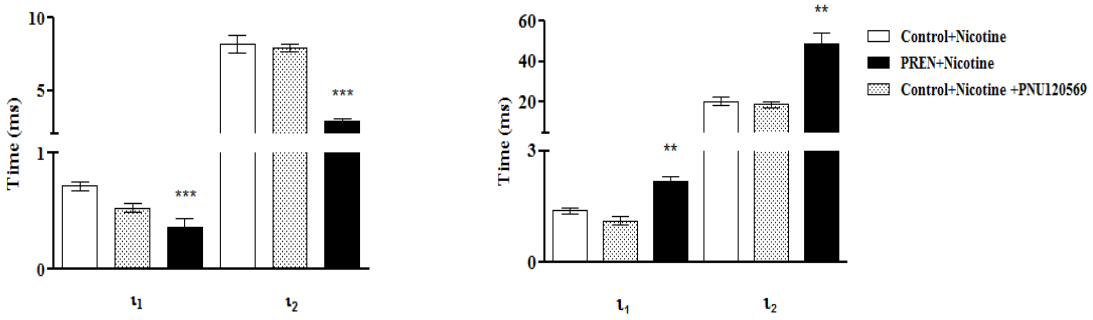
Control+Nicotine
PREN+Nicotine
Control+Nicotine +PNU120569


Time (ms)
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t1
t2
Control+Nicotine
PREN+Nicotine
Control+Nicotine +PNU120569

Figure 3.6 Prenatal nicotine exposure leads to alterations in single-channel properties of synaptic nAChRs. Representative traces and corresponding current amplitude histograms are shown for (A) control synaptosomes treated with100nM nicotine, (B) control synaptosomes activated with 1μM PNU120596 in the presence of 100nM nicotine and (C) PREN synaptosome in the presence of PNU120596 and nicotine. The channel conductance of nAChrs currents were shifted from the higher conductance level 60 Ps in control synaptosomes to the lower conductance state 10 Ps in PREN synaptosomes. The channel open peak (right peak in each histogram) is decreased in PREN synaptosomes indicating a reduction in open probability. Single-channel open and close time distributions for control (C) and PREN synaptosomes (D) and (E) and (F) respectively were fitted with 2 exponentials. (G) Bar chart illustrating the significant reduction of nAChRs channel open probability in PREN synaptosomes. (H) Bar plot showing significantly decreased open times τ_1 and τ_2 in PREN synaptosomes. (I) Bar chart showing significantly prolonged close time component (τ_2). Values are expressed as mean ± SEM from 10 animals per group ***p<0.0001, p**<0.001, *p<0.05, two-tailed, unpaired Student's t-test).

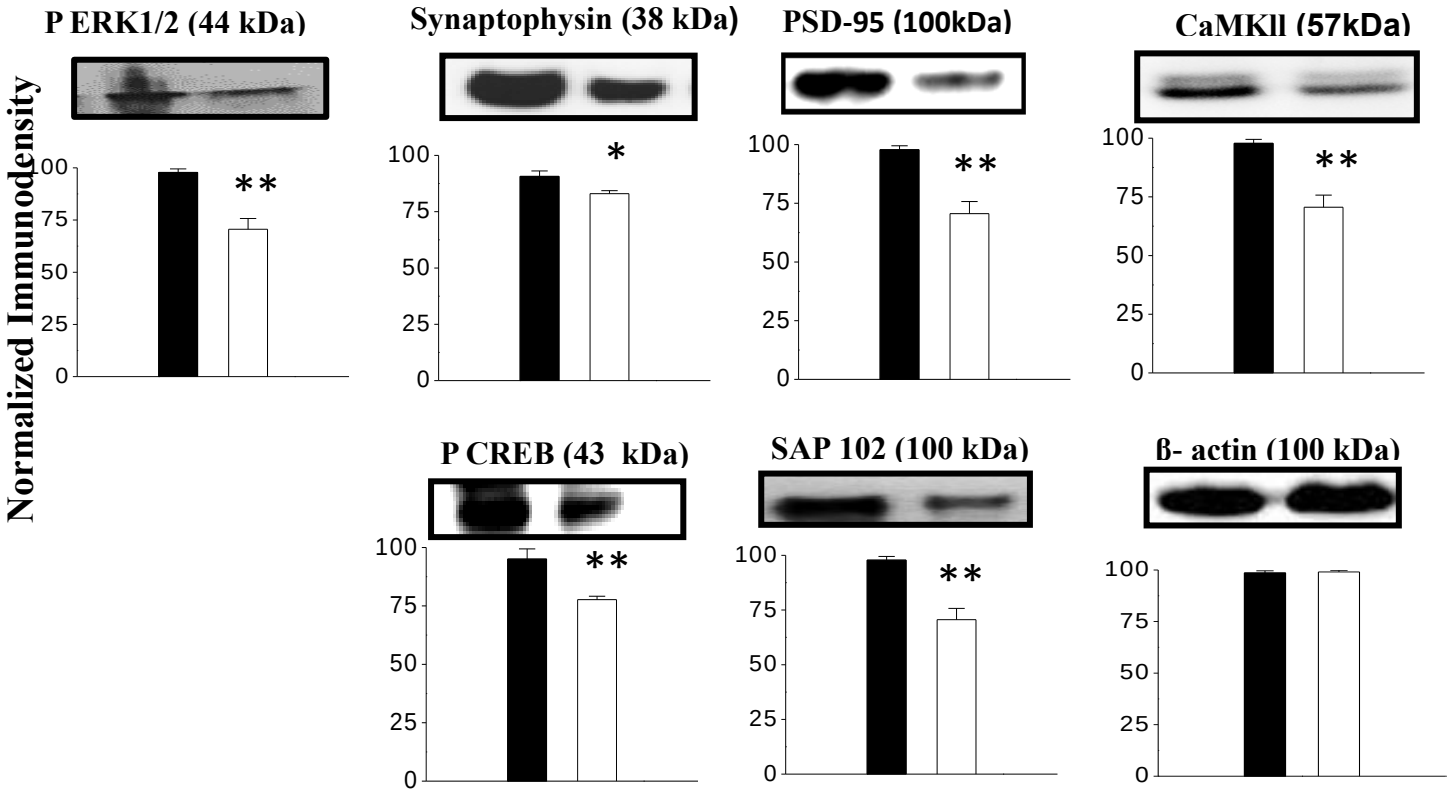

Figure 3.7 Effect of PREN on the expression of nicotinic receptor downstream signaling and their associated proteins in whole hippocampus lysate. Western blot analysis were performed to quantify the protein levels of (A) P ERK1/2, (B) Synaptophysin, (C) PSD-95, (D) CaMKII, (E) P CREB and (F) SAP102 protein 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^{**}<0.01, p^{*}<0.05, two-tailed, unpaired Student's *t*-test).

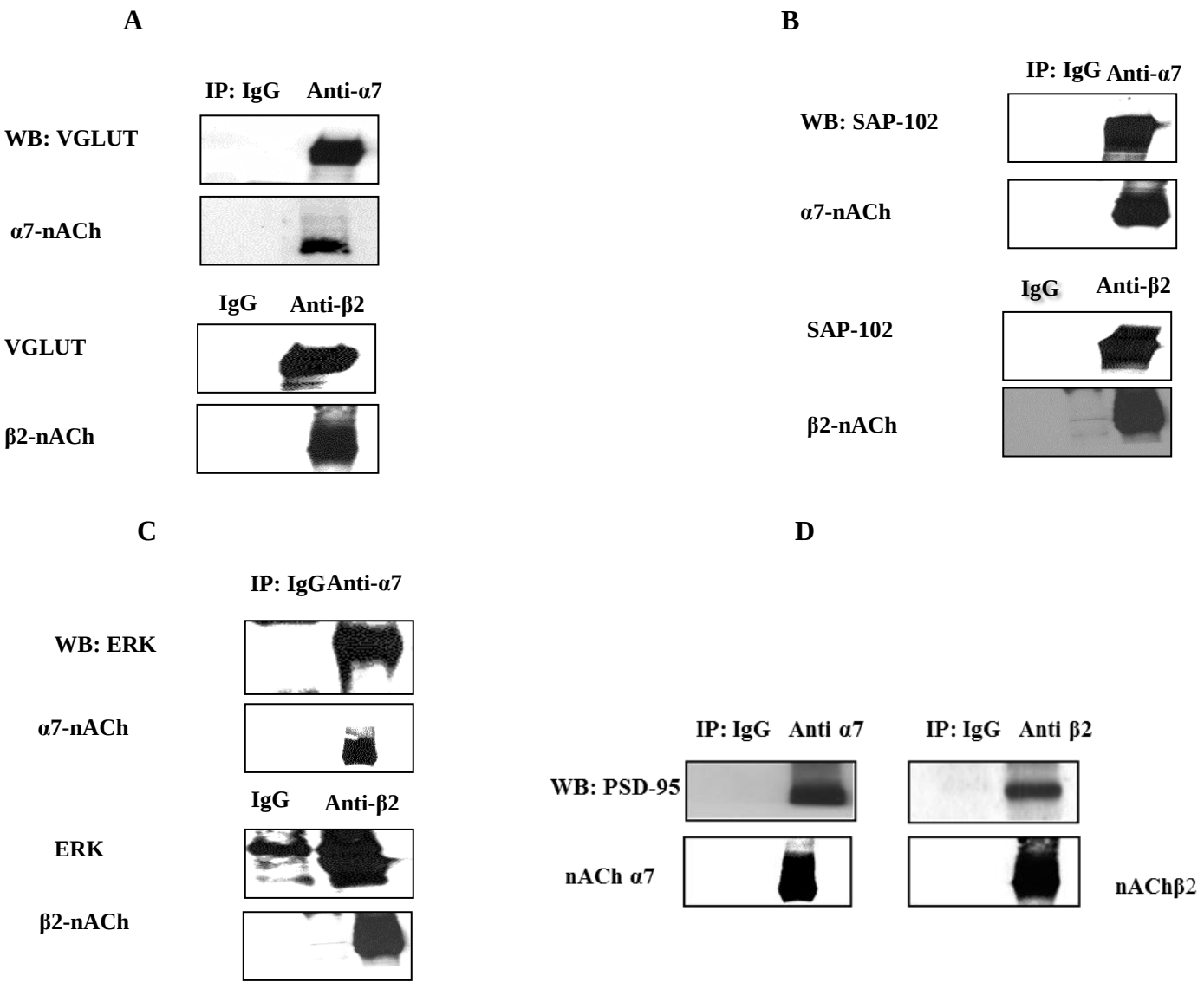

Figure 3.8 Co-immunoprecipitation of α7 &β2 nAChR with VGLUT, ERK, SAP102 and PSD-95 in the hippocampus homogenate. In the hippocampus, anti- α7 &β2 nAChR antibodies co-immunoprecipitated with (A) VGLUT, (B) SAP102, (C) ERK, and(D) PSD-95. Detection was done by performing western blot analysis using anti-VGLUT, anti-ERK, anti-SAP102 and anti-PSD-95 antibodies respectively. (F) Blots were stripped and reprobed with anti- α7 &β2 nAChR antibodies. The IgG antibody served as a negative control (n=6).

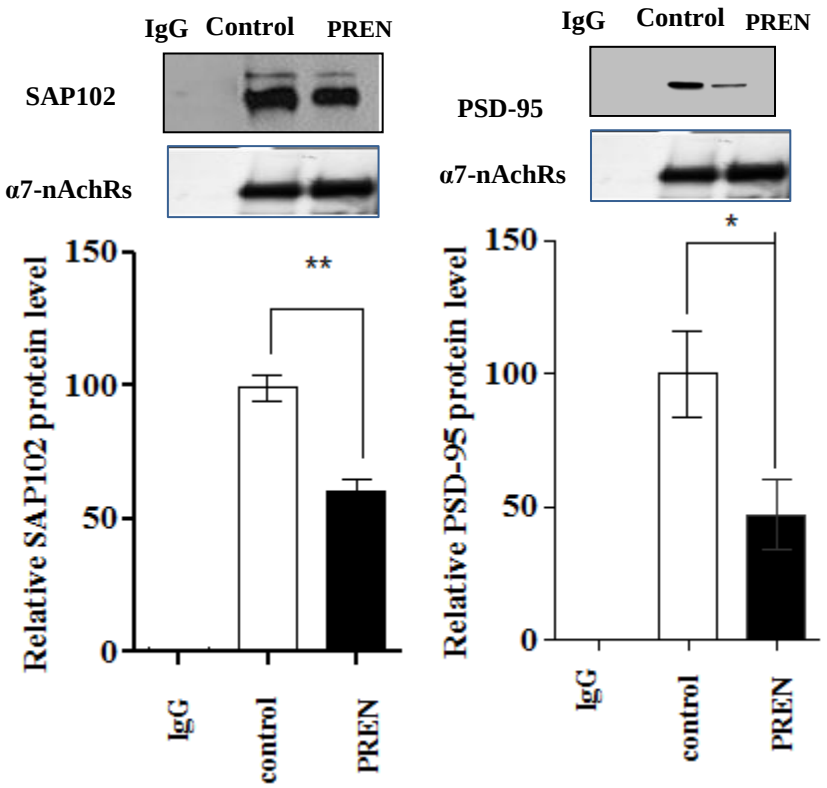

Figure 3.9 Co-immunoprecipitation of α7nAChR with (A) PSD-95, (B) SAP102, in the hippocampus was disrupted in PREN rodents. We quantified protein amounts of PSD-95 and SAP102 co-immununoprecipitated with anti- α7nAChR antibody in the homogenates prepared from control and PREN hippocampai by performing western blot analysis. Protein levels are normalized to α7nAChR and expressed as a percentage of the control group which was set as 100%. Quantity of PSD-95 and SAP102 were significantly reduced in PREN hippocampal homogenates as compared with control ($p< 0.01$and $p<0.05$, respectively, two-tailed, unpaired Student's *t*-test).

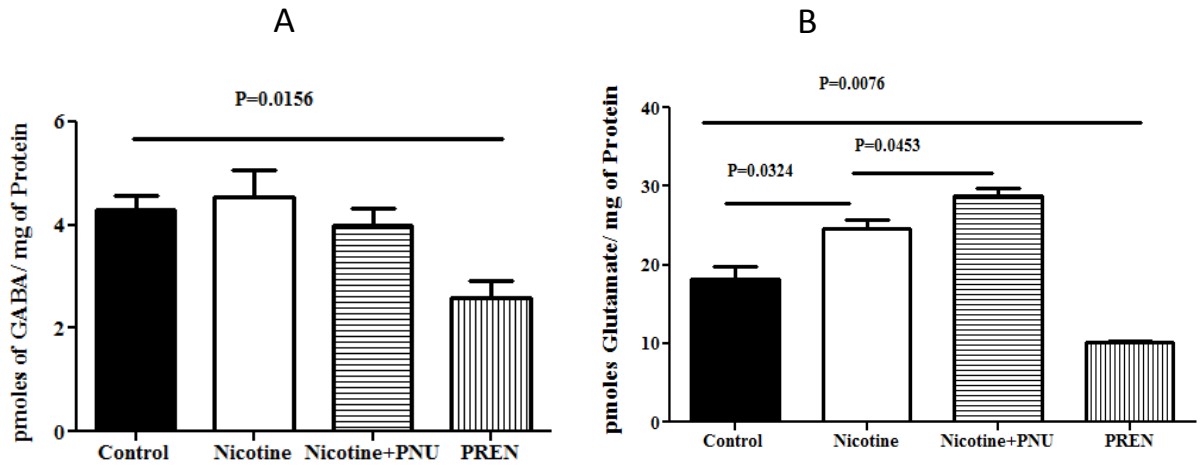

Figure 3.10 Marked increase in Glutamate and unchanged GABA levels in saline exposed rodents assessed by HPLC. (A) Bar chart shows the unchanged level in the hippocampal GABA content as detected by HPLC in saline exposed rodents treated with nicotine or PNU120596 relative to untreated control in comparison to PREN (n=6). Results are expressed as picomoles/mg of protein. **(**B) Bar chart shows the significant increase in the level of glutamate neurotransmitter measured in hippocampal region of brain of saline exposed rodents treated with nicotine and /or PNU120596 relative to untreated control slices in comparison to PREN slices (n= 6). Levels of neurotransmitter are expressed as picomoles/mg of protein.

3.5 Discussion

Children of mothers who smoke have been reported to be at higher risk for severe adverse health conditions (Pausova et al., 2007). Chronic neurobehavioral changes, such as learning and memory deficits and other conditions including stillbirth and sudden infant death syndrome, have been linked to maternal smoking during pregnancy (Ernst et al., 2001; Linnet et al., 2003; Winzer-Serhan, 2008). Studies have demonstrated that there is a strong correlation between maternal smoking and offspring cognitive outcomes, particularly in younger children (Kafouri et al., 2009). Animal studies from our laboratory and others have confirmed that prenatal nicotine exposure results in enduring learning and memory deficits that are consistent with, and an accurate model for, the cognitive deficits observed in the children of mothers who smoke (Parameshwaran et al., 2012; Vaglenova et al., 2008). The mechanisms responsible for impaired hippocampal memory function in PREN rodents are not fully understood. The major goal of this study is to elucidate the mechanisms that underlie hippocampal-dependent memories and how the nicotinic, cholinergic and glutamatergic systems interplay to regulate these mechanisms. Although several harmful substances are present in tobacco, the observed neurobehavioral alterations are mainly due to *in utero* nicotine exposure.

In the present study, we utilize a rodent model of prenatal nicotine exposure in which pregnant rats were infused with nicotine at rate of 6 mg/kg/day through a subcutaneously implanted osmotic minipump. This dose has been matched to the plasma level of nicotine in moderate to heavily smoking humans, which ranges from 25 to 75 ng/ml (Fewell et al., 2001). The results from this study demonstrate for the first time that cognitive deficits observed from prenatal

nicotine exposure are paralleled by modifications to glutamatergic hippocampal neurotransmission as a result of alterations in nAChRs.

Our recently published work demonstrated that basal synaptic transmission and LTP was impaired in PREN rat hippocampi (Parameshwaran et al., 2012). These results suggest that nicotine exposure during development affects the synaptic transmission mediated largely by glutamate receptors in the postnatal brain. The first line of analysis in this study was to evaluate the responsiveness of the glutamatergic synaptic transmission to modulation of different types of nAChRs. From our study (Figure 2C) and previous findings, it has been established that nAChRs are modulators of synaptic transmission and plasticity in the hippocampus (Welsby et al., 2006; Welsby et al., 2009a) and our findings further suggest that protein expression of α7 and β2 nAChRs are downregulated in the PREN rat hippocampus (Figure 1). These observations raise the possibility that altered expression, and perhaps function, of nAChRs contributes to observed deficits in glutamatergic synaptic transmission and plasticity.

In the first set of experiments, we utilized nicotine to activate nAChRs. It is important to determine an optimal dose of nicotine that will not cause rapid desensitization of the receptors and that will not cause a shift in fEPSPs at a given stimulus strength. In a set of pilot experiments, we identified 5 μM nicotine as the optimal dose in our experimental conditions. This dose was also used by Welsby et al. (2009) to study the changes in hippocampal LTP in rats. Pharmacological agents (nicotine 5μM, PNU120596 1μM, 5-iodo-A-85380 35nM, MLA100 μM and DMAB-AD 20 μM) were perfused during a 5 min period that overlaps the TBS induction period. The reason for this drug application is to prevent desensitization of nicotinic receptors (Figure 2A). Stimulation of the Schaffer collateral with TBS was found to produce LTP of the fEPSP in the hippocampal CA1, which returned to baseline levels within about 10 min. after the continuous

application of 5 μM nicotine; this return to baseline suggests that the induction was at least partially due to the activation of nAChRs, as opposed to being solely due to desensitization of nAChRs (Figure 2A). Drug concentrations and duration of application were optimized from preliminary experiments and previous reports (Welsby et al., 2009b).

