PRENATAL NICOTINE EXPOSURE AND MOLECULAR MECHANISMS OF

MEMORY IMPAIRMENT

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Kodeeswaran Parameshwaran

Certificate of Approval:

_____	_____
Vishnu Suppiramaniam, Chair Associate Professor Pharmacal Sciences	Muralikrishnan Dhanasekaran Assistant Professor Pharmacal Sciences
_____	_____
Daniel L. Parsons Professor Pharmacal Sciences	Kevin W. Huggins Assistant Professor Nutrition and Food Sciences

_____

George T. Flowers
Interim Dean
Graduate School

PRENATAL NICOTINE EXPOSURE AND MOLECULAR MECHANISMS OF MEMORY IMPAIRMENT

Kodeeswaran Parameshwaran

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PRENATAL NICOTINE EXPOSURE AND MOLECULAR MECHANISMS OF

MEMORY IMPAIRMENT

Kodeeswaran Parameshwaran

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Signature of Author

_____
Date of Graduation

VITA

Kodeeswaran Parameshwaran, son of Chelliah Kodeeswaran and Konesaranjitham Kodeeswaran, was born on January 01, 1968. He attended University of Jaffna in Sri Lanka, and earned his baccalaureate degree in Zoology in December 1994. In 1998 he joined University of Peradeniya in Sri Lanka and earned a masters degree in Animal Science in 2001. Parameshwaran worked as a Lecturer in the Department of Zoology, University of Jaffna from September 1996 to August 2003. In August 2003 he joined Auburn University for doctoral program in Pharmacal Sciences.

Parameshwaran was born with three other siblings, two sisters and brother. He is married to Gnanachchelvi Thirunavukkarasu and has a son Mayooran Selliah, born on April 22, 2007.

During his stay in Auburn University Parameshwaran obtained awards and prizes, including an outstanding Auburn University graduate student award in Spring 2006.

DISSERTATION ABSTRACT

PRENATAL NICOTINE EXPOSURE AND MOLECULAR MECHANISMS OF MEMORY IMPAIRMENT

Kodeeswaran Parameshwaran

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Prenatal nicotine exposure in the form of active maternal smoking is a major risk factor for several harmful effects in the children. These effects include reduced motor skills, poor IQ scores and most importantly learning and memory deficits. Several animal studies have also shown that these harmful effects could arise due to prenatal nicotine exposure. However, the physiological mechanisms underlying the learning and memory deficits have poorly been studied. In this study a rat model of prenatal nicotine exposure, in which pregnant dams received a subcutaneous dose of 6 mg/kg/day nicotine throughout gestation via osmotic mini pumps implanted beneath shoulder skin, was

utilized. Birth weights and body weights of rats after 2 weeks were measured. In addition surface righting reflex, negative geotaxis, open field activity, rotorod test, forced swim test and Y maze test were performed in young rats to assess motor coordination, activity and hippocampal based memory acquisition. Lipid peroxide, protein carbonyl and reactive oxygen species were also analyzed to assess any neurochemical changes in the hippocampus. To specifically address hippocampus dependent memory impairments observed in the young animals, detailed electrophysiological analyses were performed. The electrophysiological analyses included assessment of basal synaptic transmission and long tem potentiation (LTP) in Shaffer collateral-CA1 synapses, AMPA receptor mediated whole cell currents from CA1 pyramidal neurons and single channel synaptic AMPA receptor currents from hippocampus. Result of these studies revealed that prenatal nicotine exposure results in reduced body weight, motor coordination and strength and hippocampal memory. Results of electrophysiological studies showed decreased basal synaptic transmission, LTP, AMPA receptor mediated whole cell currents and single channel currents providing support that decreased synaptic function in the hippocampus could be a mechanism underlying cognitive deficits due to prenatal nicotine exposure. To test whether these synaptic impairments are long lasting, LTP, basal synaptic transmission, AMPA receptor mediated whole cell currents and single channel currents were analyzed in 2 months old young adult rats which showed deficits in these electrophysiological measures. This suggests that compromise in hippocampal synaptic function could be long lasting. Overall this study provides evidence for physiological mechanisms that could potently diminish memory processes in prenatal nicotine exposed animals.