We studied the effects of activation/inhibition of α7 and/or β2 nAChRs on hippocampal glutamatergic synaptic transmission and LTP in saline-exposed control and PREN animals. α7 and β2 subunit-containing nAChRs are richly expressed in the hippocampus and their physiological roles have been reported in several studies (Alkondon and Albuquerque, 2004). In these experiments, we utilized nicotine to activate nAChRs. First, we studied the changes in the fEPSPs and fiber volley amplitude in response to different stimulus strength in the presence of 5 μM nicotine. Prior to the infusion of nicotine, fEPSP recordings were performed for 15 minutes at a stimulation strength that evokes fEPSPs with 50% of the amplitude where the first population spike appear. The aim of this experiment was to identify the specific modulatory role of each of these two nAChR subtypes on hippocampal basal synaptic transmission. This was achieved by infusion of a drug (PNU120596) that acts as a potent and selective positive allosteric modulator for the α7 subtype of neural nicotinic acetylcholine receptors. The reason for using an allosteric modulator is that PNU120596 induces a form of modulator-sensitized receptors and is able to change the accumulated desensitization. For activation of β2-nAChR β2 agonist 3-[(2S)-2-Azetidinylmethoxy]-5-iodopyridine dihydrochloride (5-iodo-A) was utilized. All drugs were perfused in the ACSF. The input-output experiments were performed (after 5 min of nicotine perfusion) only if fEPSPs during the 15 min baseline recordings did not show a shift in amplitude. Our results from this study demonstrated that LTP was not induced by TBS in PREN

hippocampal slices. Assessment of basal synaptic transmission at various stimulus intensities revealed that fEPSP slope was significantly attenuated in PREN slices.

Deficits in LTP in PREN slices could be due to altered axonal depolarization, presynaptic release and/or postsynaptic mechanisms (Figure 4A). Therefore, we assessed the presynaptic fiber volley, a small deflection of the field response that precedes the postsynaptic potential and correlates with the number of presynaptic afferents activated by the stimulation pulse at different input current intensities. We found the fiber volley amplitude was significantly different between saline-control slices and PREN-control slices at various stimulus intensities (Figure 4B), indicating that the conversion of the presynaptic stimulus into axonal depolarization was affected in PREN slices. In addition, fiber volley amplitudes were significantly different among the control saline-exposed group treated with nicotine and/or PNU120596; however, no changes were seen within the PREN group after drug perfusion. Furthermore, changes were detected in PPF% among the control slices treated with nicotine and/or PNU120596 and 5-iodo-A, but no changes were observed in PREN slices (Figure 4C). 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). Paired-pulse facilitation of fEPSCs monitored in CA1 pyramidal neurons was significantly depressed after treatment with nicotine and/or PNU120596, indicating that the nicotinic modulators effect on synaptic transmission is actually due to an increase in glutamate release from presynaptic terminals and an enhancement in postsynaptic excitatory conductance. Presynaptic α7 nAChRs on the glutamatergic terminals are involved in excitatory synaptic transmission through glutamate release (Wonnacott, 1997; Wonnacott et al., 2006). This observation was consistence with our finding that nicotine and/or PNU120596 increased the rate of nicotine-triggered AMPA-sEPSCs, and affected their amplitude, in CA1 pyramidal neurons of hippocampal slices, an effect that is

blocked by nicotinic receptor antagonists (Figure 5), suggesting that PNU120596 stimulates glutamate release by enhancing presynaptic α7 nAChR activity. Overall, our results from this set of experiments suggest that nAChRs have a specific modulatory role on hippocampal basal synaptic transmission presynaptically, which was impaired in PREN rodents.

The application of 5-iodo-A is sufficient to cause a long-lasting potentiation in LTP in saline control slices. Application of the α7 nAChR agonist PNU120596 in the presence of nicotine evoked a further increase in LTP compared to a β2 nAChR agonist (Figure 3A). This increase was reduced in the presence of an α7 nAChR antagonist (MLA, 100 nM). Notably, nicotine-induced potentiation of AMPA sEPSC amplitude was sensitive to MLA (100 nM), an antagonist of the *α*7 nAChR subunit (Figure 5A). As nicotine-induced enhancement of AMPA sEPSC amplitude is associated with a decrease in the paired-pulse ratio (Figure 4C), altogether these data suggest that the α7 nAChR subunit is the major component of nAChRs located on glutamatergic presynaptic terminals neurons and that it may mediate the long-lasting excitation of pyramidal neurons. Both α7 and β2 nAChR activation are crucial for the establishment of full-sized LTP *in vivo* in the hippocampal CA3-CA1/Schaffer collateral synapses (Figures 3A & 2B), as application of DMAB-anabaseine dihydrochloride (DMAB 1 μM), which acts as an α7 nAChR antagonist and β2-nAChR agonist, impaired LTP (Figure 2B). This suggests that simultaneous activation of α7 and β2 nAChRs is required for enhancement of glutamatergic synaptic transmission and plasticity in the hippocampus and the reduced expression and function of α7 and β2 receptors PREN rodents' results in glutamatergic synaptic deficits.

Nicotinic α4β2 and α7 nAChRs are also abundantly expressed in GABAergic interneurons in the hippocampus and mediate inhibition and disinhibition of hippocampal neuronal networks (Alkondon and Albuquerque, 2002). Nicotine and/or PNU120596, therefore, might enhance

excitation in pyramidal neurons due to disinhibition in association with an increase in GABA release. However, this is not the case here, since the concentration of nicotine and PNU120596 used in this study did not change the level of GABA in rat hippocampal slices and it increases the level of glutamate release from rat hippocampal slices as found from HPLC experiments. Moreover, PNU120596s action on nicotine-triggered glutamate release was sensitive to MLA, agreeing with the implication of α7 nAChR (Figures 10A & 10B). Levels of glutamate and GABA were reduced in PREN hippocampal slices. Overall, nicotinic modulators appear to enhance excitation in hippocampal CA1 pyramidal neurons by stimulating glutamate release as mediated via presynaptic α7 nAChRs, whereas such excitation was impaired in PREN hippocampal slices.

When PNU120596, a positive allosteric modulator of α7 nAChRs, was present with nicotine in the superfusion fluid, the evoked glutamate release was increased compared to that elicited by nicotine alone. PNU120596 did not modify, *per se*, the basal release of glutamate (not shown).

To elucidate how prenatal nicotine exposure could disrupt glutamate release and to determine the relative contribution of the nicotinic receptor in regulating release and postsynaptic responses in PREN and control rats, we measured AMPA-sEPSCs from the pyramidal neurons in the CA1 region of the hippocampus utilizing the whole-cell patch clamp method. As the majority of excitatory neurotransmission in the hippocampus is mediated by AMPARs (Dingledine et al., 1999; Malinow and Malenka, 2002), α7 and β2 nAChRs, the two subtypes, have been well characterized for their presynaptic expression and regulation of neurotransmitter release (MacDermott et al., 1999, Wonnacott, 1997). The AMPA receptor-mediated excitatory synaptic transmission in the hippocampus of control saline-exposed animals is modified by nAChR modulation (Figure 5A). Results from controls showed an increase in sEPSC amplitude and

frequency with exposure to nAChR activation with nicotine, further increases in sEPSC frequency and amplitude with activation of α7 nAChRs with PNU120596 in the presence of nicotine, a decrease in sEPSC amplitude and frequency with inhibition of α7 nAChRs (exposure to antagonist MLA), and no measurable activation in PREN samples (Figure 5B). This is corroborated by a recent finding that mRNA levels of a7 nAChRs were reduced in prenatal nicotine exposed rats hippocampi (Eppolito et al., 2010). Interestingly, at higher dose of nicotine, the sEPSC amplitude and frequency in control slices diminishes (not shown). An additional complication to be considered for interpreting the mechanisms of nicotine action is the possibility that a desensitization process rather than an activation of nicotinic receptors could be the cause of the changes in sEPSCs. Indeed there is convincing evidence indicating that α7 containing nAChRs quickly desensitize upon nicotine application (Chiodini et al., 1999).

The decrease in EPSC amplitude can also result from decreased expression of synaptic AMPA receptors (Parameshwaranet al., 2012). In addition, altered quantal glutamate content can influence the mEPSC amplitude (unpublished data). In our previous study, we noted a significant decrease in the vesicular protein VGLUT, responsible for the uptake of the excitatory amino acid L-glutamate into synaptic vesicles, which may have contributed to impaired packaging of glutamate (Parameshwaran et al., 2012). Interestingly, co-immunoprecipitation studies of hippocampal tissue revealed that α7 nAChRs interact with VGLUT, PSD-95 and SAP-102 (Figure 8). Therefore, the presented evidence suggests that presynaptic terminals in PREN animals are less able to sustain release during TBS stimulation. A model consistent with this hypothesis is that the nicotinic receptor acts in the presynaptic terminal, at or near a site in the synaptic vesicle trafficking pathway, to influence neurotransmitter release. Indeed, nicotinic receptors have are associated with VGLUT and synaptic proteins which are enriched at the nerve

terminal. It is possible that α7 nAChRs can interact with these proteins at their synaptic sites and affect their synaptic localization. A deficit in vesicle release or recycling could account for the decrease in LTP induction seen in the slices from PREN animals.

We conclude that decreased AMPAR sEPSCs and amplitudes in PREN, which possibly contributed to impaired synaptic plasticity, resulted from decreased expression and function of pre- and postsynaptic nicotinic receptors. This study closely correlates with our previous results showing alteration of quantal content glutamate as well as AMPARs (Parameshwaranet al., 2012). Interestingly, GluR1 expression was found to be upregulated after nicotinic activation (Figure 5G). The decrease in frequency of sEPSCs in prenatal nicotine-exposed rodents in this study can be explained by: (i) a decrease in probability of glutamate release due to reduced presynaptic nicotinic afferents as proved by decreased FV amplitude and PPF among PREN compared to control saline exposed slices, (ii) impaired postsynaptic machinery as indicated by decreased PSD-95 and SAP102 which are interact with nicotinic receptors, in addition to downregulation of AMPAR subunit GluR1, and (iii) deficiency in vesicular packaging of glutamate due to decreased levels of VGLUT1 (Parameshwaranet al., 2012), which interacts with nicotinic receptors as revealed by immunoprecipitation studies.

Desensitization of long-term activated signaling pathway via removal of cell surface receptors and/or by alteration of ion channel properties is common. Chronic prenatal nicotine exposure might desensitize nicotinic signaling by (i) removing the surface nAChRs and/or (ii) downregulating the single-channel properties of nAChR ion channels. Our finding suggests an approximately 40% decrease in expression of α7 and a 25% reduction in β2nAChRs in PREN rat hippocampi (Figure 1). It is possible that, in addition to reduced expression, the remaining receptors localized at synapses may have altered functional properties due to PREN exposure. It is

further possible that peri-synaptic receptors may play a role in this. Therefore, to test this possibility**,** single-channel recording of synaptic nAChRs was performed utilizing isolated synaptosomes.

Alterations in intrinsic single-channel properties of synaptic AMPA receptors have been reported in our previous study (Parameshwaran et al., 2012). sEPSC amplitude and single-channel open probability are positively correlated (Kanju et al., 2008). Interestingly, in the nicotine-exposed rats, the single-channel properties of nAChRs were also altered (Figure 6), suggesting that reduced single-channel open probability and open times, coupled with increased closing times, may contribute to the deficits in single neuronal nicotinic receptor currents and, in turn, to the reduced synaptic transmission in prenatally nicotine-exposed rats. The reduced single-channel open probability of nAChRs in prenatal nicotine-exposed rats can be further explained by the fact that the nAChRs may be under the influence of tyrosine phosphorylation, which can lead to changes in receptor number and function. It has been reported that tyrosine phosphorylation can very rapidly modulate receptor function (Davis et al., 2001).

It is currently not known how prenatal nicotine exposure affects single-channel properties of nicotinic receptors. However, it is most likely due to indirect effects of nicotine through phosphorylation changes of amino acid residues in the receptor subunits, as revealed in a study showing that tyrosine phosphorylation of α7 nAChRs can lead to receptor desensitization that may have the potential of determining the channel properties (Hopfield et al., 1988). The deficits in basal synaptic transmission and LTP could be associated with alterations in nAChRs that lend a modulatory role to synaptic transmission and plasticity.

As mentioned earlier, the decline in glutamate transmission could be due a deficiency in the synaptic expression, downregulation of the overall protein levels, or both. We noted a significant reduction in the protein levels of α7 and β2nAChRs in PREN synaptosomal fractions. Furthermore, alterations in the synaptic expression of α7 and β2nAChRs, synaptophysin, SAP102 and PSD-95 seen in PREN rodents could be due to disruption of protein synthesis. In order to address this possibility, we performed Western blot experiments to measure the protein levels in the whole hippocampal homogenates (Figure 7). Remarkably, there were significant differences in PREN whole hippocampal homogenates compared to saline exposed, indicating that PREN effects α7 and β2nAChR, synaptophysin, SAP102 and PSD-95 protein synthesis. The decrease in protein levels of PSD-95 in PREN synaptosomal fractions was noticed. PSD-95 is a scaffolding protein that interacts with nAChRs, mediates downstream signaling in the neurons, and promotes the formation and function of nicotinic synapses on neurons, thereby promoting rapid trafficking of nicotinic receptors on neurons and having physiological significance for synaptic signaling. PSD-95 was also recently implicated in controlling nAChR calcium signaling (Gomez-Varela et al., 2012). Together, PSD-95 and SAP102 in the postsynaptic cell are reported to transcellularly regulate the concurrent release of transmitter from presynaptic terminals onto the neuron while stabilizing postsynaptic nicotinic receptor population under the release sites (Neff, 2009).

Rapid activation and desensitization kinetics is an intriguing characteristic of nAChRs (Seguela et al., 1993; Sudweeks and Yakel, 2000). The activation of nAChRs generates an intracellular signaling cascade that is crucial for hippocampal LTP induction. Indeed, calcium transients via α7 nAChRs has been shown to induce changes in cytoplasmic calcium levels leading to activation of a number of Ca^{2+}-dependent protein kinases, such as mitogen-activated protein kinase (MAPK) (Dineley et al., 2001) and protein kinase A (Dajas-Bailador et al., 2002). Additionally, α7

nAChRs have also been mentioned to be directly coupled to the PI3K signaling cascade (Kihara et al., 2001). Thus, the stimulation of these intracellular signals that associate with α7 nAChRs might sensitize the CA1 neurons for LTP induction (Cohen et al., 1998).

In learning and memory, the MAPK pathway is one of the main phosphorylation cascades, particularly the phosphorylation of extracellular signal-regulated kinase (ERK). It was reported that phospho-ERK (pERK) is induced in the hippocampus following long-term memory consolidation, and that inhibition of ERK pharmacologically prevents long-term memory formation in rodent learning models (Bitner et al., 2007). In contrast, *in vivo* studies have shown that an α7 nAChR agonist, given alone, can elicit enhancement of ERK1/2 and CREB phosphorylation in brain regions (Bitner et al., 2007). Another *in vitro* study has shown the lack of ERK activation in response to agonist alone in PC12 cells could be due to the rapid desensitization of α7 nAChRs. Furthermore, when PNU282987 was applied to cultured hippocampal neurons, it induced a rapid concentration-dependent desensitization seen in inward whole-cell currents and was inhibited by the α7 nAChRs antagonist MLA (Bodnar et al., 2005; Gronlien et al., 2007).

The ERK1/2 phosphorylation is downstream of CaMK II/IV (CaMK II/IV). CaMK II/IV and PKA are major kinases that are regulated by calcium entry through nAChRs and contribute to LTP in the hippocampus (Schmitt et al., 2005; Shen and Yakel, 2009). The cAMP response element binding protein (CREB) is a transcriptional factor activated by ERK1/2 and plays a vital role in LTP enhancement. Our data shows that α7 and β2nAChRs are downregulated (Figure1) and that levels of phosphorylated CaMKII, ERK1/2 and CREB in PREN rodents are decreased (Figure 7) compared to controls. These findings suggest that downstream signaling of nAChRs is affected in PREN rodents and this reduced signaling could be a major mechanism contributing to

the deficits in LTP. In future studies, we will investigate the downstream signaling by using an experimental approach that consists of activity measurement of CaMKII/IV, ERK1/2 and PKA and the analyses of synaptic plasticity in response to selective activation of kinases. The experiments proposed under this specific aim will verify and establish the role of the nAChR/MAPK pathway in altered synaptic plasticity associated with PREN exposure.

In summary, these findings strongly suggest that prenatal nicotine exposure results in potent glutamatergic impairment in the excitatory transmission in the hippocampus due to alterations in nAChRs.

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4- Developmental nicotine exposure leads to impaired N-Methyl-D-Aspartate receptor function and expression

Abstract

Smoking during pregnancy has been reported as a strong risk factor for learning and memory deficits. Consistent with these observations, animal studies from our and other laboratories have shown that developmental nicotine exposure (dNIC) results in learning and memory deficits. Nicotine exposure (6mg/kg /day), given as infusion via mini pumps to pregnant rats, is associated with cholinergic and glutamatergic dysfunctions resulting in memory deficits in pups. In association with N-Methyl-D-Aspartate receptors (NMDARs), neuronal α7/β2 nicotinic acetylcholine receptors (nAChRs) have been implicated in impaired neuronal plasticity. However, the role of synaptic NMDARs and their interaction with α7/β2 nAChRs in cognitive deficits associated with dNIC remain unknown. Here, we report that α7/β2 nAChR activation modulates synaptic NMDARs in control animals and such modulation is impaired in dNIC rodents. The NMDAR-mediated spontaneous current amplitude and frequency were significantly decreased in dNIC hippocampal slices. Deficits in LTP and basal synaptic transmission were accompanied by alterations in function and expression of α7/β2 nAChRs. The single channel open probability of synaptosomal NMDARs was significantly altered in dNIC hippocampi. In addition, there was decreased expression of NMDAR subunit NR1 and NR2a, Ephrine B2 (EphB2), Post Synaptic Density-95 (PSD-95), Brain derived neurotophic factor (BDNF) and Calmodulin Kinase-II (CaMK-II) in dNIC hippocampi. Furthermore, co-immunoprecipitation studies revealed that nAChRs complexed with NR1 and PSD-95. Analysis of within theta burst stimulation facilitation revealed a significant reduction in potentiation in dNIC rodents. These findings indicate that

nAChRs modulate synaptic NMDAR expression and function leading to decreased synaptic plasticity in dNIC rodents.

Introduction

Children of mothers who smoke have been reported to be at higher risk for severe adverse health conditions (Pausova et al., 2007). Chronic neurobehavioral changes, such as learning and memory deficits, and other conditions, including stillbirth and sudden infant death syndrome have been linked to maternal smoking during pregnancy (Ernst et al., 2001a; Ernst et al., 2001b; Linnet et al., 2003; Winzer-Serhan, 2008). Studies have demonstrated that there is a strong correlation between maternal smoking and offspring cognitive outcomes, particularly in younger children (Kafouri et al., 2009). Animal studies from our laboratory and others have confirmed that nicotine exposure during development results in enduring learning and memory deficits that are consistent with, and an accurate model for, the cognitive deficits observed in the children of mothers who smoke (Parameshwaran et al., 2012; Vaglenova et al., 2008).

Developmental plasticity in the cerebral cortex and the hippocampus, depend mainly on cholinergic input that modulates glutamatergic neuronal activity. Disruption of afferent cholinergic projections or acetylcholine process during development reduces synaptic function and plasticity (Patrick et al, 2012). It has been reported that cholinergic basal forebrain neurons damage or their innervations is an initiating event leading to extensive memory loss and neurodegeneration as in Alzheimer's disease (Auld et al., 2002). Cholinergic innervations to the hippocampus originated from the medial septal nuclei and the vertical limb of the nucleus of the diagonal band of Broca in the basal forebrain (Yoshida and Oka, 1995). These cholinergic

projections send afferents to the prefrontal cortex and several locations in the cerebral cortex (Auld et al., 2002; Yoshida and Oka, 1995) and form functional synapses in the hippocampus associated with pyramidal neurons, in addition to granules cells, interneurons, and neurons in the hippocampal hilus, during the first week of postnatal (Frotscher and Leranth, 1985). Developmental consequences of desensitization of these cholinergic fibers and the subsequent stimulation of nicotinic acetylcholine receptors (nAChRs) by cholinergic agonists (unpublished data), packaged and trafficked within synaptic vesicles, have not been well elucidated. There are several reports, however, that suggest gestational exposure to nicotine can lead to impaired learning and alter brain function (Parameshwaran et al., 2012).

Although several harmful substances are present in tobacco, the observed neurobehavioral alterations are mainly due to *in utero* nicotine exposure. Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs) and is the major psychoactive and addictive compound in tobacco. nAChRs play important roles in the formation of neuronal circuitry and the temporally defined activation of other neurotransmitter systems including the glutamate system during embryonic development. The mRNA of α7-nAChRs is expressed, and the receptors become functional, as early as embryonic day 13 (E13) in the rat fetal brain (Leslie et al., 1997). The regulatory role of nAChRs in neuronal circuitry development and synapse formation can be impaired by chronic nicotine exposure during fetal development. During early development, nicotine exposure appears to result in alterations in neuronal morphology including decreased neuronal area in the hippocampal and cortical neurons. The effects of nicotine on the developing brain are confined to certain regions. For example, the cerebellum, which develops late, does not show altered neuronal morphology. In contrast, nicotine has a rapid and long-lasting effect on hippocampal neuronal morphology with a vulnerability period that extends from prenatal to early

postnatal periods (Huang et al., 2007). Nicotine exposure studies have confirmed these morphological changes are a direct consequence of developmental nicotine exposure, as opposed to nicotine-induced placental dysfunctions or hypoxia, as similar results were observed after both pre- and post- natal chronic nicotine treatment (Roy and Sabherwal, 1994).

nAChRs have essential roles in a diversity of CNS processes, including neuronal plasticity. The α7- and β2-subunit containing nAChRs are abundantly expressed in the hippocampus (Alkondon and Albuquerque, 2004). Memory and spatial navigation are believed to be regulated by nAChRs in the hippocampus (Bancroft and Levin, 2000). Glutamate receptors are the main mediators of excitatory neurotransmission. In the hippocampus, glutamate transmission is itself modulated by other neurotransmitter systems. Presynaptic nAChRs are known to facilitate the release of glutamate. The release of glutamate mediated by presynaptic α7- and β2-nAChRs in hippocampal CA1 synapses contributes to synaptic plasticity (Ji et al., 2001; Ge et al., 2005). These findings highlight the importance of α7- and β2-nAChRs in the development of glutamatergic synapses and synaptic plasticity in the hippocampus. Glutamate acts on the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and *N*-methyl-D-aspartate receptors (NMDARs) post-synaptically, ultimately leading to downstream signaling.

Similarly, the importance of NMDAR-mediated neural transmission is demonstrated by its role in models of synaptic plasticity such as long-term potentiation (LTP) , a cellular model of memory encoding, long-term depression (LTD) (Luscher and Malenka, 2012) , and learning and memory (Rezvani, 2006). Therefore, changes in NMDAR function in the hippocampus may further contribute to cognitive deficits associated with prenatal nicotine exposure. In fact, altering their function produces similar cognitive and neurochemical alterations to those seen with prenatal

nicotine exposure (Soman et al., 2012). In addition to changes in NMDAR signaling, cognitive deficits in prenatal nicotine exposure has been associated with altered nAChR function (unpublished data). As mentioned earlier, nAChRs are involved in cognitive processes (Levin, 2002; Levin, 2012) and thus changes in nAChR function could contribute to cognitive deficits seen after prenatal nicotine exposure. However, it is unknown how prenatal nicotine exposure leads to these cognitive deficits. This study reveals that developmental nicotine exposure causes deficits directly associated with changes in nAChR function (unpublished data). In addition, if nAChR- and NMDAR-mediated processes interact, nicotinic receptors could negatively impact NMDAR-mediated cognitive processes.

Interestingly, there are many interactions between nAChR subtypes and NMDARs, these interactions are crucial for physiological and pathological functions. For instance, nAChRs mediate neuroprotection against excitotoxicity induced by NMDAR hyperstimulation in primary hippocampal culture (Dajas-Bailador et al., 2000). Presynaptic nAChR stimulation facilitates glutamatergic transmission at hippocampal synapses (Girod et al., 2000) and enhances LTP in the hippocampus (Ondrejcak et al., 2012). nAChRs activation regulates the downstream turnover of AMPA receptor GluR1 subunit (Rezvani et al., 2007).

NMDAR signaling is critically involved in learning and synaptic plasticity (Rezvani, 2006, (Malenka and Bear, 2004). NMDAR-mediated processes support both short-term and long-term alterations in neural functions underlying changes in short- and long-term memory (Luscher and Malenka, 2012). The cholinergic system is also involved in learning and synaptic plasticity; cholinergic antagonists disrupt learning (Patrick et al, 2012), and agonists such as nicotine enhance learning (Kenney et al., 2012) and synaptic plasticity as measured by LTP (Welsby et al.,

2006). Several evidence suggests that NMDAR-mediated and nAChR-mediated processes may interact during memory encoding (Alkondon et al., 2003; Risso et al., 2004) and changes in these processes may occur during prenatal nicotine exposure, leading to alterations in cognitive function. Thus, understanding how NMDAR- and nAChR-mediated processes interact to alter learning may contribute to a greater understanding of the cognitive deficits that appear in children of mothers who smoke.

In the case of pregnant smokers, the developing fetal brain receives chronic exposure to nicotine. The resulting developmental disruptions to nAChRs can have deleterious impacts on glutamatergic synaptic physiology and plasticity, culminating in impaired cognitive functions. Although impairment of hippocampus-dependent memory forms in developmental nicotine exposed rodents are widely reported, the underlying mechanisms have yet to be fully elucidated. Our initial findings (Parameshwaran et al., 2012) provide the first empirical evidence that hippocampal LTP is impaired in dNIC rodents. In addition, our results also provide evidence that basal synaptic transmission, AMPAR function, and the expression of key pre- and post-synaptic proteins were altered in the hippocampus of dNIC rodents (Parameshwaran et al., 2012). The processes that underlie modifications in these two neurotransmitter systems and that promote these deficits are currently not known.

Increasing evidence has pointed to a pivotal role of brain nAChRs in cognitive functions. However, the underlying mechanism is not fully understood. In explanation of this, we propose that nAChRs may modulate function downstream of NMDAR signaling events in the pathway of LTP. This is supported by our data demonstrating that NMDA receptor-dependent LTPs are inhibited by inhibitors of nAChRs receptors and facilitated by nicotine. In addition, it was

reported that LTP was induced by nicotine treatment even in the presence of a selective NMDA receptor inhibitor by activating downstream signaling of NMDAR (Matsuyama et al., 2000). These studies indicate that nicotinic and glutamatergic interactions have important effects on cognitive functions.

In this set of experiments, we focus our *in vitro* studies on identify means by which developmental nicotine exposure affects nAChRs, primarily the α4β2- and α7-containing nAChRs, and how these changes might influence the expression of NMDAR subunits and other proteins involved in postsynaptic signaling during the development of hippocampal neurons. It has been reported that gestational nicotine exposure results in cognitive deficits in humans (Ernst et al., 2001b). Specifically, in rats a chronic dose of 6 mg/kg of nicotine throughout pregnancy results in long-lasting effects in the performance of reflex, behavioral, and learning and retention tasks (Chistyakov et al., 2010; Parameshwaran et al., 2012; Wang et al., 2011).

In order to better understand the regulatory role that gestational exposure to nicotine has on nAChRs and the consequent effects on postsynaptic signaling in the hippocampus, we measured the synaptosomal protein expression levels of NMDAR subunits and associated synaptic proteins. Receptors, signaling molecules, and scaffolding proteins are highly modulated during developmental periods. We report that nAChRs are downregulated with prenatal nicotine at postnatal day 30 (one month old); glutamatergic NMDARs are also downregulated, and this decreased expression is concomitant with that of nicotinic receptors and multiple post-synaptic proteins. Developmental nicotine exposure also affects glutamatergic synaptic transmission and plasticity and signaling molecules associated with NMDARs. In addition, developmental nicotine

exposure alters the ERK1/2-CREB-BDNF pathway, which is involved in synaptic plasticity. Moreover, gestational exposure to nicotine affects the kinetics and function of NMDA receptors.

Materials and methods

Animals and chemicals: Surgical procedure as previously mentioned in (Parameshwaren.,et al 2007). Two days pregnant Sprague–Dawley rats weighing 150–200g (Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane on day 2 of pregnancy, small incision was made and implanted with subcutaneous osmotic mini pumps (Alzet, Model 2004, Cupertino, CA) to deliver (-)-nicotine (6 mg/kg/day, free base) or physiological saline. After all pups were born, in order to prevent postnatal exposure, osmotic mini pumps were removed under brief isoflurane anesthesia, and the wound was closed with surgical clips. Litters were culled to equal size number per dam with, if possible, an equal sex ratio. All animals were kept in a room at 22–24°C with 12 h light/dark cycles and unlimited access to food and water. Experiments were performed in one-month-old animals and utilized hippocampi collected from animals of this age. Experiments were performed in accordance with the Principles of Animal Care (NIH Publication 85–23, 1985) under animal protocols approved by the Auburn University Institutional Animal Care and Use Committee protocol number 1761). Unless specified, chemicals were purchased from Sigma (St. Louis, MO).

Preparation of hippocampal slices and synaptosomes: Transverse hippocampal slices (350 μm) were prepared as described previously (Parameshwaran et al., 2007) with some modifications. In

brief, hippocampal slices were sectioned while bathed in ice-cold dissection buffer containing (in mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 0.5 ascorbate, and 2 kynurenic acid; the buffer was bubbled with 95% CO_2 / 5% O_2 and the pH was adjusted to 7.4. Hippocampal slices were incubated for one hour in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 11 dextrose; the ACSF was bubbled with 95% CO_2 / 5% O_2. Synaptosomes were prepared per previously described methods in which hippocampi were dissected out and homogenized in homogenizing buffer (mKRBS) using a Potter homogenizer with 10 strokes (Johnson et al., 1997; Suppiramaniam et al., 2006). The mKrebs buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose and 10 mg/ml adenosine deaminase. The pH was adjusted to 7.4 and bubbled with 95% O_2 / 5% CO_2. The buffer was supplemented with 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM Benzamide to minimize proteolysis. The homogenate was filtered through a 13 mm diameter Millipore syringe filter holder which was attached to a 1 cc Tuberculin syringe followed by filtration through three layers of nylon filters (Tetko, 100 µm pore size) and finally collected in a 1.5 ml Eppendorf tube. The filtrate was then loaded into another 1 cc tuberculin syringe and forced through a pre-wetted 5 µm Millipore nitrocellulose filter followed by spinning at 1000 x g for 15 min in a microcentrifuge at 4°C. The supernatant was removed and the pellet, which contained the synaptosomes, was resuspended in 20 µl of mKRBS buffer.

Slice electrophysiology: After slicing and incubation for one hour, electrophysiological recordings were performed in recordings chambers with constant perfusion of ACSF bubbled with 95% CO_2 / 5% O_2. Field excitatory postsynaptic potentials (fEPSP) from Schaffer collateral/commissural-

CA1 synapses were recorded by stimulating CA1 stratum radiatum with bipolar electrodes and placing a recording glass electrode (1-4 MΩ) filled with ACSF approximately 200 μm from the stimulating electrode. The frequency of the test stimulation was once per 20 s. LTP experiment current intensity was set at 50% of the maximal fEPSP. In LTP experiments, after at least 15 min of stable baseline recording, theta burst stimulation (TBS) was performed via the TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ, with an interburst interval of 200 msec. LTP was measured 50-60 min post-TBS.

Single-channel electrophysiology: As previously mentioned in (Parameshwaren.,et al 2007). Incorporation of NMDA receptors from synaptosomal fractions in artificial lipid bilayers was carried out using the 'tip-dip" method. In brief, a phospholipid bilayer was formed at the tip of a polished borosilicate glass pipette (100 MΩ). The synthetic phospholipids were prepared by dissolving 1,2–diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in anhydrous hexane (Aldrich Chemical Co., Milwaukee, WI) to obtain a concentration of 1 mg/ml. Approximately 3-5 μl of synthetic phospholipids were delivered into 500 μl of bath solution containing 125 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, and 5 mM Tris HCl. The pipette solution consisted of 110 mM KCl, 4 mM NaCl, 2 mM NaHCO3, 1 mM MgCl2, 0.1 mM CaCl2, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS) and the pH was adjusted to 7.4. The bilayer formation was initiated by successive transfer of two monolayers onto the tip of the patch pipette in an asymmetric saline condition with "outside-out" configuration. After forming a stable membrane, a 3-5 μl suspension of the synaptosomes was delivered to the ECF. Following addition of NMDARs (290 nM), voltage was applied to evoke single-channel activity. Single-channel currents were filtered at 2 kHz and digitized at 5 kHz (Mini-digi, Molecular Devices)

with pClamp 9 software (Molecular Devices) and saved to a computer hard disk. Only data exhibiting long stretches of single-channel current transition without baseline drifts was chosen for quantitative analysis. The all-points current amplitude histograms were constructed and fitted using the Gaussian method to identify individual conductance levels. The single-channel open probability was computed from the area under the current-amplitude histogram. Log-transformed dwell time count histograms were constructed and fitted with the variable-metric fitting method to identify distinct open and close times.

Western blot analysis: For Western blotting, hippocampal cell lysates were resolved with 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 either anti-α7-nAChR antibody (1:1000; Cell Signaling Technology), anti-β2-nAChR antibody (1:1000; Cell Signaling Technology), anti-β-actin (1:1000; Cell Signaling Technology), anti-NR2A (Cell Signaling, USA), anti-NR2B (Cell Signaling, USA), anti-NR1 (Cell Signaling, USA), p-CAMKII, p-CREB, BDNF or PSD-95 (Santacruz, USA) antibodies overnight at 4 °C. Membranes were probed with the corresponding anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibodies for 1 h (1:5000; Cell Signaling Technology, 1:5000; Cell Signaling Technology) and developed using enhanced chemiluminescence (Super Signal West Femto ECL reagent (Pierce Biochem). All immunoreactive bands were scanned with a desktop scanner and were subsequently quantified by densitometric analyses using Quantity One 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 represents the mean ± SEM. Significance was determined using a two-tailed Student's *t*-test.

Co-immunoprecipitation studies: Male Sprague-Dawely rats (one month of age) rats were euthanized and the hippocampus was dissected and immediately frozen on dry ice. Tissues were homogenized as described previously (Shonsey et al., 2010) 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* (4°C) to remove cellular debris, and the protein content of the supernatants was determined using the BCA protein assay (Pierce, Rockford, IL). α7-nAChRs and β2-nAChRs were immunoprecipitated using anti-α7-nAChR and anti-β2-nAChR antibodies or mouse-IgG (Invitrogen) (negative control), and preconjugated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described above. To examine interactions between nAchRs and NR1 and PSD-95 in the hippocampus homogenates, Western blots were performed using the following antibodies: anti-NR1 (1:1000; Cell Signaling Technology), and anti- PSD-95 (Cell Signaling Technology 1:1000). The blots were stripped and reprobed with anti-α7-nAChR and anti-β2-nAChR antibodies (Cell Signaling Technology 1:1000). To further examine these interactions, NR1 and PSD-95 proteins were immunoprecipitated using anti-NR1 and anti-PSD-95 antibodies or mouse-IgG (Invitrogen) (negative control), and preconjugated with protein A/G PLUS agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) as described above. Western blots were then performed using the nAChRs antibodies. The blots were then stripped and reprobed with anti-NR1 and anti-PSD-95 antibodies.

Whole-cell patch clamp recordings: After the two hour incubation period, slices were transferred to a recording chamber perfused with ACSF solution saturated with 95% CO_2 / 5% O_2 at a rate of 2 ml/min Individual hippocampal slices were visualized by Nomarski differential interference contrast optics with an Olympus BX51WI (Olympus,USA) microscope equipped with a water

immersion lens. Recordings from CA1 pyramidal neurons were made with patch pipettes (5–7 MΩ) 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; the solution pH was adjusted to 7.3 with KOH and the osmolarity was adjusted to 280–290 mOsm. To conserve G protein-mediated responses, GTP was added to 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 (≥ 2 GΩ before breaking into whole cell mode) were achieved. While using the whole-cell voltage-clamp technique, to isolate NMDA-receptor-mediated quantal events (sEPSCs) from CA1 pyramidal neurons 4 µM 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and 30 µM bicuculline methiodide (BMI) were added to the ACSF. Current output was low-pass filtered (2 kHz) and sampled at 10 kHz. Voltage was clamped at -40 mV and the signal was continuously digitized (Digidata 1200) and monitored (Clampfit 9 Software, 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 a student *t*-test. Results are presented as mean ± SEM.

Statistical analysis: Results are given as mean ± SEM. Statistical analysis was performed by using one-way ANOVA with Dunnet test as an appropriate post-hoc test at the significance level $p < 0.05$. All statistical analysis was performed using Prism-V software.

Results:

Prenatal nicotine exposure impairs synaptic plasticity in the Schaffer collateral synapses in one-month old hippocampi

We examined LTP, an accepted cellular correlate of memory encoding, in SC synapses in control and dNIC acute slices by inducing long-term potentiation with theta-burst stimulation (TBS). Our TBS protocol involved 5 trains of 10 bursts of 4 pulses at 100 HZ with an interburst interval of 200 msec. TBS is 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 the average slope, as a percentage of baseline over 55–60-min interval post-TBS showed diminished LTP maintenance in developmentally nicotine-exposed rats (Fig. 1A; 106 ± 5.63%; $p<0.05$, n = 6), whereas the controls showed stable maintenance of LTP (142.79 ± 6.32%; $p<0.05$, n = 6). These data demonstrate that developmental nicotine exposure is associated with impaired synaptic plasticity, suggesting that this impairment may account for the memory deficits. Because LTP was reduced in dNIC rats, we next studied whether impaired induction was a determining factor by analyzing fEPSPs elicited during TBS. 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 dNIC slices compared to control slices (Fig. 1B; $p<0.001$; n=6). We then normalized the first pulse of fEPSPs from 2^{nd}, 3^{rd}, 4^{th} and 5^{th} TBS to that of the 1^{st} TBS to assess whether subsequent TBS resulted in facilitation in dNIC slices. Our results revealed that there was significant facilitation with subsequent TBS in the control whereas such facilitation was largely impaired in dNIC slices (Fig. 1C; $p<0.001$; n=6), suggesting that excitatory

postsynaptic potentials (EPSPs) mediated by the activation of NMDARs are diminished in dNIC slices. These results affirm that nicotine exposure during development results in a depression of depolarization during TBS and thereby is a factor for impairment of LTP in nicotine-exposed rats.

Developmental nicotine exposure alters the synaptosomal nicotinic receptors expression

The most abundant nAChRs in the brain are composed of the homomeric α7 or the heteromeric α4β2 subtype. The nAChRs are known to modulate the strength of synaptic plasticity in the hippocampus (Fujii et al., 1999; Ji et al., 2001). Increasing evidence suggests that NMDAR-mediated and nAChR-mediated processes may interact during learning (Dajas-Bailador et al., 2000 (Alkondon et al., 2003; Risso et al., 2004) and that changes in these processes may occur in prenatal nicotine exposure that involve altered cognitive function. Therefore, using Western blot analysis, we next studied whether nicotine exposure during the prenatal brain developmental period could alter the expression of these receptors in the hippocampus. A reduction in nicotinic receptors expressions were found in prenatal synaptosomal fraction from rodents exposed to nicotine (6mg/Kg) as compared to an equivalent volume of the vehicle (Fig. 2.). We found that (Fig.2. 102.0 ± 1.518, for controls and 50.11 ± 2.890, 80.46 ± 1.804 for α7 and β2-nAChRs respectively in dNIC, *** $p< 0.0001$, ** $p< 0.001$ suggesting ~50% decreased expression of α7 and ~20% reduction in β2-nAChRs in dNIC rodents synaptosomes .