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1. INTRODUCTION

In the mammalian brain nicotinic acetylcholine receptors (nAChRs) are one of the major mediators of excitatory neurotransmission. The nAChRs are ionotropic receptors that form ligand gated, cation permeable ion channels (Romanelli et al., 2007) and widely distributed in the central (Nashmi and Lester, 2006) and peripheral nervous tissues and in the neuromuscular junctions of somatic muscles (Salpeter and Loring, 1985). Activation of receptors modifies the state of neurons through movements of cations causing a depolarization of the plasma membrane, which results neuronal excitation. In addition, entry of calcium ions through nAChRs acts, either directly or indirectly, on vital intracellular cascades which moderate neuronal function, survival and even death (Kalamida et al., 2007). While nAChRs are normally activated by the endogenous neurotransmitter acetylcholine, these receptors can also be activated and modulated by several chemical compounds (Romanelli and Gualtieri, 2003). One such potent compound is nicotine, an alkaloid found in tobacco plant. Nicotine is one of the widely abused substances in the form of tobacco smoking and chewing.

Nicotine is the major psychoactive component in the tobacco (Di Matteo et al., 2007) and tobacco smoking is widespread resulting in health complications and economic impacts. Smoking during pregnancy is a major concern in public health and it is

estimated about half of the tobacco smoking women continue to smoke during their pregnancy which results in about half a million of births per annum with infants exposed to toxic substances from tobacco smoke during their fetal life (Ebrahim et al., 2000; Martin et al., 2003). Exposure to nicotine in developing fetus results in serious health complications including abortion, stillbirth, low birth weight, sudden infant death syndrome (SIDS), cognitive deficits, and various other neuropsychological disorders.

Epidemiological studies have revealed that maternal smoking is a strong risk factor for abortion (Ness et al., 1999), low birth weight and preterm delivery (Windham et al., 2000) stillbirth and infant mortality (Wisborg et al., 2001; Hogberg and Cnattingius, 2007) and even smokeless tobacco use during pregnancy increases stillbirth risk, with a risk at least as great as that associated with maternal cigarette smoking (Gupta and Subramoney, 2006). In addition infants who were exposed to nicotine during their fetal life have a high risk of dying of SIDS (Milerad and Sundell, 1993; Rajs et al., 1997; Neff et al., 2004). Studies have shown that nicotine inhibit cellular progesterone synthesis which could be a contributing factor for abortion or preterm birth (Gocze et al., 1999). This is supported by experimental evidence that show rats exposed to nicotine prenatally become apneic more rapidly than unexposed animals (Fewell et al., 2001). In addition, neonatal rats prenatally exposed to nicotine show reduced tolerance for hypoxia caused by impaired heart rate control (Slotkin et al., 1997). Nicotine increases maternal blood pressure and heart rate, with a concomitant reduction in uterine blood flow. Physiological effects of nicotine on fetal growth seem to be a culmination of both the vasoconstrictive

effects of nicotine on the uterine and potentially the umbilical artery (Lambers and Clark, 1996). Though maternal physiological effects may potentially contribute to poor fetal growth, fetal/infant death could also, to a greater extent, be due to harmful effects of nicotine on cardiac and respiratory functions and the central control of these functions. It has been found that in prenatal nicotine exposed animals neurochemical mechanisms regulating heart rate response to hypoxia is impaired and thus a link between prenatal nicotine exposure and an exaggerated bradycardia during hypoxia may contribute to SIDS (Neff et al., 2004). Thus alterations in maternal and fetal physiology cause poor fetal growth, early delivery and possible death.

One of the major harmful and potentially long lasting consequences of prenatal exposure of nicotine is the neurobehavioral alterations in the offspring. Since nAChRs are expressed early in the development of CNS and these receptors regulate several neurodevelopmental processes (Levin et al., 1996) persistent, early and potent modulation of these receptors, as in the case of maternal smoking, could result in potential harmful effects in the neurodevelopmental processes. In particular structural changes, in terms of cell number and size, have been observed in hippocampus of rodents which were exposed to prenatal nicotine (Roy et al., 2002). The mammalian hippocampus is a vital region that controlling formation of memory (Squire et al., 2004). These correlate with studies that show prenatal nicotine exposed animals as well as children show impaired cognition. Children whose mothers smoked during pregnancy show attention deficit hyperactivity disorder (ADHD) and results in deficient motor

scores, verbal comprehension skills, design memory, problem solving and various other cognitive tasks (Gusella and Fried, 1984; Sexton et al., 1990; Cornelius et al., 2001; Linnet et al., 2003; Huizink and Mulder, 2006). These studies observations were supported by animal studies in which prenatally nicotine exposed rats showed increased anxiety and decreased cognitive abilities (Sobrian et al., 2003; Vaglenova et al., 2004). Thus these studies indicate memory processes in the hippocampus are compromised in animals that were exposed to nicotine prenatally.