Synaptic currents mediated by NMDARs are modified in rats exposed to developmental nicotine exposure

In the previous study, we reported that AMPAR-mediated synaptic currents were diminished in three-weeks-old animals that were prenatally exposed to nicotine (Parameshwaran et al., 2012). AMPAR synaptic currents are critical for basal synaptic transmission and LTP due to the fact that in Shaffer collateral-CA1 synapses and other major synapses these receptors contribute the majority of the excitatory synaptic transmission. As a result, NMDARs are critically involved in learning (Rezvani, 2006) and synaptic plasticity (Malenka and Bear, 2004). Therefore, we next assessed NMDAR-mediated whole-cell synaptic currents of CA1 pyramidal cells in prenatal-nicotine-exposed rodent slices (in the form of sEPSCs). In prenatal-nicotine-exposed animals, NMDAR-mediated sEPSCs showed a reduction in amplitude and an increase in the interevent interval (Fig. 3. B and C, Table 1;, n=8). A leftward shift in the amplitude cumulative fraction plot and a rightward shift in the interevent interval cumulative fraction plot (Fig.3.D and E) indicate reductions in frequency and amplitude of sEPSCs in dNIC rodents. Furthermore analysis of decay times (τ_d) revealed reductions in the nicotine exposed rats (Table 1). Nicotinic receptor stimulation, either with nicotine alone or with PNU120596 (after GABA block with 2 µM picrotoxin) in saline-exposed control animals, was found to induce a characteristic increase in NMDA receptor-mediated sEPSCs in hippocampal Schaffer collateral-CA1 synapses, shown in Figure3. Spontaneous EPSC frequency and amplitude was significantly increased by nicotine (5 µM), with further larger increases with PNU120596 (1 µM), and reductions induced by MLA (100 nM). These sEPSCs for nicotine and PNU showed respective decay times of 81.28± 2.611, 92.14 ± 6.048 and 141.8 4.331, 155.3 5.743 ms, which differ significantly from decay times of baseline sEPSCs: 64.19 ± 3.121and 128.7 ± 4.055 ms respectively. A rapid desensitization was

observed during the application of higher concentrations of nicotine (30μM). Our data showed that in dNIC rodents, neither nicotine nor PNU affected the deficits in mean current frequency of NMDAR-sEPSCs to any significant extent (Fig.3).

In summary, these results suggest NMDARs themselves are modulated pre- and post synaptically by nicotinic receptors, which are impaired in gestational nicotine exposure rodents. In addition, developmental nicotine exposure induced deficits in NMDA synaptic currents in offspring and might contribute to the cognitive deficits. The results also suggest reduced presynaptic glutamate release and a reduced number of NMDAR and nicotinic receptors in the synapses.

Effects of dNIC on single-channel properties of synaptosomal NMDARs

Alterations in single-channel activity could result in alterations in overall synaptic currents and LTP (Ambros-Ingerson and Lynch, 1993; Parameshwaran et al., 2007; Wijayawardhane et al., 2008). Therefore, we analyzed synaptic NMDAR single-channel currents from synaptosomes reconstituted in lipid bilayers. Results show that channel open probability (Po) in nicotine exposed rats was reduced (4.59 ±1.4%) compared to the controls (31.043 ± 3.2%; Fig. 4A, n=5, $p<0.05$). In addition, the channel conductance was reduced in dNIC from 40ps to 16ps. Furthermore, analyses of dwell times revealed open times (τ_o) were reduced and close times were increased (τ_c) in nicotine exposed rats (Table 2). These results suggest that alterations in single-channel properties of NMDARs may play a role in deficient sEPSCs and LTP in nicotine-exposed rats.

Developmental nicotine exposure is associated with altered NMDA expression and PSD-95, EphB2 and ERK1/2 signaling

Nicotine can modulate the NMDARs by directly activating cell signaling cascades activated by NMDARs. For instance, nicotine has been shown to induce cell signaling cascades involving PKA activation and CREB phosphorylation. CREB is a transcriptional factor activated by ERK1/2 and plays a vital role in LTP enhancement (Chiamulera et al., 2008; Welsby et al., 2006). The ERK1/2 phosphorylation is downstream of CaMK II/IV. Therefore, we next studied whether nicotine exposure during the prenatal brain developmental period could alter the expression of NMDARs in the synaptosomes. Results of the Western blot analysis illustrate that NR1 and NR2A receptors were down regulated, whereas no significant change in NR2B was detected in the synaptosomes of these rats (Fig. 7A; $p^{***}<0.001$; n=6 CTL, 8 dNIC and $p^{**}<0.01$; n=7 CTL, 8 dNIC) respectively). The downregulation of the NR1 subunit will result in a decrease in the number of NMDARs available for synaptic transmission in the mature brain.

ERK1/2 is implicated in synaptic physiology and is specifically associated with strengthening of LTP in the hippocampus (Morozov et al., 2003). This kinase is also involved in the nicotinic enhancement of LTP in the CA1 region of the hippocampus (Welsby et al., 2009). In addition, it is well documented that calcium influx through NMDAR or L-VGCC activates Ras-MEK-ERK1/2 signaling. The activation of ERK1/2 may further upregulate the transcription factor CREB and CREB-mediated gene transcription. These molecular events in the nucleus are required for neuronal function, such as neuroplasticity, differentiation and survival (Peltier et al., 2007). When proteins levels were analyzed it was found that the activated (phosphorylated) forms

of ERK1/2, CAMKII and CREB were reduced in nicotine exposed rat synaptosomes (Fig. 5; $p^{**}<0.01$, $p^{*}<0.05$, $p^{*}<0.05$, n=6 respectively).

PSD-95 is a synaptic scaffolding protein and a member of the membrane-associated guanylate kinase family that is highly abundant in the postsynaptic density that supports the surface expression of AMPA and NMDARs at synapses and other characteristics of synaptic transmission and plasticity. Moreover, PSD-95 was found to affect NMDARs channel properties by reducing the desensitization of NMDAR responses and enhancing NMDAR channel opening characteristics (Lin et al., 2006). This postsynaptic marker was significantly reduced as assessed by Western blots of PSD-95 (Fig. 5; $p^{***}<0.001$, n = 8).

The EphB family of receptor tyrosine kinases is abundant at excitatory synapses and has a crucial role during synapse and spine formation and maintenance (Aoto and Chen, 2007; Klein, 2009). EphB2, a receptor tyrosine kinase that binds and phosphorylates NMDARs, it controls NMDAR subunit localization and function at synapses trafficking (Nolt et al., 2011). In addition, EphB has been reported to modulate NMDAR-mediated calcium influx. Furthermore, activation of EphBs leads to a direct association between EphB and the NMDAR NR1 subunit (Dalva et al., 2000). We found that the level of EphB2 is reduced in dNIC-exposed rodents (Fig. 5; $p^{**}<0.01$, n = 8).

These findings ascertain that prenatal nicotine exposure is associated with alterations in NMDA expression, associated proteins and ERK1/2 signaling.

Nicotinic receptor interacts with NMDAR subunits and their scaffolding proteins

Since dNIC exposure impaired the surface expression of NR1and PSD-95 proteins and affected their synthesis, we hypothesized that nicotinic receptors may physically interact with these proteins and mediate their cellular trafficking. To determine if nicotinic receptors complex with NMDARs subunits and their associated proteins, we conducted co-immunoprecipitation experiments using homogenates from the hippocampus. Nicotinic receptors subunits α7 and β2 antibodies were sufficient to immunoprecipitate the NR1 and PSD-95 proteins (Fig.6. A and B, n=6).

Effects of dNIC on nicotinic receptor interactions with NMDAR subunits and their scaffolding proteins

In order to test whether dNIC exposure can impair the interaction of nicotinic receptor with NR1 and PSD-95 and thereby reduce their synaptic membrane availability, we performed co-IP experiments in control and in dNIC-exposed acute hippocampal slices using anα7-nAChR antibody. We found a significant decrease in the co-immunoprecipitated protein levels of NR1and PSD-95 proteins from dNIC slices compared to control (Fig 8. A and B, 100.0 ± 5.217 and 100.0 ± 6.893, for controls and 60.59 ± 3.866, 47.08 ± 13.33 for dNIC hippocampal homogenates; (**p<0.01 and p^{*}<0.05 respectively, n=6).

Figures:

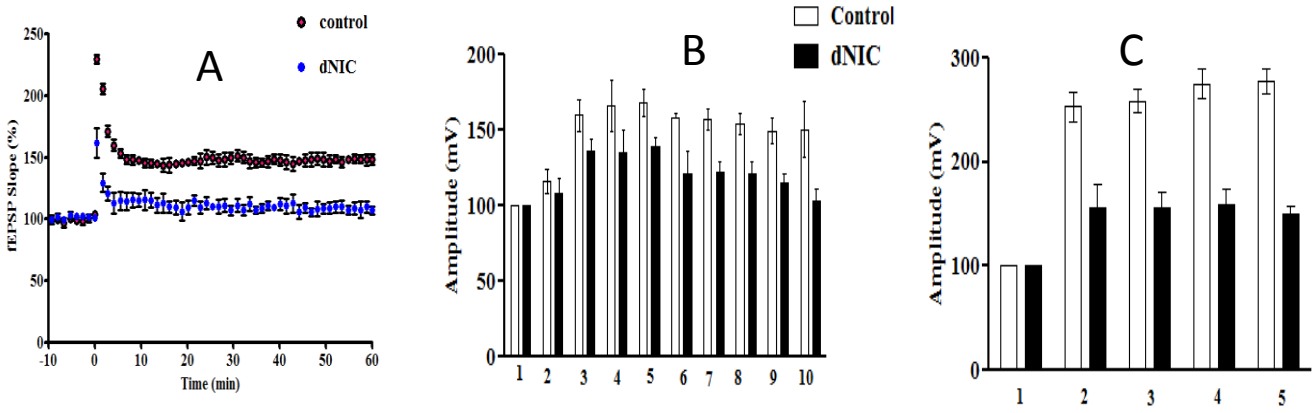


Figure 4.1 Effect of developmental nicotine exposure on hippocampal LTP. (A) LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP in dNIC acute hippocampal slices were reduced compared to control slices. Normalized fEPSPs slopes of 60 min post-TBS averaged 140.6% ± 4.297% for control and 109.5% ± 2.187% for dNIC slices, ($p^{**} < 0.001$; n= 6). (B). 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 dNIC treated slices ($p<0.001$; n=6). (c) In the control but not in dNIC slices, tetanic facilitation occurred with each successive train ($p<0.001$; n=6).

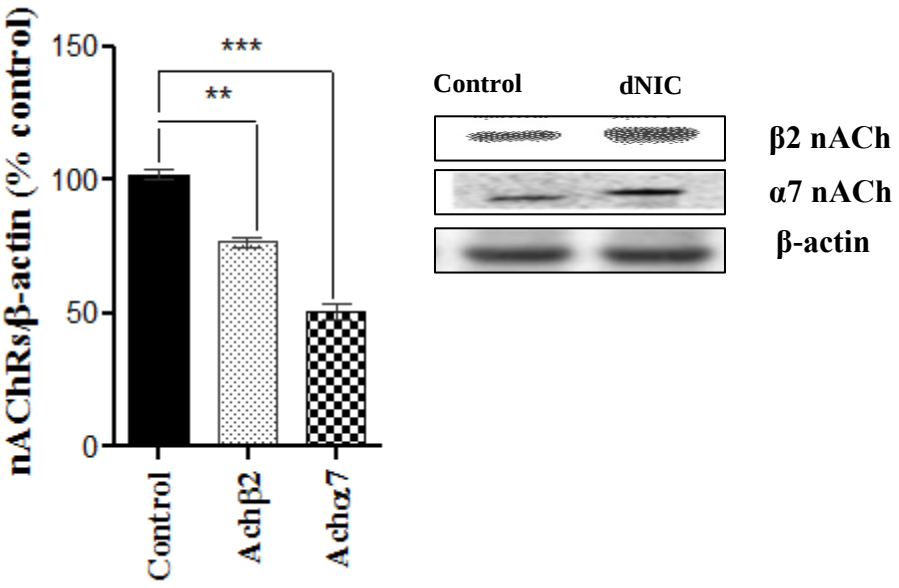
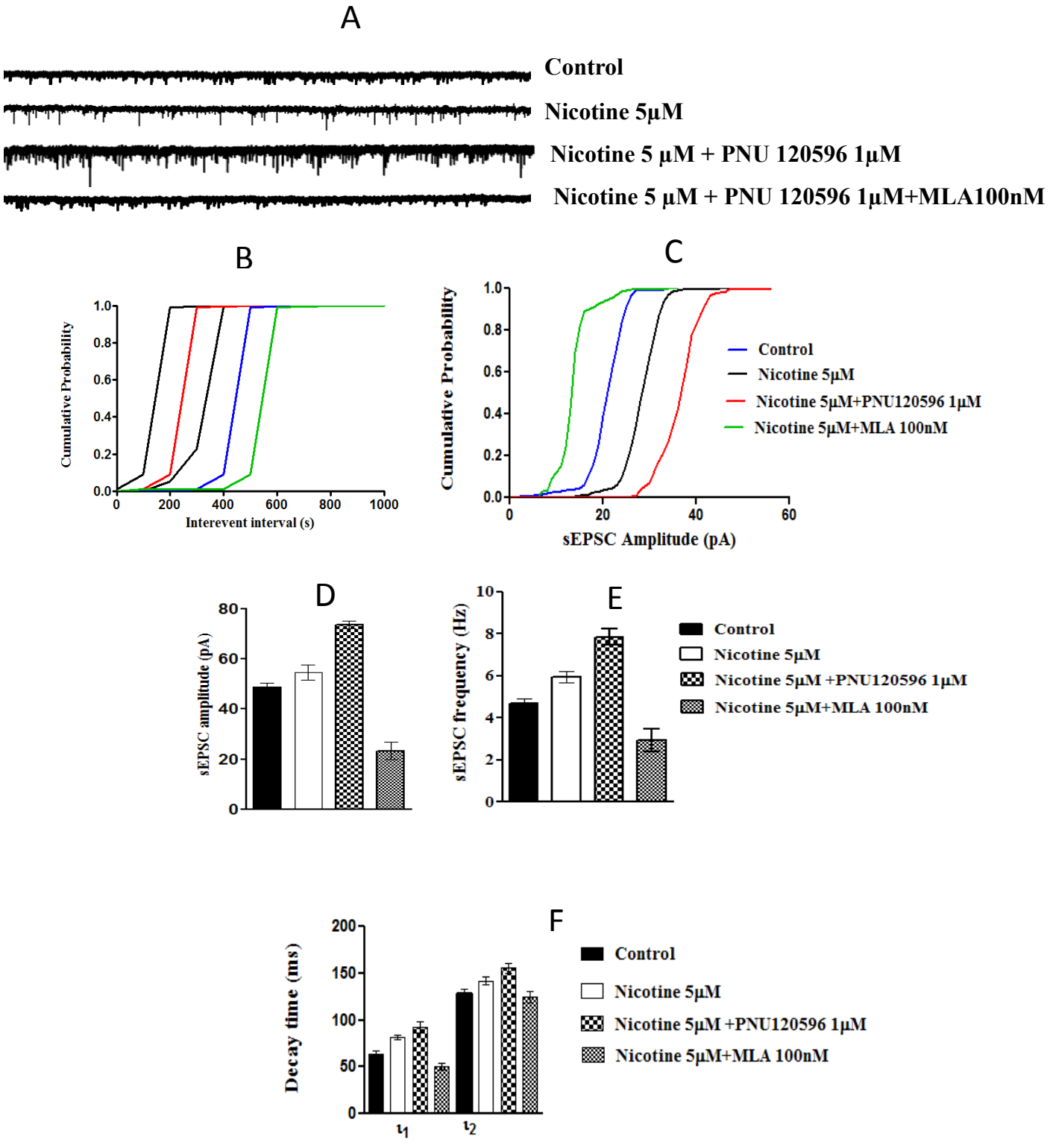


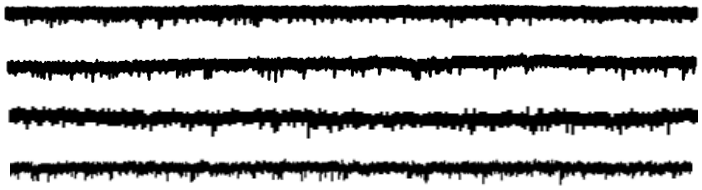
Figure 4.2 Effect of developmental nicotine exposure on protein levels of synaptosomal α7-nAChR and β2-nAChR in dNIC offspring rodents. Western blot analysis shows α7-nAChR expression was significantly down regulated by 50% following developmental nicotine exposure (***P<0.001, n=8 CTL, 8 dNIC). Whereas, the level of β2-nAChR expression in the nicotine group was significantly decreased by 20% (**P<0.01; n=8 CTL, 8 dNIC). Values are the mean±SEM relative to saline-exposed controls. Protein levels in the control and nicotine exposed rodents' synaptosomes lysate. The density of α7-nAChR and β2-nAChR protein levels are normalized to β-actin and expressed as a percentage of the control group which was set as 100%. Typical Western blots for each treatment are shown in band pairs above the bars (left band: control; right band: dNIC). Abbreviation CTL=control, dNIC= developmental nicotine-exposed, n=the number of pups used for each bar, two-tailed, unpaired Student's t-test.

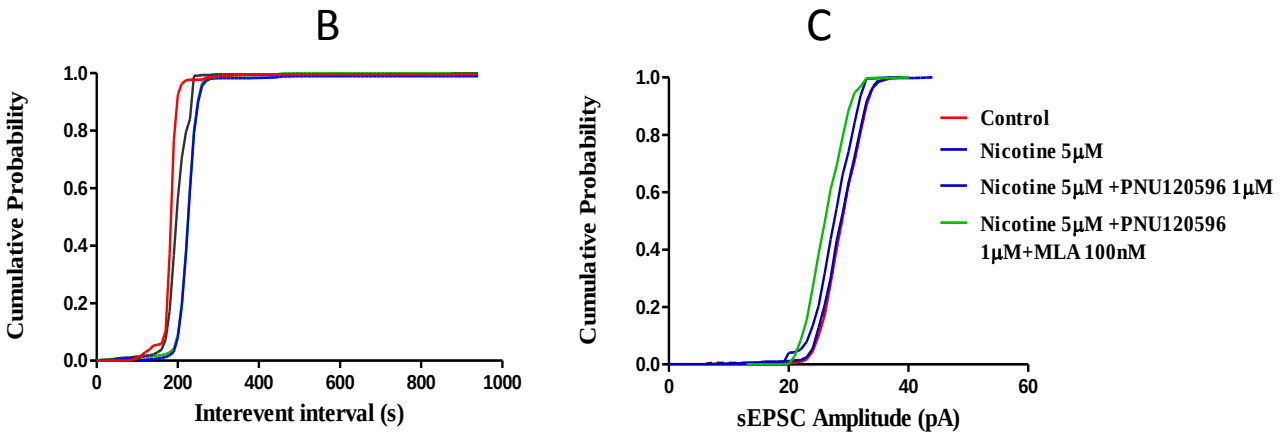
Developmental saline exposed rodents group (Control)

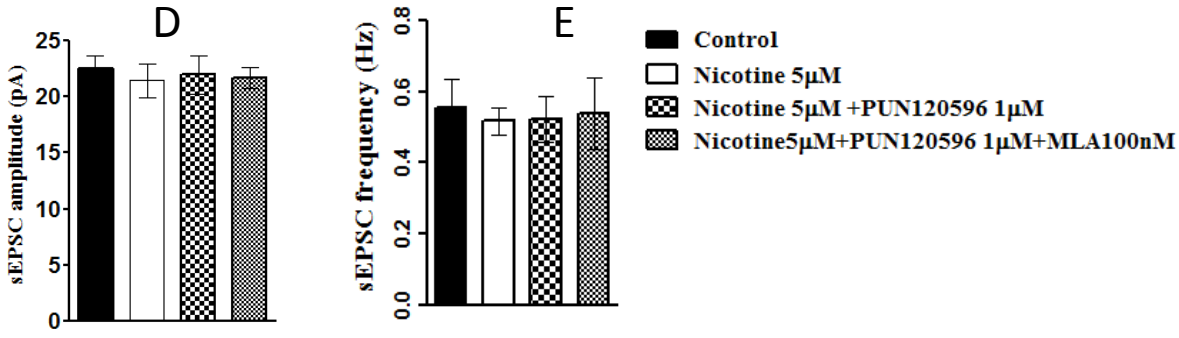


Developmental nicotine exposed group

A







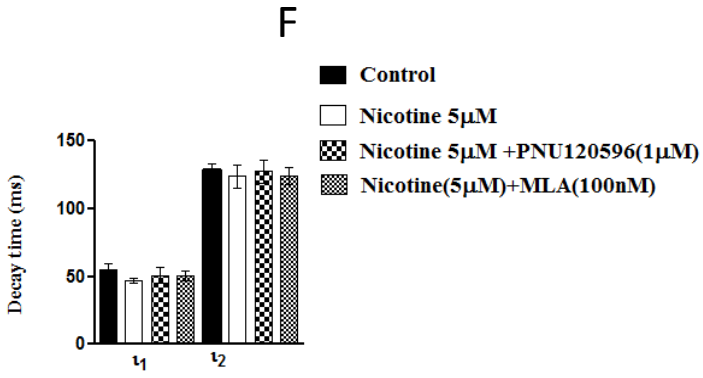


Figure 4.3 Effect of nicotinic receptor activation on NMDARs-mediated whole cell synaptic currents. (A) Sample sweeps show NMDARs-mediated sEPSC recorded at -40 mv membrane potential from a representative neuron showing increase in amplitude and frequency, in saline-nicotine treated slices and further increase with PNU120596 compared to control- saline slices and with no change in dNIC among the four groups. (B) Cumulative probability of distributions of amplitude shows a significant shift of nicotine treated curve to the right from the controls, suggesting increase in amplitude compared to dNIC group which shows no change. (C) Cumulative probability of distributions of interevent interval shows a significant shift of nicotine and /or PNU-saline treated curve to the left from the control-saline treated, implying increase in frequency of sEPSCs in the saline treated group curve compared to dNIC group which shows no change in interevent interval among the four PREN group. (D and E) Bar chart shows the significant increase in mean amplitude and frequency of NMDARs-mediated sEPSCs in nicotine and/or PNU –saline treated slices than that of saline-control ($p< 0.01$; n= 6) compared to dNIC group which shows no significant change. (F) The decay times of NMDARs-mediated sEPSCs were fitted with two terms. Decay times were depict a significant increase in NMDARs-mediated sEPSCs decay times in nicotine and /or PNU saline-treated slices than that of control (Student's *t*-test, $p<0.05$; n=6) compared to dNIC group which shows no change in decay time among the four dNIC group ($p< 0.01$; n= 6).