In the mammalian hippocampus the major neuronal circuitries and the excitatory synapses therein are glutamatergic. The glutamatergic synapses are one the major excitatory types in the hippocampus as well as in the entire CNS. These synapses are activated by presynaptic release of the neurotransmitter glutamate, which binds to the postsynaptic glutamate receptors. The glutamate receptors are categorized into ionotropic and metabotropic. The ionotropic glutamate receptors are further classified into three subtypes, *N*-methyl-*D*-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and kainite receptors, based on their specific pharmacological, ion channel and functional properties (Dingledine et al., 1999). Of these NMDA receptors and AMPA receptors are vital for synaptic plasticity processes that are believed to regulate learning and memory at the level of synaptic organization. In particular activity dependent insertion or removal of AMPA receptors are essential for two types of long lasting changes in synaptic efficacies, long term potentiation (LTP) and long term depression (LTD). In addition to their role in synaptic plasticity these receptors

are also believed to be important for basal synaptic transmission, synaptic integrity and synaptogenesis. Despite this the hippocampal synaptic plasticity and transmission have not been studied in prenatal nicotine exposed animals.

In this context the current study was designed to determine the changes in hippocampal based memory, activity and motor function in a rodent model of prenatal nicotine exposure. In addition this study tests a central hypothesis that hippocampal synaptic plasticity and synaptic transmission, in particular that mediated by AMPA receptors, are altered in prenatal nicotine exposed animals.

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2. REVIEW OF LITERATURE

1. Nicotine and nicotinic acetylcholine receptors

Nicotine is a hygroscopic nitrogenous base alkoloid (Fig. 2.1) found naturally at high concentrations in the tobacco plant *Nicotiana tabacum* (Dawson et al., 1960). Nicotine would freely penetrate skin and has been shown to efficiently cross placenta (Luck et al., 1985) and blood brain barrier (Ohno et al., 1979). Nicotine burns at a temperature below its boiling point, and its vapors combust at 95 °C in air despite a low vapor pressure (Jackson, 1941). Because of this property, when a cigarette is smoked most of the nicotine is burned and enough is inhaled to provide the desired effects. The amount of nicotine absorbed rely on many factors, including the type of tobacco smoked, whether the smoke is inhaled, the amount inhaled, and whether a filter is used. If tobacco is taken in the nose, as in the case of snuff, or chewed, the amount released into the body tends to be much greater than smoked tobacco. The major metabolite is cotinine when nicotine is metabolized in the liver by cytochrome P450 enzymes (Hammond et al., 1991).Nicotine acts on the nAChRs by increasing their activity in small concentrations (Hogg et al., 2003). Nicotinic acetylcholine receptors are ionotropic receptors that form ligand gated ion channels and their opening is triggered by the neurotransmitter acetylcholine (ACh), but they are also opened by their potent agonist nicotine

(Itier and Bertrand, 2001). In contrast muscarinic ACh receptors functions with a second messenger. Though nAChRs are present in many tissues in the body, they are highly expressed in the CNS, peripheral nervous system and in the neuromuscular junctions of

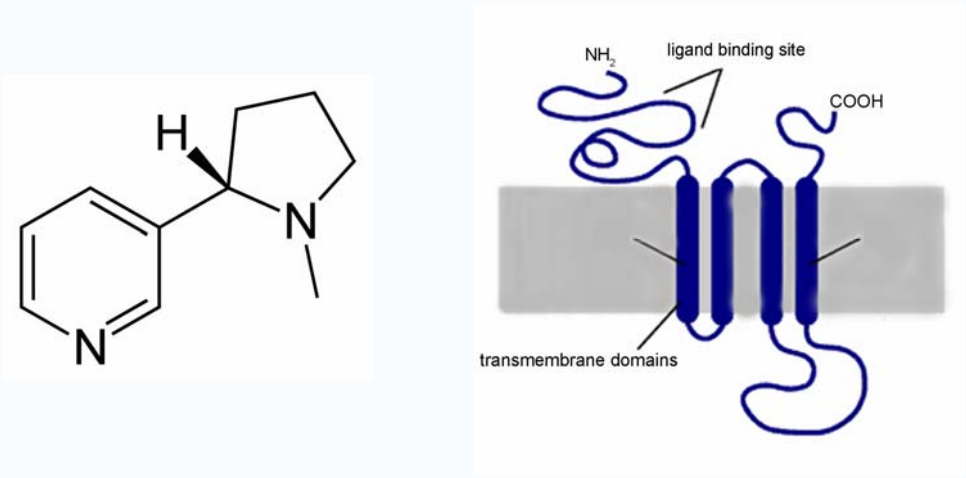

Figure 2.1. Structures of nicotine (left) and nAChR subunit (right).

somatic muscles. Nicotinic receptors are made up of five subunits arranged symmetrically around the central channel opening pore and have a molecular mass of about 280 kDa. Subunits have four transmembrane domains and N terminus and COOH terminals (Fig. 1, right). Twelve types of nicotinic receptor subunits, α2 through 10 and β2 through 4, combine to form pentamers (Fig. 2.2). The subunits are somewhat similar to one another, especially in the hydrophobic regions (Cooper et al., 1991; Lindstrom, 1997; Itier and Bertrand, 2001). The sites for binding ACh are on the outside of the α subunits near their N termini. When the agonist binds, the α subunits become more similar to the other subunits, the channel becomes more symmetrical, and a pore with a diameter of about 0.65 nm opens. Nicotinic AChRs may exist in different interconvertible

conformational states. Binding of nicotine stabilizes the open and desensitized states. Opening of the channel allows positively charged ions, in particular, sodium and calcium, to enter the cell (Cooper et al., 1991; Karlin, 2002; Unwin, 2005). The main conductance levels vary between 25-48 pS and the conductance depends on the actual subunit composition and localization (Mathie et al., 1991; Kuryatov et al., 1997). This activation of receptors by nicotine modifies the state of neurons through two main mechanisms. On one hand, the movements of cations cause a depolarization of the plasma membrane, which results in an excitation, particularly of neurons, but also by the activation of other voltage-gated ion channels. On the other hand, the entry of calcium acts, either directly or indirectly, on different intracellular cascades leading, for example, to the regulation of the activity of some genes or the release of neurotransmitters (Hogg et al., 2003; Dajas-Bailador and Wonnacott, 2004).

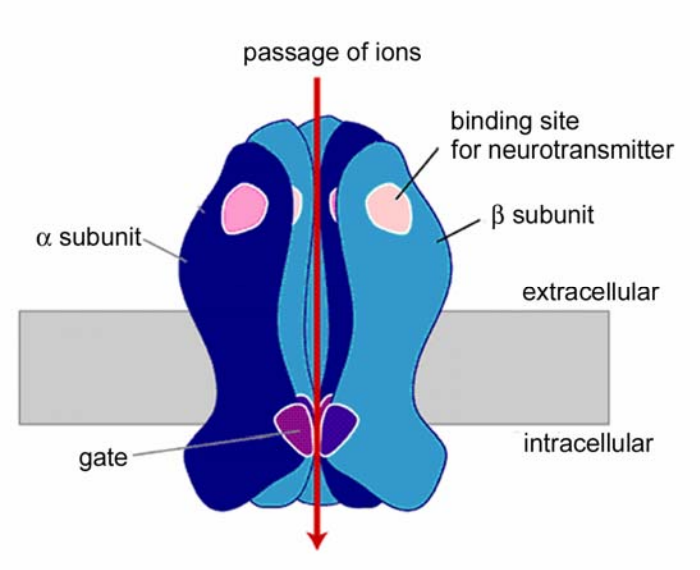

Figure 2.2. Structure of nAChR

Nicotine also causes increased release of adrenaline. The release of adrenaline causes changes in vital physiological changes such as, increases in heart rate, blood pressure and respiration, as well as higher blood glucose levels (Grenhoff and Svensson, 1989; Mancia et al., 1990; Omvik, 1996; Haass and Kubler, 1997). In addition, nicotine increases dopamine levels in the reward circuits of the brain (Staley et al., 2006). Studies have shown that smoking tobacco inhibits monoamine oxidase (MAO) that breaks down monoaminergic neurotransmitters such as dopamine, in the brain (Fowler et al., 2003). Studies in the rat suggest that the mesolimbic dopamine system is central to the reinforcing effects of nicotine (Corrigall et al., 1994). The mesolimbic system projects from the ventral tegmental area of the midbrain to the nucleus accumbens and is known to be involved in reinforcement for other drugs of abuse, such as cocaine. High-affinity binding of labeled nicotine to cell bodies and terminal fields of the mesolimbic neurons and expression of mRNAs for α3, α4, α5 and β2 subunits in mesolimbic neurons has been demonstrated (Clarke and Pert, 1985). Nicotine increases firing of ventral tegmental area neurons and facilitates release of neurotransmitters in this system (Benowitz, 1996).

Addiction is a complex behavioral phenomenon with causes and effects that range from molecular mechanisms to social interactions. Ultimately, the process of drug addiction begins with molecular interactions that alter the activity and metabolism of the neurons that are sensitive to that drug. Over time, this alters the properties of individual neurons and circuits, which leads to complex behaviors such as dependence, tolerance, sensitization, and craving (Koob et al., 1997; Nestler and Aghanjanian, 1997). In the case of tobacco products, the principal addictive component is nicotine.