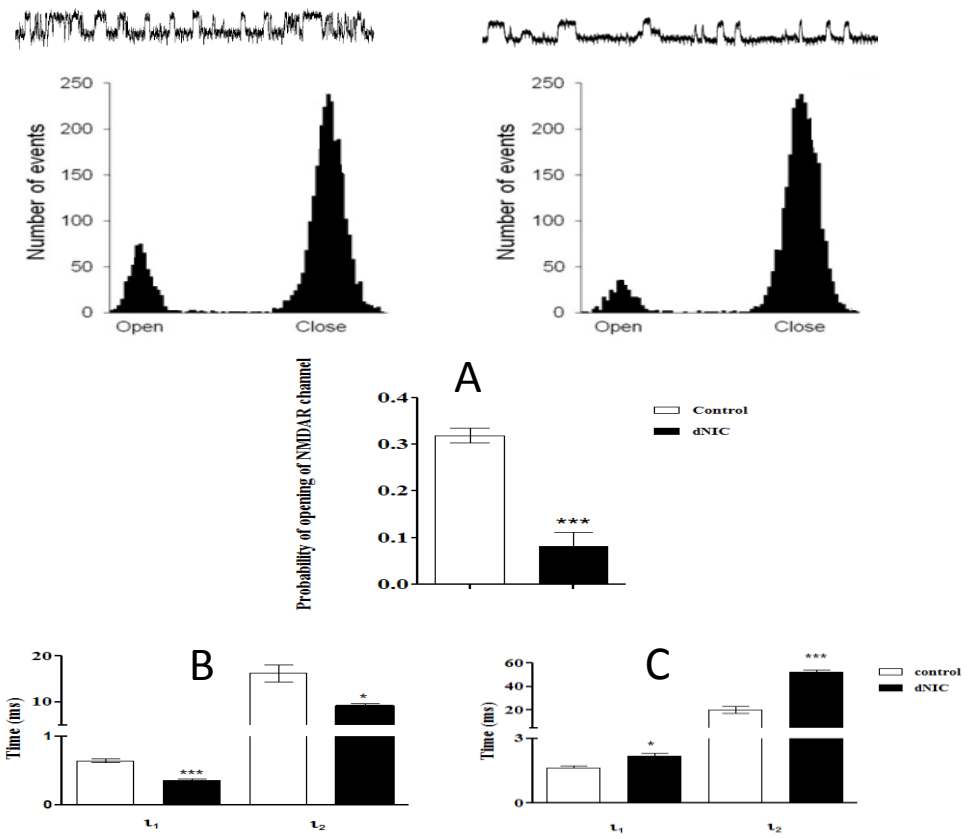


Figure 4.4 Developmental nicotine exposure is associated with alterations in the single channel properties of hippocampal synaptic NMDARs. (A) Bar plot showing significantly reduced channel open probability in developmental nicotine-exposed rats compared to the controls. Representative traces and amplitude histogram shown above illustrate the reduced channel activity (upward deflections) in the d (NIC) trace. (B) Bar plot illustrating significantly reduced open times (τ1 and τ2) in nicotine-exposed rats (C) Bar plot showing significantly increased close times in developmental nicotine-exposed animals compared to saline exposed rodents. (n = 5, $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$ versus control by two tailed student's t test).

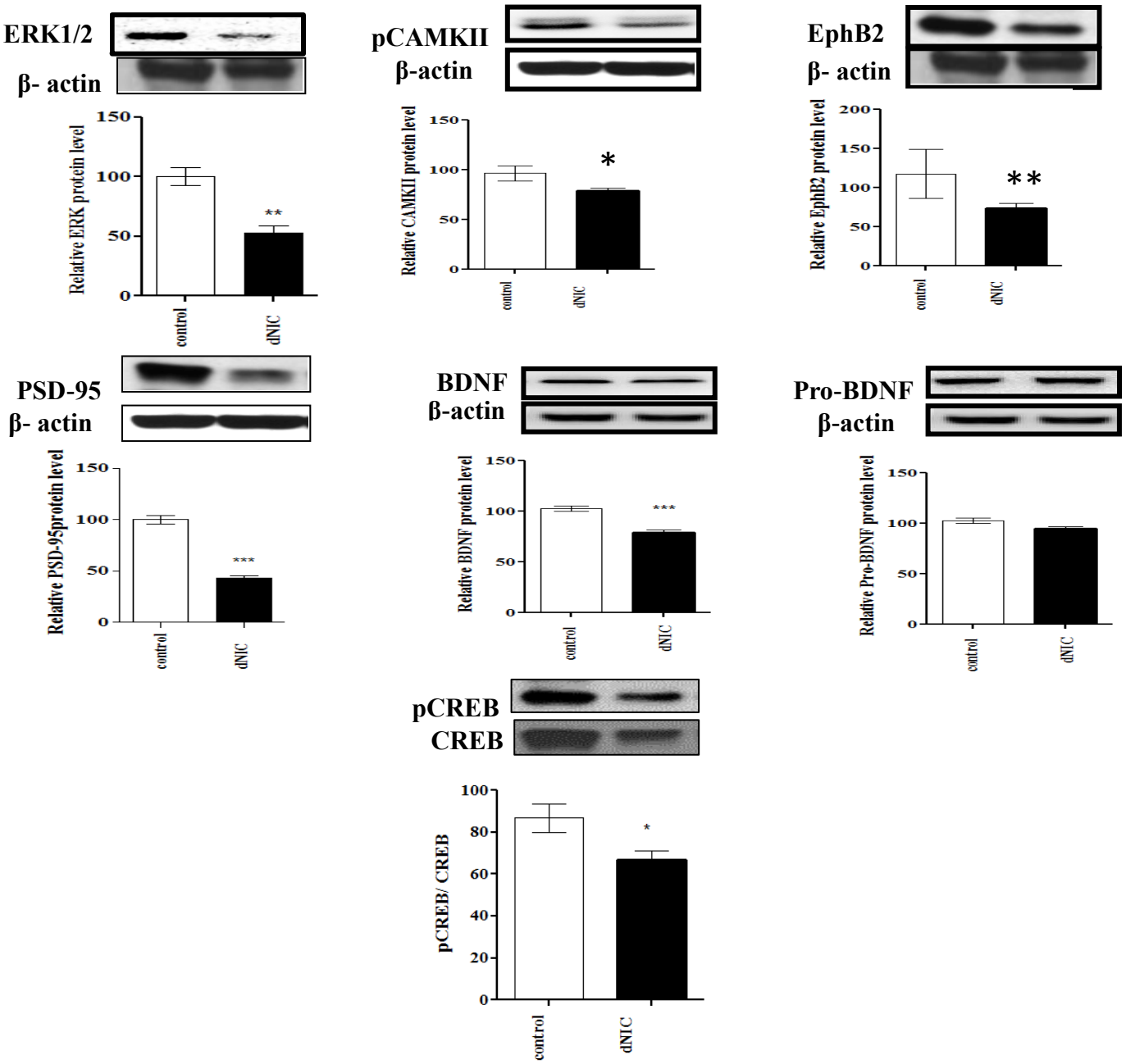


Figure 4.5 Effect of developmental nicotine exposure on regulation of hippocampal postsynaptic markers (ERK1/2, PSD95, BDNF, Ephβ2, CREB and CaMKII) in rodents. (A) Western blot analysis shows ERK1/2 expression was significantly downregulated by 56% following prenatal nicotine exposure (**P<0.01, n=8 CTL, 8 dNIC). (B) The level of PSD95 expression in the nicotine group was significantly decreased by 44% (***P<0.001; n=8 CTL, 8 dNIC). (C) Ephβ2 expression was significantly down regulated (**P<0.01; n=6 CTL, 6 NIC). (D)

CaMKII and CREB expressions were significantly down regulated (*****P<0.05; n=8 CTL, 8 dNIC). (E) BDNF expression decreased by 24% (*******P<0.001; n=6 CTL, 6 NIC).(F) Pro-BDNF expression no significant change was found. Values are the mean±SEM relative to saline-exposed controls. Protein levels in the control and nicotine exposed rodents' synaptosomes. Protein levels are normalized to β-actin and expressed as a percentage of the control group which was set as 100%. Typical Western blots for each treatment are shown in band pairs above the bars (left band: control; right band: dNIC). Abbreviation CTL=control, dNIC= developmental nicotine-exposed, n=the number of pups used for each bar, two-tailed, unpaired Student's t-test.

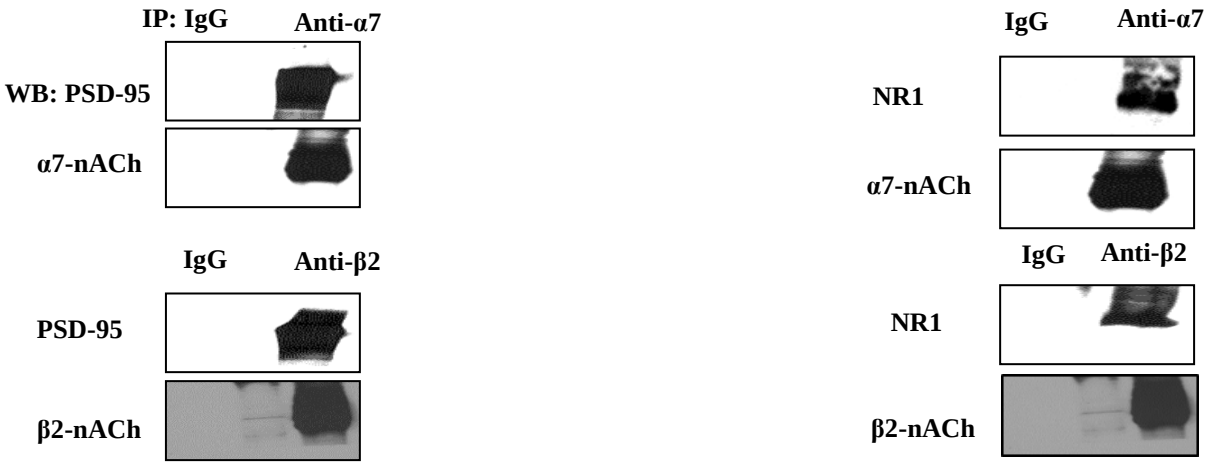


Figure 4.6 Co-immunoprecipitation of nAChR with NR1 and PSD-95 in the hippocampus homogenate. In the hippocampus, anti- α7 & β2nAChR antibody co-immunoprecipitated with (A) NR1 and (B) PSD-95. Detection was done by performing western blot analysis using anti-NR1 and anti- PSD-95 antibodies respectively. (F) Blots were stripped and reprobed with anti- α7 & β2nAChR antibodies. The IgG antibody served as a negative control (n=6).

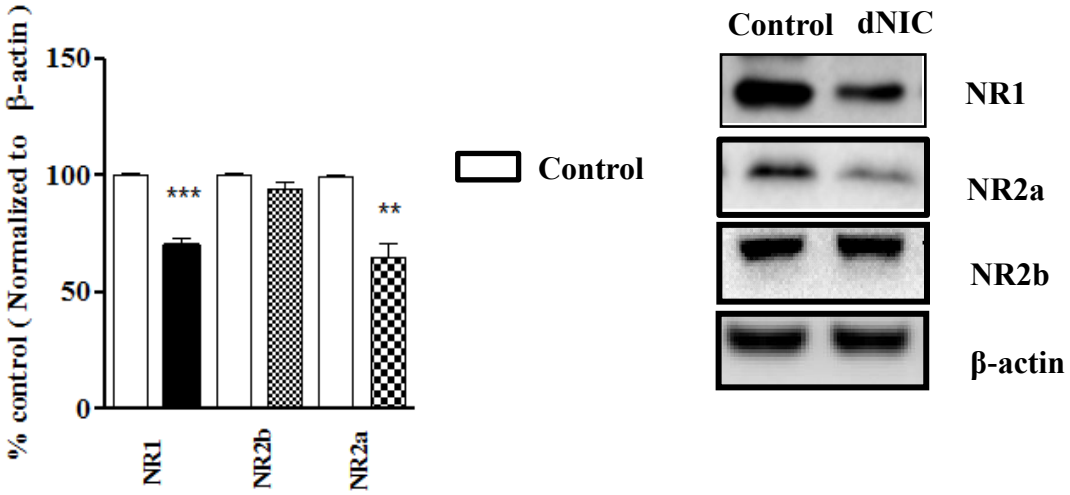


Figure 4.7 Developmental nicotine exposure influences NMDAR subunits (NR1, NR2a, NR2b) in the developing rat synaptosomes. (A) Western blot analysis shows a significant nicotine exposure-reduce the expression of NR1(30% decrease from control) (***P<0.001; n=6 CTL, 8 dNIC), and NR2b expression shows no significant regulation presence of nicotine exposure (n=6 CTL, 8 dNIC). Whereas NR2a expression was 38% decrease from control (**P<0.01; n=7 CTL, 8 dNIC). Values are the mean±SEM relative to saline-exposed controls. 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 beside the bar graphs. (left band: control; right band: dNIC). Abbreviation CTL=control, dNIC=nicotine-treated, n=the number of pups used for each bar, two-tailed, unpaired Student's t-t.

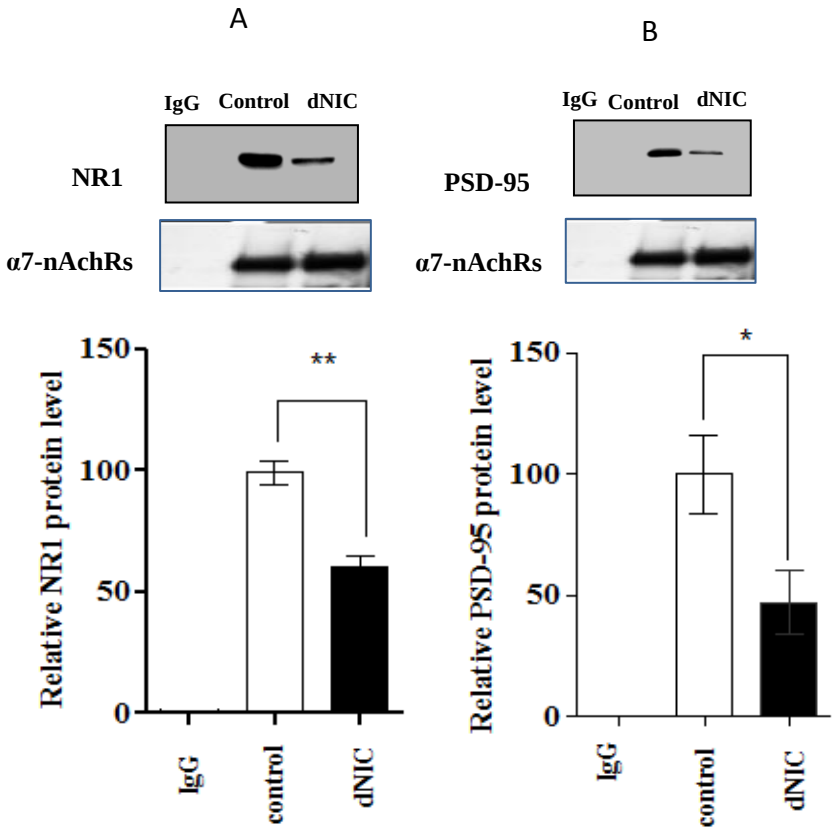


Figure 4.8 Co-immunoprecipitation of α7-nAchRS with (A) NR1 and (B) PSD-95.in the hippocampus was disrupted by developmental nicotine exposure. We quantified protein amounts of NR1 and PSD-95 co-immunoprecipitated with anti- α7-nAchRS antibody in the homogenates prepared from control and dNIC hippocampi by performing western blot analysis. Protein levels are normalized to α7-nAchRS and expressed as a percentage of the control group which was set as 100%. Quantity of NR1 and PSD-95 were significantly reduced in dNIC hippocampal homogenates as compared with control (p^{**}<0.01 and p^{*}<0.05 respectively, n=6, two-tailed, unpaired Student's *t*-test).

Table 1: Properties of NMDA mediated sEPSCs recorded from CA1 pyramidal neurons in the hippocampus of control and prenatal nicotine exposed rats.

	Control	Nicotine	Nicotine+PNU	MLA	dNIC	Nicotine	Nicotine+PNU	MLA
Amplitude (pA)	**50.60 ± 2.612**	**67.92 ± 3.899***	**86.21 ± 3.242***	**23.28 ± 3.378*****	**22.55 ± 1.129**	**21.44 ± 1.482**	**21.94 ±1.742**	**21.67 ± 0.9122**
Frequency (Hz)	**4.697 ± 0.2075**	**5.933 ± 0.2603***	**7.838 ± 0.3823***	**2.937 ± 0.5339****	**3.697±0.2075**	**3.933 ± 0.3180**	**3.938 ± 0.2819**	**3.637 ± 0.1637**
Decay time (ms):								
τ_{d1}	**64.19 ± 3.121**	**81.28 ± 2.611***	**92.14 ± 6.048**	**50.38 ± 3.431**	**48.86 ± 5.811**	**46.61 ± 1.830**	**50.81 ± 5.896**	**50.38 ± 3.431**
τ_{d2}	**128.7 ± 4.055**	**141.8 ± 4.331***	**155.3 ± 5.743**	**124.4 ± 6.133**	**132.7 ± 4.333**	**123.8 ± 8.595**	**127.3 ± 8.460**	**124.4 ± 6.133**

*p<0.05, **p<0.01 or ***p<0.001 versus control by two tailed student's t test

Table 2: Single channel properties of synaptic NMDARs in the control and prenatal nicotine exposed rats.

	Control	dNIC
Open time (ms):		
τo_1	**0.6436±0.02924**	**0.3594±0.01744***
τo_2	**16.27 ± 1.847****	**9.233 ± 0.3930** *
Close time (ms):		
τc_1	**1.628 ± 0.09548**	**2.167 ±0.1453***
τc_2	**20.26 ± 2.916**	**52.37±1.994*****
Conductance	**40 Ps**	**16Ps**
PO	**31.043 ± 3.2%**	**4.59 ±1.4%**

$^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$ versus control by two tailed student's t test

Discussion:

This study is based upon recently published work demonstrating that basal synaptic transmission and LTP is impaired in dNIC-exposed rat hippocampi (Parameshwaran et al., 2012). These results suggest that nicotine exposure during development affects the synaptic transmission mediated largely by glutamate receptors in the postnatal brain. Previous findings in our laboratory and others have established that nAChRs are modulators of synaptic transmission and plasticity in the hippocampus. Therefore, nicotinic–glutamatergic interactions may have important implications in cognitive functions.