A common feature of many addictive drugs, including nicotine, is that they increase dopamine (DA) levels in the nucleus accumbens (NAcc) at the same concentrations that are achieved in serum during self administration (Stolerman and Jarvis, 1995; Dani and Heinemann, 1996; Dani and De Biasi, 2001; Staley et al., 2006). The principal dopaminergic projections to the NAcc arise from neurons in the ventral tagmental area. Evidence that NAcc DA levels are important in reward has come from ventral tagmental area lesion studies and microperfusion of the NAcc with DA receptor antagonists, both of which result in reduced self administration of many addictive drugs, including nicotine (Balfour, 1991; Corrigall and Coen, 1991; O'Neill et al., 1991; Corrigall et al., 1992; Corrigall et al., 1994; Vezina et al., 1994; Museo and Wise, 1995; Louis and Clarke, 1998). Although some drugs of abuse alter DA metabolism or reuptake to increase DA levels in the NAcc, nicotine alters the activity of ventral tagmental area neurons to enhance DA release. It should be noted that there are important differences in cellular and behavioral effects of changing DA levels by these two mechanisms. Interestingly, nicotine appears to preferentially stimulate activity in and release from DA neurons in the mesoaccumbens but not the nigrostriatal system, despite the fact that these cells have many other properties in common (Imperato et al., 1986; Mereu et al., 1987; Benwell and Balfour, 1997). Although there is strong evidence linking NAcc DA levels and reward, several recent studies suggest that this may be indirect. A more complex and less direct role for DA has been hypothesized, suggesting that DA signals novelty or reward expectation rather than reward itself (Schultz et al., 1997; Berke and Hyman, 2000; Di Chiara, 2000; Dani and De Biasi, 2001). In a recent study, rats were equipped

with intracranial self-stimulation devices in midbrain dopamine areas. In these individuals, self-stimulation of the reward centers caused elevation in NAcc DA levels during the learning period, but these increases were not seen in response to self-stimulation even 30 min later (Garris et al., 1999). Thus, regulatory processes appear to control DA release. The inhibition of action-potential driven DA release in the striatum by physiologically relevant nicotine concentrations suggests that cholinergic mechanisms may be important in these control mechanisms (Zhou et al., 2001). Although addiction likely involves the convergence of many CNS effects, the importance of the DA system provides a focus for many studies.

2. Effects of nicotine on the developing brain and offspring

The nAChRs appear to have functional roles during brain development as periods of transient high receptor density have been observed in the frontal cortex, hippocampus, cerebellum and brainstem in humans during mid-gestation and neonatal periods (Court and Clementi, 1995; Court et al., 1995; Court et al., 1997). Presence of both choline acetyltransferase (ChAT) and nAChRs during early development suggest important roles for nicotinic signaling in brain development. ChAT activity has been detected as early as 8 weeks of gestation (Candy et al., 1985; Perry et al., 1986) while the earliest reported binding of [^{3}H]-nicotine so far has been demonstrated in whole brain homogenates from 12 week old fetuses (Cairns and Wonnacott, 1988). The presence of nAChRs in the brain during development is of considerable interest, especially in view of the adverse effects of maternal smoking on fetal development including behavioural abnormalities and

cognitive deficits in addition to other harmful effects to the offspring. Nicotine is readily transferred to the fetal compartment throughout pregnancy and the fetuses of smoking mothers are in fact exposed to higher nicotine concentrations than the mother.

Tobacco smoking during pregnancy affects a large number of people in the US and worldwide and the proportion of pregnant women who smoke is estimated to be 20 - 25 %, resulting in about 800,000 babies prenatally exposed to tobacco smoke born annually in the US (Martin et al., 2003). *In utero* exposure to tobacco smoke results in low oxygen and high carbon monoxide blood levels, reduced placental function and increased vasoconstriction. These adverse effects deprive the fetus periodically of oxygen, and several chemical compounds, including nicotine, that are in tobacco smoke and reach the fetus (Ernst et al., 2001). In addition, maternal smoking is associated with an increased risk of perinatal morbidity such as low birth weight, perinatal mortality and Sudden Infant Death Syndrome (SIDS) (Haglund and Cnattingius, 1990; Mitchell et al., 1993; Ernst et al., 2001). Nicotine is the major psychoactive component in tobacco and believed to be causative compound for tobacco use and addiction.