Some evidence suggests that NMDAR- and nAChR-mediated signaling may interact during learning. Changes in these processes may occur in prenatal nicotine exposure involving altered cognitive function. In the current study is reported that NMDA receptor-dependent LTP is inhibited by inhibitors of nAChRs and that nicotine facilitates hippocampal synaptic transmission and induces LTP. In addition, it was reported that activation of nicotinic receptors with nicotine was able to maintain the induction of LTP in the presence of a selective NMDA receptor inhibitor by activating the downstream signaling of NMDARs (Matsuyama et al., 2000). Thus, understanding how NMDAR- and nAChR-mediated processes interact to alter learning will contribute to greater understanding of cognitive deficits in children born to mothers who smoke.

In the present study, we propose to investigate whether nicotine exposure during development may modulate the function and/or downstream expression of NMDAR signaling events in the pathway of LTP, the prevalent cellular correlate of memory (Malenka, 1994; Lynch, 2004; Citri and Malenka, 2008), through nAChR alteration which is abundantly expressed in the hippocampus.

This study demonstrates that developmental nicotine exposure impairs synaptic NMDAR function and that this contributes to the cognition deficits that appear in children of mothers who smoke. We used a sensitive and proven animal model which exhibits behavioral and cognitive impairments associated with dNIC exposure (Parameshwaran et al., 2012). Our experiments reveal two main findings: (1) Developmental nicotine exposure resulted in impaired synaptic transmission by altering the single-channel properties of synaptic NMDA through alteration of nAChRs function, and (2) NMDAR and nAChR-mediated processes may interact during learning and memory.

Therefore, our results suggest that altered expression and function of nAChRs have deleterious impacts on glutamatergic synaptic physiology and plasticity, leading to cognitive dysfunction as a result of developmental nicotine exposure.

Developmental nicotine exposure impairs LTP in offspring rodent hippocampi

Our recent study demonstrated that nicotine exposure during fetal development decreases the protein expressions of α7- and β2-nAChRs in rodent synaptosomes. These results raise the possibility that altered expression and function of nAChRs may have deleterious impacts on glutamatergic synaptic physiology and plasticity, culminating in impaired cognitive functions.

In the current study, theta burst stimulation demonstrated diminished LTP maintenance in developmental nicotine-exposed rats (Fig. 1). These data demonstrate that nicotine exposure during development is associated with impaired synaptic plasticity, which is responsible for the memory deficits.

As shown earlier in our unpublished data, and in agreement with the data obtained from other reports, exposure with PNU 120569 in the presence of nicotine, or 5-iodo in saline exposed control slices showed enhanced LTP in the CA1 hippocampal region. The results of our study indicate that the enhancement in LTP observed in control slices was due to the activation of nicotinic receptors. This effect was reduced by the application of MLA or macylamine. The administration of nicotine and/or PNU120569 to developmental nicotine-exposed slices failed to induce hippocampal LTP. The present findings provide direct evidence highlighting the importance of nAChRs in the development of glutamatergic synapses and synaptic plasticity in the hippocampus.

Within TBS facilitation analysis revealed a significant reduction of potentiation in dNIC-exposed hippocampal 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 dNIC slices. This suggests that excitatory postsynaptic potentials (EPSPs) mediated by the activation of NMDARs, which are critically involved in learning and synaptic plasticity (Malenka and Bear, 2004) are diminished in dNIC slices.

Developmental nicotine exposure attenuates NMDAR-mediated synaptic currents in offspring rodent hippocampi

From our previous study, recordings of sEPSCs from CA1 neurons in postnatal hippocampal slices showed marked reduction in the amplitude and frequency of AMPAR-mediated EPSCs after prenatal nicotine exposure, indicating that there may be selective loss or downregulation of AMPARs at those synapses (Parameshwaran et al., 2012).

Similarly, in the mammalian hippocampus, NMDARs are involved in synaptic plasticity. The downregulation of the NR1 subunit (Fig. 7) will decrease the number of NMDARs available for synaptic transmission in the mature brain. Therefore, we next assessed NMDAR-mediated synaptic currents in dNIC-exposed rodent hippocampal slices. Our results revealed that developmental nicotine exposure decreases the NMDARs-EPSCS in CA1 hippocampal pyramidal cells. The mean current amplitude and frequency of NMDA-mediated sEPSCs were significantly attenuated in dNIC slices compared to saline-exposed control. Interestingly, administration of nicotine and/or PNU120569 did not restore the impairment in the NMDAR-mediated sEPSCs mean current amplitude and frequency, whereas the NMDARs-mediated synaptic currents amplitude and frequency in saline-exposed control slices were affected by this treatment (Fig. 3). An explanation for this observation is that nicotine acts presynaptically on α7 nAChRs to enhance, or elicit glutamate release and that the released glutamate acts on postsynaptic NMDARs. Furthermore, it acts post-synaptically to enhance the activity of NMDAR. NMDARs are both ligand and voltage gated (Clarke and Johnson, 2008). Therefore, binding of glutamate to an NMDAR is inadequate to gate calcium ion influx alone as the membrane needs simultaneous depolarization to remove the magnesium block in the ion pore. This depolarization, along with glutamate binding, will trigger an influx of calcium through NMDAR ion channels (Clarke and Johnson, 2008). Glutamate binding to AMPARs are thought to mediate the necessary depolarization for NMDAR activation (Rao and Finkbeiner, 2007). However, it was reported that nAChRs may act in a similar manner to AMPARs (Gould and Lewis, 2005). This was supported by the fact that, specifically in hippocampus, postsynaptic currents were present in pyramidal cells even after antagonism of NMDARs and AMPARs. These residual currents were completely inhibited by broad-spectrum nAChR antagonism by *d*-tubocurarine, and partially inhibited by α7-

specific antagonists MLA and α-bungarotoxin (Hefft et al., 1999). These data demonstrate the contribution of nicotinic receptors in activating glutamatergic receptors. As shown in our previous study, there was impairment in function and expression of AMPARs contributing to the cognitive deficits in the prenatal nicotine exposed animals and the decreased expression and function of nicotinic receptors may further explain the deficits. The presence of nAChRs postsynaptically (Alkondon et al., 2003), may have a contribution to the depolarization required for NMDA activation in the CA1-CA3 region.

This consistent with our studies demonstrating that, NR1 subunit interacts with nicotinic receptors as revealed by immunoprecipitation experiments. Thus, nAChRs may contribute to the activation of NMDAR signaling by providing the postsynaptic depolarization necessary for NMDAR activation. In support of this notion, immunocytochemical evidence that α7nAChRs are prevalent at the postsynaptic sites of glutamatergic synapses in the hippocampus and neocortex, along with electrophysiological evidence for postsynaptic nicotinic currents in the neocortex and hippocampus, has incited speculation that the α7nAChR allows for an activation of NMDARs post-synaptically (Levy et al., 2006).

Developmental nicotine exposure attenuates single-channel currents of synaptic NMDARs in offspring rodent hippocampi

In the above-mentioned study, it was demonstrated that nicotine exposure during development decreases the NMDARs-EPSCs in CA1 hippocampal pyramidal cells. The mean current amplitude of single-channel currents is a product of single-channel conductance (g) and the probability of opening. Therefore, a reduction in conductance or opening probability will reduce

the mean current amplitude of the single synaptic receptors (Kanju et al., 2007). In this experiment, the single-channel data obtained by performing single-channel recordings of NMDAR current using synaptosomes isolated from saline-exposed control and dNIC-exposed acute hippocampal slices, indicated that developmental nicotine exposure reduces channel open probability, conductance, and mean open time while increasing the channel closing time (Figure 4). These outcomes support the fact that prenatal nicotine exposure significantly reduces the NMDA-mediated synaptic transmission by modulating single channel kinetics of synaptic NMDARs.

As a result of altering the single-channel properties of synaptic NMDARs, the amplitude of the spontaneous currents will be decreased if any of previous mentioned parameters is reduced for single synaptic receptors. This alteration leads to modification in synaptic transmission. Therefore, due to the decrease in the conductance and opening probability, it is not surprising that there is impairment in LTP in offspring rodents exposed *in utero* to nicotine due to the alteration of single synaptic receptor properties. In addition, a decline in the single-channel NMDAR open time can result in the fast decay of synaptic current observed in NMDAR-sEPSC measurements in developmental nicotine exposed hippocampal slices.

Developmental nicotine exposure reduces NMDA subunits and synaptic protein in offspring rodent synaptosomes.

The NR1 receptor subunit of the heteromeric NMDA receptor is the critical subunit for the ionophore function of gated calcium ions. The NR1 subunit has multiple isoforms that have different pharmacologic functions (Clarke and Johnson, 2008). NMDA receptors lacking the

NR1 subunit are unable to gate calcium and thus do not function efficiently. This altered receptor mechanism reduces NMDA-mediated neural transmission and consequently synaptic plasticity.

The decline in NMDAR-sEPSCs synaptic current amplitude could be also due to decreased synaptic expression of NMDARs (Conti and Weinberg, 1999). Because the NR1 subunit of the NMDA receptor is critical to full receptor activity and assembly, a reduction of NR1 in the hippocampus in dNIC-exposed rodents suggests a functional impairment in glutamatergic transmission at the NMDAR, resulting in synaptic plasticity modification.

In addition, we also found a decrease in protein level of NR2A receptors in dNIC-exposed rodent hippocampi, yet no significant change in NR2B protein levels compared to control rodents (Figure 7). In the hippocampus, the NR2A subunit is believed to be involved in the induction of LTP, whereas the NR2B subunit contributes to the formation of LTD (Toyoda et al., 2005). Both contain the binding-site for the neurotransmitter glutamate. In the present study we identify alterations in the molecular signaling pathway that is strongly linked to LTP and glutamatergic receptor expression and translocation. We found a significant decrease in the phosphorylated state of hippocampal CREB and ERK1/2 MAP kinase (Fig. 5), both of which have been shown to activate transcription factors involved in learning and memory. In addition, we observed a significant decrease in the protein level of PSD-95 in dNIC-exposed rodent synaptosomal fractions. PSD-95 is a scaffolding protein that promotes clustering and synaptic expression of AMPARs (Bats et al., 2007), nAChRs, and NMDARs. PSD-95 can also affect NMDAR channel properties by reducing the desensitization of NMDAR responses and enhancing NMDAR channel openings (Lin et al., 2006; Lin et al., 2004). Similarly, the reduction in the protein level of PSD-95 in dNIC synaptosomal fractions can explain the decrease in the synaptic expression of NR1

and NR2A NMDAR subunits. Moreover, it could explain the reduction in single-channel NMDAR open probability and open time.

Nicotinic receptors interact with NMDAR subunits and their scaffolding proteins

Nicotinic stimulation enhances the synaptic transmission and modulates NMDARs-EPSC in control hippocampal slice. In addition, dNIC impaired the expression of NR1, NR2A and PSD-95 proteins and synaptic plasticity. Therefore, it is probable that nicotinic receptors can interact with these proteins at their synaptic sites and affect their synaptic localization. In order to test this hypothesis, we studied the interaction between nicotinic receptors and NR1 and PSD-95 by performing co-immunoprecipitation studies in the hippocampus. Our data revealed that nicotinic receptors form complexes with NR1 and PSD-95 (Figure 6). Interestingly, this interaction is significantly disrupted in dNIC rodents (Figure 8). Therefore, it is reasonable to assume that prenatal nicotine exposure is responsible for disturbing the interaction of nicotinic receptors with NMDARs and their associated proteins, resulting in impaired synaptic expression.

Developmental nicotine exposure modifies NMDA cell signaling in offspring rodent hippocampi

Nicotine can also modulate the NMDARs by directly activating cell signaling cascades activated by NMDARs. Both nAChRs and NMDARs gate calcium influx (Jahr and Stevens, 1993; Letz et al., 1997). It is possible that nicotine modulates NMDAR, thus enhancing learning and memory, by gating a sufficient amount of calcium to directly activate learning-related cell signaling cascades (Dajas-Bailador and Wonnacott, 2004). For instance, nicotine has been shown to induce

cell signaling cascades involving PKA activation and CREB phosphorylation. The cAMP response element binding protein (CREB) is a transcriptional factor activated by ERK1/2 and plays a vital role in LTP enhancement (Rao and Finkbeiner, 2007; Welsby et al., 2006). One of the mechanisms by which nAChRs may also modulate learning is through activation of the extracellular regulated kinase 1/2 (ERK1/2) cascade, a member of the mitogen activated protein kinase family of proteins that are involved in synaptic plasticity and hippocampus-dependent learning (Welsby et al, 2006). Consistent with published results from other lines of research, inhibition of ERK1/2 blocked the nicotine enhancement of LTP (Welsby et al, 2009). The ERK1/2 phosphorylation is downstream of calmodulin-dependent protein kinases II/IV (CaMK II/IV). Calcium influx from NMDA receptors is necessary for the activation of CaMKII. Similarly, CaMK II/IV and PKA are major kinases regulated by calcium entry through nAChRs contributing to LTP in the hippocampus (Shen and Yakel, 2009). Impairment of LTP in dNIC rodents may be explained by the down regulation of nAChRs (Figure 2) and decreased levels of phosphorylated CaMKII, ERK1/2 and CREB in dNIC rodents (Figure 5), compared to controls. These findings suggest that downstream signaling of nAChR is affected in dNIC rodents, and this reduced signaling could be a major mechanism contributing to the deficits in LTP. It is possibly that the disturbance to the interaction of NMDA with nAChRs and/or synaptic proteins in dNIC-exposed rodent hippocampi could underlie a hypoglutamatergic state in the CA1 region of offspring hippocampi. Clearly, more work is needed to understand how nAChRs and NMDARs interact and affect learning and synaptic plasticity.

Developmental nicotine exposure modulates NMDA subtypes of glutamatergic receptors via modulating transcription factors

Nicotine may alter the components of glutamatergic receptor stoichiometry and functional properties in adults after prenatally nicotine exposure (Wang et al., 2011). In order to understand how developmental nicotine exposure might affect neuronal communication and result in learning and memory deficits in children born of mothers who smoke, we investigated the expression of NMDARs. These receptors are present on the postsynaptic membrane and are necessary for strengthening synapses and facilitating long-term potentiation of synaptic inputs (Malenka and Bear, 2004; Malinow and Malenka, 2002).

In order to evaluate the possible modulation of the glutamatergic system as a result of gestational nicotine exposure, we studied the *in vitro* effect of developmental nicotine exposure on the expression levels of the NMDAR subunits NR1, NR2a and NR2b, which are essential for functional NMDARs in rodents and are the most important subtypes of glutamate receptors involved in memory. Our results suggest that developmental nicotine exposure can affect the glutamatergic signaling system throughout postnatal development by reducing the availability of NMDARs and their signaling components. The persistent postnatal depression of the NR1 subunit, which is essential for NMDAR assembly and function, indicates that nicotine alters the biophysical properties of NMDARs. dNIC exposure decreases the NR2a and NR1 subunits of NMDARs, though NR2b subunit expression was found to be unchanged (Figure 7). Consistently, phosphorylation of Ca2+/calmodulin-dependent protein kinases II (CAMKII) at the threonine 286 residue was found to be reduced in dNIC-exposed hippocampal rodents, (Figure 5). Similarly, the phosphorylation-induced activation of the transcription factor CREB (cAMP response element-

binding protein) was reduced significantly in synaptosomes from dNIC hippocampi. Both CAMKII and CREB activation was found to induced in the presence of nicotine via nAChRs (Jackson et al., 2009). CREB activation in the nucleus is known to regulate the transcription of the BDNF and c-fos genes, which have shown to be crucial for maintaining the learning and memory function of the hippocampus. Along the same lines, we found a substantial decrease in the expression of BDNF protein in dNIC rodents (Figure 5). The decrease in BDNF was accompanied by no change in the level of pro-BDNF, which acts as a precursor for BDNF.

Conclusion:

In association with NMDARs, neuronal α7 nAChRs have been implicated in neuronal plasticity as well as neurodevelopmental neurological disorders. However, the role of NMDARs and their interaction with α7 nAChRs in these physiological and pathophysiological processes remains unknown. Here, we report that nAChRs modulate NMDAR expression and the structural plasticity of glutamatergic neurons during the postnatal period of heightened cholinergic function. Developmental nicotine exposure markedly decreased cell surface NMDAR and synaptic protein expression as well as synaptic plasticity in offspring rodents. Moreover, whole-cell patch clamp showed a significant increase in synaptic activity mediated by NMDAR spontaneous EPSCs through activation of nicotinic modulation in control but no alteration in the frequency and amplitude of NMDAR spontaneous EPSCs in developmental nicotine exposure offspring rodents, suggesting the selective enhancement of postsynaptic NMDARs upon activation of nAChRs. Furthermore, developmental nicotine exposure reduces activation of the CAMKII/CREB/BDNF pathway and may subsequently result in modulation of NMDAR subunit expression. Also, nicotine exposure in utero reduces the CREB activity through the PKA/CAMKII pathway, which may impair synaptic plasticity by modulating the excitatory transmission. Taken together, these

findings indicate that nAChRs modulate NMDAR function and implicate synaptic nAChR/NMDAR interactions in synaptic development and plasticity. In addition, these results suggest that nicotinic modulation of NMDA receptor-mediated responses is important for maturation of hippocampal synapses. Conversely, chronic exposure to nicotine may lead to nAChR desensitization, and therefore, disrupt synaptic plasticity during this critical period.

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5- Prenatal nicotine exposure leads to long lasting alterations in synaptic physiology involving expression of α7 and β2 nicotinic receptors subunits and cell signaling in the hippocampus of adult rats.

Abstract:

Maternal smoking is associated with long-lasting cognitive deficits in children. The current study was performed to investigate effect of prenatal nicotine exposure on excitatory synaptic physiology and cellular signaling in the adult hippocampus using a rodent model of prenatal nicotine exposure (PREN), where nicotine is infused (6 mg/kg/day) via mini-pumps to pregnant dams, resulted in cognitive deficits in off spring. Prenatal nicotine exposure resulted in a decrease in long term potentiation (LTP) and an increase in long term depression (LTD). In nicotine exposed rats, presynaptic pool of vesicles docked close to release sites were diminished. Moreover, reduced levels of α7 and β2 subunits containing nicotinic receptors and signaling event of ERK1/2-CREB-BDNF pathway, which is involved in neuronal function were observed in nicotine exposed rats. These findings suggest that alterations in the nicotinic receptors and their signaling mediate the long lasting changes in the hippocampal excitatory synaptic physiology in prenatal nicotine exposed rodents.

Introduction:

Nicotinic acetylcholine receptors (nAChRs) represent a group of pentameric ligand gated ion channels that generate excitatory neurotransmission when activated. These receptors are widely distributed in the mammalian central nervous system and perform essential functions, including regulation of the activity of other neurotransmitter systems (Dani and Bertrand, 2007). The nAChRs are expressed early and reach their maximal expression levels during prenatal brain development (Adams et al., 2002). Developmental roles of nAChRs encompass spatio-temporally defined regulation of neuronal morphogenesis, cell survival and cell death (Ballesteros-Yanez et al., 2010; Hory-Lee and Frank, 1995; Lauder and Schambra, 1999; Orr-Urtreger et al., 2000). In the developing hippocampus nAChRs mediate spontaneous excitatory neuronal firings. Such neuronal firings, propagating as wave-like activation oscillations, were also observed in other developing regions of central nervous system (Bansal et al., 2000; Feller, 1999; Hanson and Landmesser, 2003). During development, presynaptic modulation by nAChRs activates the silent synapses in the hippocampus and thereby strengthens the glutamatergic transmission (Maggi et al., 2003). These reports highlight the essential role of nAChRs during hippocampal development and in the excitatory synaptic physiology in this brain region.

Abnormal nAChR activity during neurodevelopment can, therefore, cause long lasting changes in physiological and anatomical processes regulated by these receptors. Such abnormal activation of nAChRs occurs in cases of tobacco use (primarily in the form of smoking) during pregnancy, affecting the developing fetal brain. Smoking during pregnancy is quite high with about 20% of women aged between 18 and 44 years reported current smoking, making the current estimates to 1 in 7 pregnancies in the United States (2009; Tong et al., 2009). Several clinical reports had established that cigarette use during pregnancy is a high risk factor for adverse health

conditions in children (Blood-Siegfried and Rende, 2010; Ernst et al., 2001; Murin et al., 2011; Pavic et al., 2011; Ruckinger et al., 2010; Rydell et al., 2012). Specifically, prenatal nicotine exposure leads to deficits in tasks that require learning, memory, and problem-solving in children (Cornelius et al., 2001; DiFranza et al., 2004). Many of the behavioral consequences, including cognitive impairments, were found to be long lasting (Murin et al., 2011; Weissman et al., 1999), which emphasize the need for better understanding of the underlying pathophysiology of prenatal nicotine exposure resulting in enduring cognitive impairments.