3. Prenatal Nicotine induced neurological changes

Several epidemiological studies focused on the relationship between maternal smoking and the associated long-term behavioral problems with it (Ernst et al., 2001; Batty et al., 2006). Many of these studies have focused on cognitive performance, hyperactivity and attention deficits and increased risk of drug abuse in school-age children. The most clearly established consequence of maternal smoking during

pregnancy is decreased birth weight (Lumley, 1987). Studies have observed strong dose dependent weight decreases. Full-term babies born to mothers who smoked during pregnancy weight 170 to 250 g less than the average newborn (Hardy and Mellits, 1972; Conter et al., 1995). Babies born to heavy smokers weighted about 377 g less at birth (Wang et al., 2002). Therefore, smoking is most likely causally related to decreased birth weight. Though some reports suggest no further impairment of body growth later in life, the initial differences in height might extend into adolescence (Lassen and Oei, 1998). However, low birth weight increases the risk of adverse outcomes and could therefore contribute to health and behavioral problems associated with maternal smoking.

Most epidemiological studies have concluded that maternal smoking is a predictor of lower cognitive function in the offspring (Lassen and Oei, 1998; Cornelius et al., 2001; Batstra et al., 2003; Breslau et al., 2005; Batty et al., 2006). However, most recent studies have concluded that the correlation between maternal smoking during pregnancy and cognitive performance in the offspring has been overestimated (Lambers and Clark, 1996; Breslau et al., 2005; Batty et al., 2006). Thus the current knowledge suggests that the effects of maternal smoking during pregnancy on cognitive function in exposed children later in life might be limited and that smoking might be an indicator of decreased cognitive performance later in life.

Attention deficit hyperactivity disorder (ADHD) is one of the common behavioral disorders in children with a prevalence rate of 3-5% (Faraone and Doyle, 2000). Though the establishment of a causal link between gestational exposure to tobacco smoke and

ADHD has been difficult several studies revealed that children of mothers who smoked during pregnancy are at greater risk for ADHD (Linnet et al., 2003). In addition, a large population based study revealed a three-fold higher risk for ADHD (Linnet et al., 2005). Altogether, the vast majority of epidemiological studies conclude an increased risk of ADHD, conduct disorder, or behavioral problems in children after exposure to prenatal maternal smoking.

A hallmark for chronic nicotine exposure in adult and developing animals is the increased expression of nAChR binding sites which indicates the successful delivery of nicotine to the brain (Tizabi et al., 2000; Vaglenova et al., 2004). In particular, heteromeric α4/β2-containing nAChRs, which form most of the high affinity nicotine binding sites in the brain, are upregulated (Nguyen et al., 2004; Staley et al., 2006). Most studies suggest that upregulation of nAChRs is not due to increased mRNA expression for nAChR subunits (Marks et al., 1992; Pauly et al., 1996; Miao et al., 1998; Huang and Winzer-Serhan, 2006), but due to posttranscriptional regulation and increased receptor stabilization (Peng et al., 1994; Buisson and Bertrand, 2002; Wang et al., 2002; Sallette et al., 2005).

Chronic developmental nicotine exposure can alter hippocampal and cortical morphology, resulting in decreased neuronal area and increased packing densities in hippocampal principal cells and cortical neurons. The effects of nicotine on neuronal morphology are a direct consequence of developmental nicotine exposure and not an indirect effect resulting from nicotine-induced placental dysfunctions or hypoxia because

similar results are found after pre- and postnatal chronic treatment (Roy and Sabherwal, 1994; Roy et al., 2002; Huang et al., 2007). However, these effects are area specific, cerebellum, which develops late, did not show altered neuronal morphology whereas nicotine has a rapid and long-lasting effect on hippocampal neuronal morphology with vulnerability from prenatal to postnatal ages (Huang et al., 2007).

Nicotinic receptors are neuroprotective in conditions that challenge neuronal survival (Staley et al., 2006). Particularly, the heteromeric α4/β2 nAChR is associated with increased neuronal survival but the homomeric α7 receptor is believed to mediate increased cell death in immature neurons (Zoli et al., 1995; Laudenbach et al., 2002). Two *in vitro* studies found that nicotine exposure alone is sufficient to induce increased numbers of apoptotic cells in immature hippocampal progenitor cells or embryonic brain slices (Berger et al., 1998; Roy et al., 2002). Though some reports suggest developmental chronic nicotine alone does not seem to be neurotoxic to the immature brain (Machaalani et al., 2005; Huang et al., 2007) nicotine could potently interfere with development of neuronal circuitaries.