Previously, we reported that in young rats prenatal nicotine exposure resulted in mood disorders, deficits in spatial memory, reduced LTP in the hippocampus, and decreased basal synaptic transmission and synaptic currents gated by AMPARs (Parameshwaran et al., 2012). The processes that impose enduring changes in cognition need further investigation. Hence, in this study, we have performed LTP and LTD experiments in two months old rats to investigate the long-lasting effects of prenatal nicotine exposure in synaptic plasticity. These extensive electrophysiological studies were complemented with the measure of protein levels to detect changes in α7, β2 subunits containing nAChRs, CREB, BDNF, CAMKII and ERK1/2, a member of mitogen activated protein kinases (MAPK).

It is increasingly becoming evident that functional significance of the α7 nAChR can be attributed not only to modulation of neuronal excitability and neurotransmitter release, but also to its high Ca^{2+} permeability and it is contribution with biochemical signaling pathways (Berg and Conroy, 2002; Dajas-Bailador and Wonnacott, 2004). One of the most significant role that is involved in learning and memory, phosphorylation cascades of mitogen-activated protein kinase (MAPK) pathway, specifically, the phosphorylation of extracellular signal-regulated kinase

(ERK). Phospho-ERK (pERK) is reported to enhanced in hippocampus and other brain areas following long-term memory consolidation, Many studies have shown α7 nAChR activation can lead to enhance ERK1/2, CREB and AKT phosphorylation and activation associated with cognitive processing, in hippocampus (Bitner et al., 2007; Nakayama et al., 2002; Nakayama et al., 2001). Interestingly, our previous study (unpublished data) reveals the protein interaction between α7 nAChR and ERK1/2, which was failed to show such interaction with β2 nAChR subunit. Suggesting that α7 nAChR plays significant role in activating ERK pathway.

Reduce levels of ERK expression in PREN could be due to the rapid desensitization of α7 nAChRs which leads to reduce the influx of Ca^{2+} necessary for initiating downstream signaling. This is consistence with a study, When PNU-282987 applied to cultured hippocampal neurons, evoked a rapidly desensitizing (milliseconds) inward whole-cell current that was concentration-dependent and blocked by the antagonist MLA (Bodnar et al., 2005).

Considerable evidence has shown a retrograde signaling effect on synaptic transmission where the depolarization induced postsynaptic BDNF release can signal reverse to the presynaptic cells to increase the transmitter release probability (Magby et al., 2006). In addition, BDNF and the ERK pathway has been shown to be involved in the enhancement of immature CA3 - CA1 connections by associated network activity in the hippocampus (Mohajerani et al., 2007). This neurotrophin has emerged as key in many neuronal function, for instance, synaptic activity, transmitter release (Lohof et al., 1993; Stoop and Poo, 1995) and long-term regulation of synapse maturation (Wang et al., 1995). BDNF also seems to play an important role in long-term potentiation (LTP) in the hippocampus, and may also be involved in the long-term maintenance of

LTP (Korte et al., 1998). From a different aspect, BDNF also attenuated synaptic fatigue at CA1 synapses induced by HFS (Gottschalk et al., 1999).

Apart from excitatory transmission, BDNF was also shown to modulate inhibitory (GABAergic) synaptic transmission, but in a different way. In general, BDNF has been shown to down-regulation of chloride transport and its presynaptic effects leading to decrease the efficacy of inhibitory synaptic transmission by (Tanaka et al., 1997). Thus, BDNF seems to give strength to the glutamatergic excitatory synapses and weaken GABAergic inhibitory synapses, subsequently lead to increase in neuronal activity.

From these perspective, there are substantial evidences suggest a connection between the BDNF and α7- nAChRs in hippocampal neurons, BDNF is reported to result an increase in α7- nAChR number and clustering (Kawai et al., 2002; Massey et al., 2006). It was mentioned that BDNF act as synaptic modulator, which can change the synaptic activity and morphology. Because of the actions of downstream intracellular kinases and effector proteins triggered immediately after BDNF binding to tyrosine kinase B (TrkB) receptors (for review, see Poo, 2001). In this regard, we suggest that α7- nAChR down regulations might drive chronic changes in BDNF-mediated responses that are required for a precise and rapid modulation of synaptic function. In the other hand, it was reported that BDNF elevates surface and intracellular pools of alpha7-nAChRs on cultured hippocampal neurons (Massey et al., 2006).

The signal transduction mechanisms that mediate the synaptic actions of nicotinic receptors in the hippocampus remain unknown. The cyclic AMP response element-binding protein (CREB), a transcription factor known for its role in synaptic plasticity, appears to be an important downstream mediator for BDNF function (Finkbeiner et al., 1997). Both CAMKII and CREB

activation was found to induced in the presence of nicotine via nAChRs (Jackson et al., 2009). CREB activation in the nucleus is known to regulate the transcription of the BDNF and c-fos genes, which have shown to be crucial for maintaining the learning and memory function of the hippocampus. In the present study, we determined the signaling event of ERK1/2-CREB-BDNF pathway, which is involved in neuronal function, was altered in the hippocampus by prenatal nicotine exposed rodents. We also tested whether PREN can enhance the synaptic fatigue due to alteration in nicotinic receptors signaling pathways. Our results suggest that alteration in the nicotinic function and expression lead to modulation of synaptic fatigue in the hippocampus and signaling pathway.

However, it unclear which signaling cascades are involved in that long-term effect and whether this effect is associated with functional modifications of nicotinic receptors responses. Knowledge about these issues is expected to elucidate whether a common pathway downstream of nicotinic receptor activation is responsible for configure a mechanism involved in the adaptation to local changes in neuronal activity that occur in the hippocampus during learning and memory formation. Our results widen the fundamental mechanisms by which PREN influences synaptic transmission and synaptic plasticity in the rodents. Because alterations on α7 nAChRs levels, and disruption of BDNF function in the hippocampus have been involved in cognitive deficits PREN rodents.

Materials and Methods

*Animals and Chemicals***:** Timed pregnant Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were implanted with osmotic minipumps (Alzet, Cupertino, CA), filled with nicotine (free base diluted with sterile physiological saline) under isoflurane anesthesia, beneath the skin to deliver a subcutaneous dose of nicotine at a rate of 6 mg/kg/day. The osmotic minipumps were filled with sterile physiological saline in control rats. Osmotic minipumps were removed once the pups were born so that prenatal nicotine exposure was limited from ~ day 3 of pregnancy to birth. On the day of parturition after all pups were born, osmotic minipumps were removed under brief isoflurane anesthesia, and the wound was closed with surgical clips. The day-after parturition was considered day 1 (PND 1), and litters were culled to ten pups per dam with, if possible, an equal sex ratio. Rats were randomly chosen among different litters for the experiments. Rats were housed under standard conditions (12 hr light/dark cycle with free access to food and water). Experiments were performed in 2 month old animals, and from hippocampi collected from animals of this age. All the experimental procedures involving live animals were performed as per NIH guidelines and the protocol approved by the Auburn University Institutional Animal Care and Use Committee (IACUC). Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Preparation of Hippocampal slices: Transverse hippocampal slices (400 µm) were prepared as described previously (Parameshwaran et al., 2012; Parameshwaran et al., 2007). Briefly, hippocampal slices were sectioned while bathed in ice cold dissection buffer containing (in mM): 85 NaCl, 2.5 KCl, 4 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 0.5 ascorbate and 2 kynurenic acid; bubbled with 95%CO_2/5%O_2, pH 7.4. Hippocampal slices were

incubated for one hour in artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, $CaCl_2$ 2.5, 1 NaH_2PO_4, 26 $NaHCO_3$ and 11 dextrose; bubbled with 95%CO_2/5%O_2.

*Slice electrophysiology***:** Following incubation, electrophysiological recordings were performed in a recording chamber with continuous perfusion of ACSF purged with 95%CO_2/5%O_2. Field excitatory postsynaptic potentials (fEPSP) from Schaffer collateral/commissural-CA1 synapses were recorded by stimulating CA1 stratum radiatum with bipolar electrodes and placing a recording glass electrode (1-4 MΩ) filled with ACSF ~ 200 µm from the stimulating electrode. The frequency of the test stimulation was after every 20 s. For LTP and presynaptic vesicle draining experiments current intensity was set at 50% of the amplitude at which the initial population spikes began to appear. In LTP experiments, after at least 15 min of stable baseline recording, 3 high frequency stimuli (HFS; 100 pulses, 100 Hz) were delivered every 20 s. LTP was measured 55-60 min post HFS. LTD was induced by two low frequency stimuli (LFS; 900 pulses at 1 Hz), separated by 10 min, preceded by 15 min of baseline recordings and measured at 55-60 min from the end of second LFS. Stimulation intensity was set at ~60% (during LFS) or 40% (at all other times excluding LFS) of the amplitude at which initial population spikes began to appear.

*Western blots***:** Western blot experiments were performed using standard procedures. In brief, hippocampi were isolated and homogenized in a lysis buffer that contained protease and phophatase inhibitors. Samples containing equal amounts of protein were resolved in 12% SDS-PAGE, transferred to PVDF membranes, blocked and incubated with anti-goat pERK1/2 (1:500,

Santa Cruz Biotechnology, Santa Cruz, CA), total ERK1/2 (1:1000; Santa Cruz), β2-nAChR (1:500; Santa Cruz), α7-nAChR (1:1000; Santa Cruz), pCAMKII (1:1000; cell signaling), PDNF (1:1000; cell signaling), or β-actin (1:1000; Cell Signaling, Danvers, MA) overnight at 4°C. Membranes were then washed and incubated with appropriate HRP-conjugated secondary antibodies. Bands were then detected with an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL).

Data analysis: The fEPSP and single channel data were analyzed with LTP Mini Analysis and pClamp 9 programs respectively. Western blot bands were quantified by imageJ (free program available from NIH) program. Data were subjected to two-way ANOVA or *t* test as appropriate with statistical significance defined at $p<0.05$. Data are presented as mean±S.E.M.. More details on the analysis of the electrophysiological data are provided in our recent report (Parameshwaran et al., 2012).

Results:

Prenatal nicotine exposure impairs synaptic plasticity in the Schaffer collateral synapses in the adult hippocampus.

Our research has previously shown that in young rats, prenatal nicotine exposure resulted in decreased LTP (Parameshwaran et al., 2012). Previous studies have also demonstrated morphological alterations in the hippocampus and impaired hippocampal based memory tasks in adult rats subjected to nicotine exposure during prenatal developmental period (Roy and Sabherwal, 1998; Roy et al., 2002; Vaglenova et al., 2008). Therefore, in this study we questioned whether LTP deficits persist to adult age and whether other forms of synaptic plasticity such as LTD are also altered. LTP was measured 55-60 min after conditioning with 3 HFS interspaced at 20 s. The average fEPSP slope, computed as a percentage of the baseline, exhibited impaired maintenance of LTP in nicotine exposed rats compared to control rats (Fig. 1 A and C; $p<0.001$, $n=5$). In separate set of experiments we measured LTD. To elicit LTD in hippocampal slices prepared from two-month old control and nicotine exposed rats, two trains of 900 pulses were delivered with a 10 min interval. This protocol induced a long-term reduction of the fEPSP slope by >20% from the baseline in control slices and >45% in nicotine group slices. LTD when measured 55-60 min after the completion of second LFS was different in the nicotine exposed group compared to the controls (Fig. 1B and D; $p<0.001$, $n=4$). These results demonstrate that nicotine exposure during the prenatal period is associated with reduced LTP and pronounced LTD in adult rats.

Potentiation during HFS was not altered by prenatal nicotine exposure.

The form of LTP studied here in CA1 synapses is N-methyl-D-aspartate receptor (NMDAR) dependent (Bashir et al., 1991). Sufficient depolarization of membranes to expel voltage dependent Mg^{2+} block of NMDARs is a vital event during HFS. Strength of induction and production of LTP critically depends on the strength of depolarization phase during HFS (Bliss and Collingridge, 1993). Since LTP was reduced in nicotine exposed rats, we next studied whether impaired induction was a determining factor by analyzing fEPSPs elicited during HFS. This analysis revealed that amplitudes of fEPSPs elicited by each pulse within HFS were not different between control and nicotine exposed rats (Fig. 2 A-D; $p>0.05$, $n=5$). Furthermore, when measured by the amplitude of the first EPSP elicited within HFS, each subsequent HFS resulted in similar potentiation (Fig. 2 E and F; $p>0.05$, $n=5$). These results affirm that prenatal nicotine exposure did not result in a depression of depolarization during HFS.

Prenatal nicotine exposure is associated with increased synaptic fatigue.

Impaired synaptic plasticity and basal synaptic transmission also suggest the possibility of deficient presynaptic mechanisms. This hypothesis was tested by investigating the release probability of the readily releasable pool (RRP) of vesicles. The synaptic response to a high frequency short train of pulses (40 pulses at 100 Hz) is believed to be a measure of RRP quanta, which is morphologically represented by the docked vesicles (Cabin et al., 2002).While the control and nicotine data exhibited a similar pattern, decreasing fEPSP amplitude with successive pulses within the train, fEPSP amplitudes of nicotine rats were lower than the controls (Fig. 4;

$p<0.01$, n=5). This finding asserts that presynaptic release mechanisms involving the RRP vesicles are impaired in nicotine exposed rats.

Prenatal nicotine exposure was associated with altered nAChR expression and ERK1/2 signaling.

The nAChRs are known to modulate the strength of synaptic plasticity in the hippocampus (Fujii et al., 1999; Ji et al., 2001). The α7 and β2 subunits containing nAChRs are the most predominant types in the brain (Wada et al., 1989) and expressed postsynaptically in the hippocampal pyramidal neurons (Drever et al., 2011). Therefore, we next studied whether nicotine exposure during prenatal brain developmental period could alter the expression of these receptors in the hippocampus. Results from western blot analysis illustrate that the receptors were downregulated in hippocampus of nicotine exposed rats (Fig. 7A; $p<0.05$, n=4). ERK1/2 is implicated in synaptic physiology and specifically associated with strengthening of LTP in hippocampus (Morozov et al., 2003). ERK1/2 was also found to be involved in the nicotinic enhancement of LTP in the CA1 region of hippocampus (Welsby et al., 2009). Results indicate that the activated (phosphorylated at Thr 202/Tyr 204) form of ERK1/2 was reduced in nicotine exposed rat hippocampus (Fig. 7; $p<0.01$, n=4). These findings ascertain that prenatal nicotine exposure is mediated by alterations in nAChR expression and ERK1/2 signaling.

Figures:

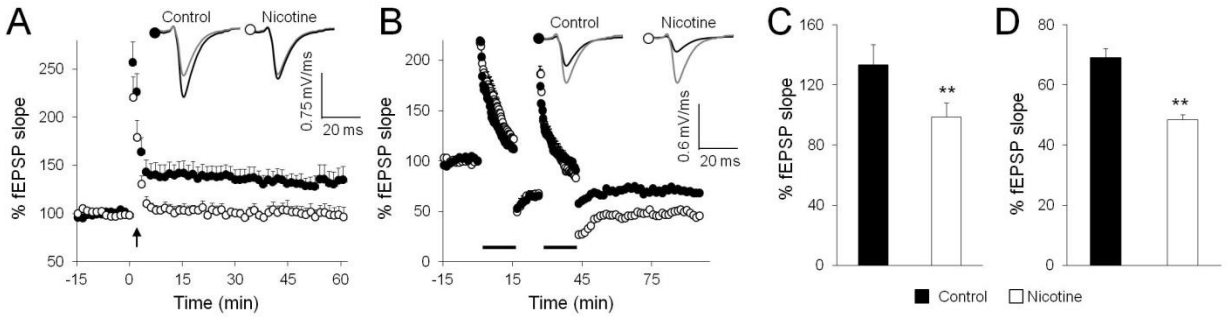

Figure 5.1: Decreased LTP and increased LTD in the hippocampus of prenatal nicotine exposed rats.

(A and C) LTP induced by HFS (upward arrow) was reduced in nicotine exposed rats (open circles and bar) compared to the controls (filled circles and bar). Representative traces collected during baseline recordings (gray) overlaid on traces collected 55-60 min post HFS (black) are illustrated for both control and nicotine data. n=5 rats and 2 slices/rat, $p<0.01$, two-tailed student's t-test.

(B and D) LTD induced by two LFS (thick black lines) and measured at 55-60 min after the second LFS was increased in nicotine exposed rats. Representative traces shown include control and nicotine data collected during baseline recordings (gray) overlaid on traces collected during the 55-60 min LTD measuring period (black). n=4 rats with 2 slices/rat, $p<0.001$, two-tailed student's t-test.

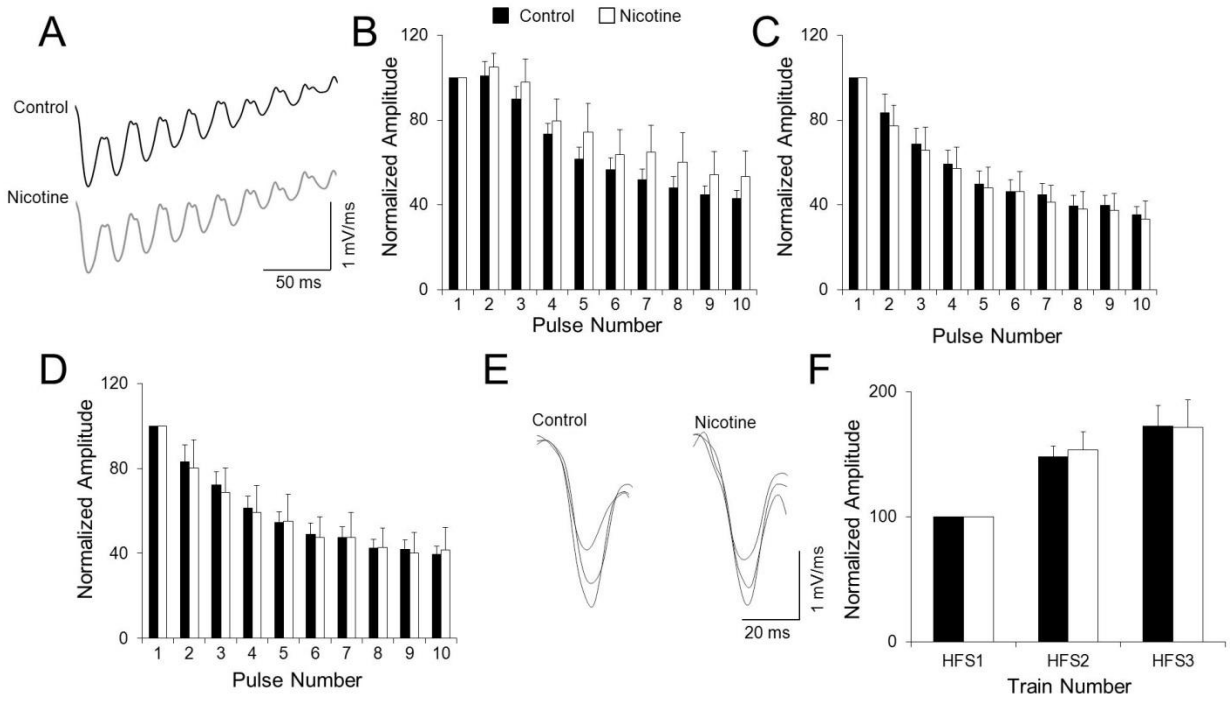

Figure 5.2: Prenatal nicotine exposure did not impair processes associated with the induction of LTP.

(A) Sample traces of multiple fEPSPs evoked during the first HFS are illustrated for control (black trace) and nicotine (gray traces). Only the initial segments with 9 fEPSPS are shown for clarity.

(B-D) Facilitation of the fEPSPs within individual HFS was computed by normalizing the amplitude of the first 10 fEPSP with the amplitude of the first fEPSP. When compared control (filled bar) and nicotine (open bar) data were not different. B, C and D represent the analysis of the first, second and third HFS respectively. n=5 rats with 2 slices/rat, $p>0.05$, two-way ANOVA (group X stimulus number) with Tukey posthoc test.

(E) Representative traces illustrating facilitation with successive HFS. Traces of the first fEPSP for each of the three HFS are overlaid together. Both control and nicotine data show similar facilitation.

(F) Group data for between HFS facilitation. Nicotine data (open bars) was not different from control data (filled bars). n=5 rats with 2 slices/rat, $p>0.05$, two-way ANOVA (group X HFS) followed by Tukey test.

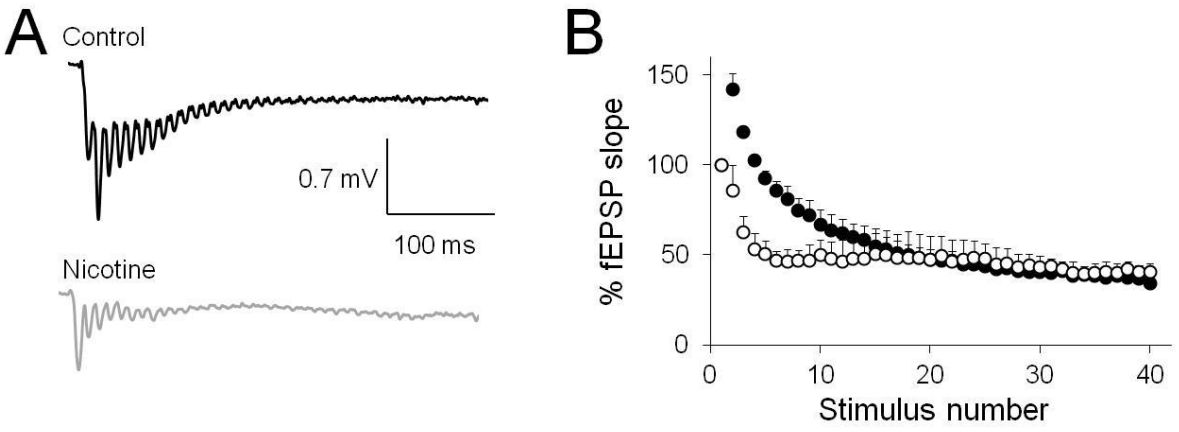

Figure 5.3: Impaired patterning of presynaptic RRP vesicle release in prenatal nicotine exposed rats.

(A) Sample traces showing impaired RRP vesicle in nicotine exposed rats (gray) compared to the control (black trace).

(B) Prenatal nicotine exposed rats showed synaptic fatigue as illustrated by the rapid reductions in fEPSP slopes (open bars) during the train stimulation (100 Hz, 40 pulses) applied to drain the RRP vesicles. n=5 with2 slices/rat, $p<0.01$, two-way ANOVA (group X pulse) with Tukey test.

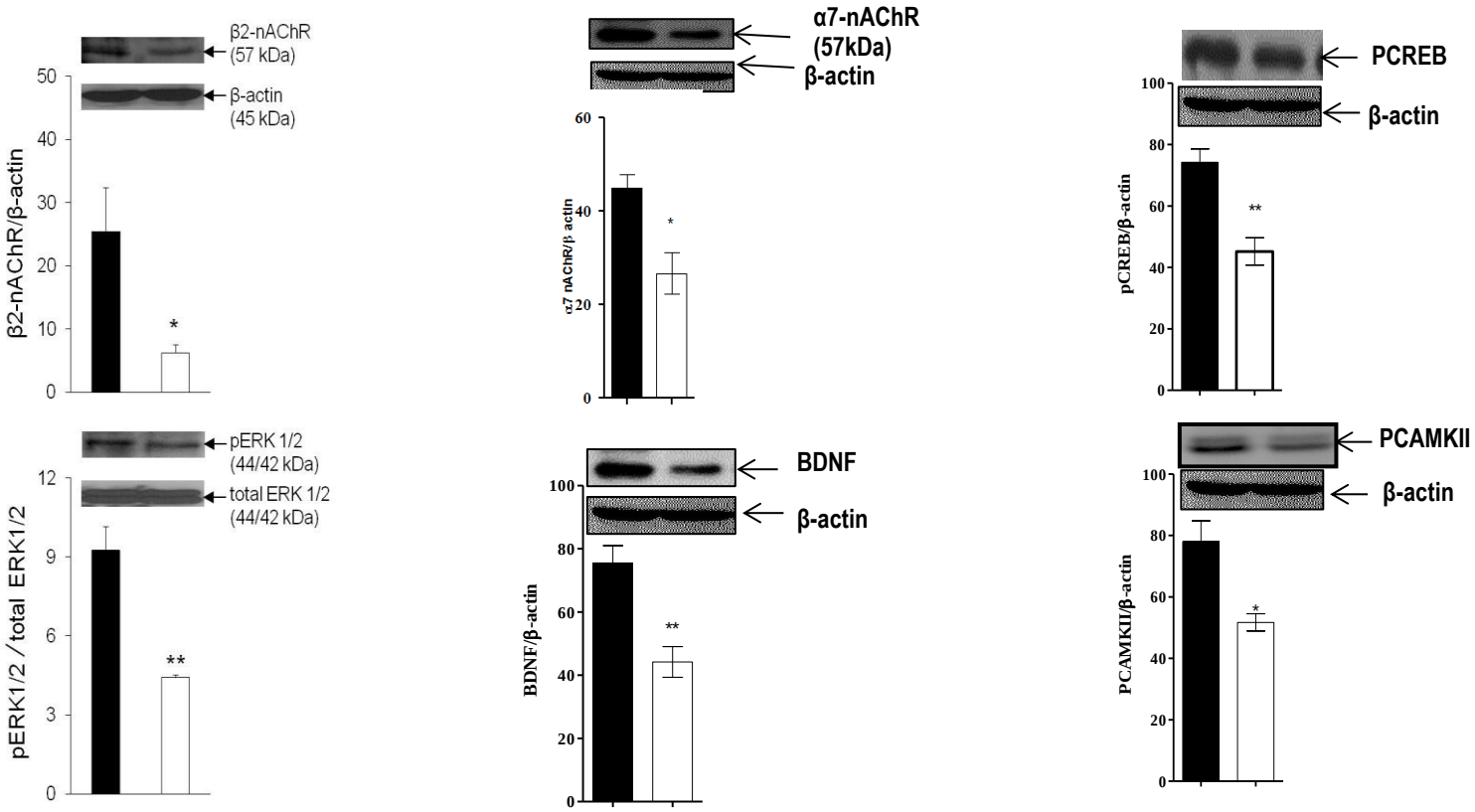

Figure 5.4: Reduced protein expression levels in the hippocampus of prenatal nicotine exposed rats.

(A) Immunoblot detection and quantization shows that α7 and β2-nAChRs are downregulated in the hippocampus of nicotine exposed rats. n=4, $p<0.05$, $p<0.05$ two-tailed students t test.

(B) Decreased protein expression of pERK1/2, pCREB, pCAMKII and BDNF in prenatal nicotine exposed rats. n=4, $p<0.01$, $p<0.05$, $p<0.01$, $p<0.05$ respectively two-tailed students t test.

Discussion:

In this study, we demonstrate that alterations in synaptic plasticity and reduced protein levels of nAChRs, and it is signaling as possible cellular physiological and molecular mechanisms underlying the long lasting cognitive impairments associated with prenatal nicotine exposure. Our findings reveal strong reduction of LTP and increased LTD in the hippocampus of nicotine exposed rats. The strong reduction in LTP conforms to our recently published findings that prenatal nicotine exposure results in reduced LTP in young rats (Parameshwaran et al., 2012). The findings reported in this study, particularly the decline in LTP, are in agreement with the established findings that cholinergic glutamatergic interactions can modulate synaptic plasticity (Aigner, 1995; Drever et al., 2011; Ovsepian et al., 2004; Placzek et al., 2009), which has been physiologically implicated in memory (Hoffman et al., 2002; Huerta and Lisman, 1995). α7and β2-nAChRs are predominantly located in the pre and postsynaptic loci of pyramidal cells and are permeable to Ca^{2+} ions (Drever et al., 2011). Thus, reduced protein levels of nAChRs in the nicotine exposed rats may contribute to reduced Ca^{2+} influx into the pyramidal neurons. Under such circumstances, intracellular Ca^{2+} dynamics may be altered contributing to downstream alterations in cellular signaling resulting in reduced LTP. It is interesting to note that, a recent study reported that in the CA1 region pulsed release of acetylcholine induced synaptic enhancement by recruitment of AMPARs in the dendritic spines (Fernandez de Sevilla et al., 2008). Most importantly chronic effects of the reduced nAChRs levels may be a critical factor imposing persistent modification of Ca^{2+} dynamics in the postsynaptic neurons. This could also be a reason for the reduced pERK1/2 levels and such changes can impair LTP (Sweatt, 2004; Thomas and Huganir, 2004). The factors that favored pronounced LTD in the nicotine exposed hippocampus are not clear. The types of LTP and LTD studied here require the activation of

NMDARs (Malenka and Bear, 2004). Results of this study indicate that potentiation during HFS was similar in control and nicotine exposed rats. This implies that a deficiency in NMDAR mediated induction during HFS may not be a major factor for decreased LTP in the adult nicotine exposed rats, in contrast, in the young rats, analysis of within theta burst stimulation facilitation revealed a significant reduction in potentiation in PREN rodents (unpublished data). The similar fEPSP data points in control and nicotine data during LFS, also suggests that alterations in NMDAR function did not mediate the elevated LTD in nicotine exposed rats.

The mEPSCs are true synaptic currents resulting from release of single neurotransmitter vesicles. These currents are essential to maintain the balance in dendritic protein synthesis and thereby conserving synaptic integrity (Sutton et al., 2004). In the CA1 region, LTP and LTD resulted in increased and decreased amplitude of mEPSCs respectively (Isaac et al., 1996). These finding imply that synaptic plasticity is associated with changes in quantal size and highlights the physiological relationship between synaptic plasticity and spontaneous neurotransmitter release. Previous findings also showed LTP was associated with increased probability of neurotransmitter release and was accompanied by long lasting enhancement of vesicle release from RRP (Lauri et al., 2007; Lisman and Raghavachari, 2006; Stanton et al., 2005). Therefore, the synaptic fatigue in terms of reduced RRP of vesicles observed in nicotine exposed animals may reduce the efficacy of presynaptic release associated with LTP and impair its production. Furthermore, presynaptic nAChRs are known to regulate spontaneous currents in the CA1 neurons (Alkondon and Albuquerque, 2002). Hence, disrupted presynaptic nAChR signaling, a notion supported by our findings of reduced expression of α7 and β2-nAChRs in nicotine exposed hippocampi, may be an underlying mechanism for altered neuronal function.

As evident from unpublished data, prenatal nicotine exposure resulted in altered postsynaptic functions also. The reduced basal synaptic transmission, LTP deficits, reduced AMPAR EPSC decay times in one month old (unpublished data) and three weeks (Parameshwaran et al., 2012) animals exposed with nicotine prenatally, and altered single channel properties of AMPARs are suggestive of severe depression of AMPA receptor function and expression. Interestingly, these alterations in the synaptic function are still persisting with adult animal exposed prenatally with nicotine (data not shown). In our previous study, we established that protein expressions of vesicular glutamate transporter 1 (VGLUT1), synaptophysin, AMPAR subunit GluR1, postsynaptic density-95, and p(Ser 845) GluR1 were reduced in the hippocampus of younger rats that were exposed nicotine during their prenatal developmental period (Parameshwaran et al., 2012). The exact mechanism through which prenatal nicotine exposure causes long lasting down regulation of excitatory synaptic physiology in hippocampus remains to be completely elucidated. In our immunoprecipitation studies revealed the nicotinic receptors interact with VGLUT and PSD-95, in which these interactions is disturbed in PREN. Nicotinic signaling is important for conversion of silent synapses (synapses without functional AMPARs) to active ones (Maggi et al., 2003). Reduced functional properties of AMPARs and decreased presynaptic release reported in this study suggests the changes in the levels of these proteins may persist to the adult age. Our results are supported by a previous study which showed nicotine exposure to immature rat hippocampus resulted in persistent decrease of synaptic efficacy in Schaffer collateral-CA1 synapses (Maggi et al., 2003). Continued prenatal nicotinic activation and withdrawal of exposure following birth may result in premature strengthening followed by weakening of AMPAR mediated transmission in hippocampus. Development of nicotine dependence has indeed been suggested to lead to compensatory decreases in number and/or function of the AMPARs to

counter prolonged stimulatory effects of nicotine on reward pathways (Kenny et al., 2003). Extended nicotine exposure decreased AMPA receptor immunoreactivity in the ventral tagmental area and nucleus accumbens (Lee et al., 2002) indicating sustained exposure may decrease AMPAR expression. Disruptive developmental modulation of nicotinic receptors followed by abrupt withdrawal that persist for a long time may therefore be the basic process, downregulating excitatory synaptic physiology, activation of nicotinic receptors presynaptically induce calcium influx, which is required for activation of CAMKII and ERK1/2 signaling and synaptic protein expressions in the hippocampus of rats subjected to prenatal nicotine exposure. The mechanisms that link the parallel declines in α7 and β2-nAChRs and ERK1/2 are not clear, in our unpublished data indicates that the interaction between α7 nAChRs and ERK, but not β2-nAChRs subunit. CAMKII was reported to regulate the number of docked vesicles, loss of CAMKII results in synaptic fatigue (Hojjati et al., 2007). Thus, activation of pre-synaptic CAMKII is important for synaptic augmentation. Both CAMKII and CREB activation was found to elevated in the presence of nicotine via nAChRs (Jackson et al., 2009). CREB activation in the nucleus is known to regulate the transcription of the BDNF and c-fos genes, which have shown to be crucial for maintaining the learning and memory function of the hippocampus.

In addition, BDNF and the ERK pathway has been shown to be involved in the enhancement of immature CA3 - CA1 connections by associated network activity in the hippocampus (Mohajerani et al., 2007). This neurotrophin has emerged as key in many neuronal function as in LTP in the hippocampus, and may also be involved in the long-term maintenance of LTP (Korte et al., 1998). From a different point of view, BDNF also attenuated synaptic fatigue at CA1 synapses induced by HFS (Gottschalk et al., 1999). In this study we reported that the signaling event of ERK1/2-CREB-BDNF pathway, which is involved in neuronal function, was altered in

the hippocampus by prenatal nicotine exposed rodents. In addition, our finding indicates that PREN can enhance the synaptic fatigue due to alteration in nicotinic receptors and their signaling pathways.

Alteration in synaptic plasticity may be due to reduced signaling downstream of nAChRs or could be a product of general decline in nicotinic and glutamatergic synaptic activity in nicotine exposed rats. Another strong possibility is the alteration in other regions such as medial septum, where are both nAChRs subunits are expressed, may also affect the synaptic physiology in the hippocampus. Hence further research focusing on changes in other nAChR functions may provide more insights on the mechanisms regulating presynaptic release in the hippocampus of animals that received nicotine exposure during pregnancy.

In conclusion, findings of this study provide a mechanistic support for the cognitive deficits observed in adult prenatal nicotine exposed rats. We propose reduced nAChR receptor expression and modified nicotinic signaling as a consequence of prenatal nicotine exposure followed by long term postnatal withdrawal as potential mechanisms for disrupted excitatory synaptic physiology in the hippocampus. Furthermore, such synaptic deficits may underlie the long lasting cognitive deficits observed with prenatal nicotine exposure. Glutamatergic transmission in the hippocampus and in brain reward regions has been implicated in mood disorders and nicotine addiction. Hence findings of this study may have implications for other behavioral aberrations, such as mood disorders and pronounced susceptibility for nicotine addiction, observed in animal models and in humans with prenatal nicotine exposure.

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6-summary and Conclusion:

Tobacco smoking during pregnancy remains a significant public health issue in spite of several awareness programs. It is estimated that 20 to 25 % pregnant women smoke, resulting in birth of more than 800, 000 babies exposed to prenatal tobacco smoke annually in the US. Nicotine is a natural agonist of nicotinic acetylcholine receptors (nAChRs), which play important roles during development and plasticity. Activation of nAChRs promotes synaptic contacts during a critical period of development in the hippocampus. Cholinergic innervations in the mammalian brain commence prenatally and prenatal nicotine exposure (PREN) interrupts the normal development of the nicotinic cholinergic system and its regulation of glutamatergic system. Chronic administration of nicotine during development has been reported to result in changes in nicotine binding in the brain. Apart from the molecular changes, PREN is believed to cause neuronal death and morphological changes in specific brain regions like hippocampus, which play a critical role in learning and memory processes. Accordingly, forms of hippocampus dependent memories are also impaired in PREN rodents. The mechanisms responsible for impaired hippocampal memory function in PREN rodents are not fully understood. The major goal of this study is to elucidate the mechanisms that underlie hippocampus dependent memories and how the nicotinic-cholinergic and glutamatergic systems interplay to regulate these mechanisms. This project was utilized a variety of electrophysiological, biochemical and molecular biological to elucidate the mechanisms of memory loss in PREN rodents. Results from this project will greatly enhance the understanding of the complex interplay between the nicotinic and glutamatergic systems and how it would be impaired by prenatal nicotine exposure. The findings can contribute to the development of effective therapy to improve the health of children whose mothers smoked during pregnancy. Based on our results from these studies, demonstrated that alterations in

nAChR expression and function lead to modified glutamatergic transmission in the hippocampus resulting in memory deficits in PREN rodents. Our finding indicates that long term-potentiation (LTP), decreased with concomitant deficits in basal synaptic transmission in PREN which were not rescued in the presence of nicotinic modulators due to modified in nicotinic receptors. In contrast, in saline exposed animals, nicotinic receptor modulation perfused into hippocampal slices has been shown to promote LTP and synaptic transmission. Therefore, nicotine exposure during development has a long lasting effect on the excitatory synaptic transmission and synaptic plasticity in the hippocampus.

We conclude that decreased AMPA sEPSCs and amplitudes in PREN, which possibly contributed to impaired synaptic plasticity, resulted from decreased expression and function of pre- and postsynaptic nicotinic receptors. This study closely correlates with our previous results showing alteration of quantal content glutamate as well as AMPA receptors. Interestingly, GluR1 expression was found to be upregulated after nicotinic activation. The decrease in frequency of sEPSCs in prenatal nicotine-exposed rodents in this study can be explained by: (i) a decrease in probability of glutamate release due to reduced presynaptic nicotinic afferents as proved by decreased FV amplitude and PPF among PREN but not in control saline exposed slices, (ii) impaired postsynaptic machinery as indicated by decreased PSD-95 and SAP102 which are interact with nicotinic receptors, in addition to downregulation of AMPA subunit GluR1, and (iii) deficiency in vesicular packaging of glutamate due to decreased levels of VGLUT, which interacts with nicotinic receptors as revealed by immunoprecipitation studies.

Untimely activation of nAChRs by nicotine results in short-term and long-term consequences on learning and behavior. In this study, the aim was to determine how prenatal nicotine exposure modified glutamatergic signaling in the hippocampus during postnatal development. We investigated regulation of both nAChRs and glutamate receptors for AMPA and NMDA in one-month-old rodents after a temporally restricted exposure to saline or nicotine for the whole period of pregnancy *in utero*. We analyzed postsynaptic density components associated with NMDAR signaling: calmodulin (CaM), CaM Kinase II (CaMKII), postsynaptic density-95 (PSD95), as well as ERK1/2. In postnatal one-month-old rodents, there was significantly reduced expression of NMDAR subunits NR1, NR2a, but not NR2b. CaMKII, and PSD95 were also significantly downregulated, together with ERK1/2. This reduced expression of NMDARs and signaling proteins was concomitant with reduced levels of nAChR expression in the hippocampus, indicating that α7-α4β2 nAChR may influence glutamatergic function in the hippocampus of one-month-old rodents. However, up regulation of CaMKII and ERK were reported with acute nicotine treatment. Our results suggest that prenatal nicotine exposure can impair the glutamatergic signaling system throughout postnatal development by reducing the availability of NMDAR and/or their signaling components. The persistent depression, in adults, of synaptic plasticity and α7 and β2 subunits containing nicotinic receptors and signaling event of ERK1/2-CREB-BDNF pathway, which are involved in neuronal function indicates that nicotine may alter glutamate receptor functional properties in adults after prenatally-confined nicotine exposure, suggesting that alterations in the nicotinic receptors and their signaling mediate the long lasting changes in the hippocampal excitatory synaptic physiology in prenatal nicotine exposed rodents.

In summary, these findings strongly suggest that prenatal nicotine exposure results in potent glutamatergic impairment in the excitatory transmission in the hippocampus due to alterations in nAChRs