Early reports have shown prevalence of cognitive deficits in the young age group after prenatal exposure to nicotine; decreases in motor scores and verbal comprehension was found in 13-months-old offspring (Gusella and Fried, 1984), and reduced auditory perception has also been reported (Saxton, 1978). Decreased cognitive function has also been found in older children after in utero exposure to nicotine. For example a lower score on general cognitive functioning was found in 3-year-old children whose mothers

smoked 10 or more cigarettes throughout pregnancy as compared to children whose mothers quit smoking during pregnancy (Sexton et al., 1990). In 6-year-old children who were exposed to prenatal nicotine deficits in sustained attention, response inhibition, and memory, and lower overall cognitive function and receptive language were observed (Gusella and Fried, 1984). A dose–response relationship between maternal smoking during pregnancy and lower scores on arithmetic and spelling tasks of children between 5.5 and 11 years of age was found in a Dutch birth cohort (Batstra et al., 2003). Similarly, a study that compared a group of 6–17-year-old boys with ADHD with their first-degree relatives and healthy control subjects showed that children whose mothers smoked during pregnancy had significantly lower IQ scores than children whose mothers did not smoke (Milberger et al., 1998). In addition, another study showed that prenatal nicotine exposure was significantly related to deficits in verbal learning and design memory, problem solving, and a slower response in eye-hand coordination in 10-year-olds (Cornelius et al., 2001). More specific cognitive deficits in infants born to mothers who smoked during pregnancy have also been found. At 6–11 years of age, children prenatally exposed to nicotine were shown to have central auditory processing and visual-perceptual processing deficits (Gusella and Fried, 1984).

The neurobehavioral effects of *in utero* exposure to nicotine in animals have been described in a recent review (Ernst et al., 2001). Results showing an enhanced locomotor activity related to in utero nicotine exposure across species (rats, mice, and guinea pigs), and cognitive impairment, such as attention and memory deficits in performance on various maze tasks are of particular interests and parallels with the human studies. In

particular, animal studies have demonstrated deficits in learning tasks in prenatally exposed animals, which is in line with the cognitive deficits found in psychiatric disorders such as ADHD (Ernst et al., 2001). Thus, the effects on neurobehavioral outcome in animal studies parallels and supports the results from human studies.

4. Hippocampus, glutamate receptors and synaptic plasticity

The hippocampus is a unique and vital region found in each of the cerebral hemispheres. The hippocampus is a part of the forebrain, located in the medial temporal lobe and forms a part of the limbic system (Fig. 2.3). Although the precise role of hippocampus is still debatable, there is general agreement that it has an essential role in the formation of new memories about experienced events. In addition the hippocampus has been implicated in both spatial and contextual learning. The Morris water maze task, a well established test of spatial navigation, is sensitive to hippocampal damage (Morris et al., 1982) and fornix damage (Devan and White, 1999; Pouzet et al., 1999). Damages in hippocampus and fornix also disrupt contextual fear conditioning (Phillips and LeDoux, 1995; Maren and Fanselow, 1997; Bannerman et al., 2001). The neurotransmission in the major excitatory circuitries are mediated by glutamate receptors. The vitality of glutamategic neurotransmission in the hippocampus is exemplified in several neurological diseases with dementia.

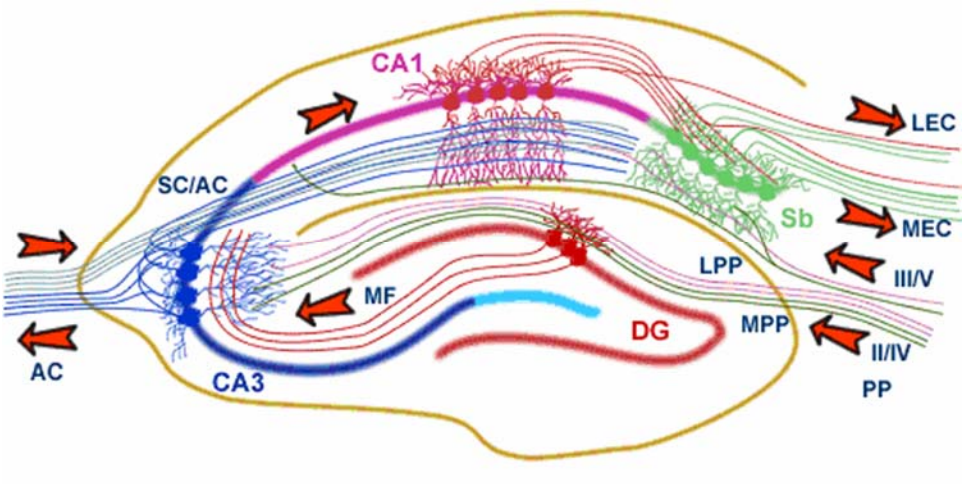

Figure 2.3. Section of hippocampus showing major regions and pathways.

In the mammalian hippocampus excitatory neurotransmission is mainly mediated by glutamate receptors. Based on the mechanism of channel activation, glutamate receptors are divided into ligand gated ionotropic and metabotropic receptors. The ionotropic glutamate receptors are ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the brain. The three pharmacologically defined classes of ionotropic glutamate receptor were originally named after selective agonists — N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate. Analysis of receptor structure and function revealed that glutamate receptors are composed of an agonist-binding domain, three transmembrane domains, a cytoplasm facing loop domain, an N-terminal domain, and a cytoplasmic C-terminal domain (Fig. 2.4) (Mayer and Armstrong, 2004). Once the correct topology for GluRs was established, it was possible find that in combination the S1S2 segments constituted the agonist-binding core (Stern-Bach et al., 1994). Further

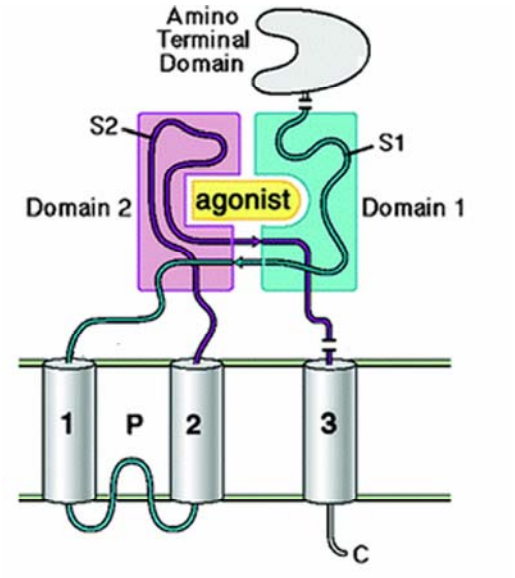

Figure 2.4. Structure of a glutamate receptor subunit

research involving homology modeling, combined with site-directed mutagenesis, supported the conclusion that the S1S2 segments form the agonist-binding site in AMPA, kainate, and NMDA receptors (Paas et al., 1996; Swanson et al., 1997; Foucaud et al., 2003). Such studies resulted in the establishment of the domain organization of GluR subunit, in which the agonist-binding core is a discrete structure, joined to the N-terminal domain and the ion channel pore by polypeptide linkers of currently unknown structure. It is now widely accepted that four subunits assemble to form functional glutamate receptors.

The ~400-amino acid polypeptide segment that makes up the N-terminal domain is a major determinant of subtype-specific assembly within GluR gene families (Ayalon and Stern-Bach, 2001; Meddows et al., 2001). In the case of the AMPA receptor GluR4 and NR1 subunits, the N-terminal domain forms dimers in solution (Kuusinen et al.,

1999; Meddows et al., 2001). Modeling and functional tests by site-directed mutagenesis established that this domain has a fold similar to that of leucine-isoleucine-valine-binding protein (LIVBP) and the agonist-binding domain in G–protein–coupled glutamate receptors (mGluRs). The structure of the N-terminal domain is expected to be distinct from the structure of the agonist-binding core of GluRs because in LIVBP and mGluRs, the two globular domains are connected by three β-strands instead of the pair of β-strands found in the GluR glutamate- and glycine-binding cores (Sack et al., 1989; Kunishima et al., 2000). In the case of NMDA receptors, functional experiments have established that Zn^{2+} and the noncompetitive antagonist ifenprodil bind to the N-terminal domain to modulate ion channel gating via an allosteric mechanism (Masuko et al., 1999; Paoletti et al., 2000; Zheng et al., 2001; Perin-Dureau et al., 2002).

The S1 and S2 polypeptide sequences that make up the agonist-binding core are interrupted by insertion of two membrane-spanning segments which in combination with a pore helix and pore loop make the narrowest part of the pore (Kuner et al., 1996; Kuner et al., 2001; Panchenko et al., 2001; Wang et al., 2002). The pore is sufficiently wide to allow Na^+ and K^+ ions to pass the narrowest segment in their hydrated state and/or the pore loops in GluRs are flexible enough to permit both Na^+ and K^+ ions during permeation. The C terminus of GluRs varies in length from around 20 to 500 amino acids. It interacts with numerous cytoskeletal proteins and is important for receptor trafficking (Scannevin and Huganir, 2000; Sheng et al., 2001). The C-terminal domain in NMDA receptors is much larger than that in AMPA and kainate receptors.

The AMPA receptor subunits (GluR1-4) occur in two alternatively spliced versions, flip and flop, that are encoded by exons 14 and 15 positioned just prior to domain 3 (Sommer et al., 1990; Monyer et al., 1991). Flip variants are abundant before birth and continue to be expressed in adult rats, whereas flop variants are in low abundance before the eighth postnatal day and are up-regulated to about the same level as the flip forms in adult animals. The flip forms of most subunits desensitize more slowly than the flop forms. In the primary transcript of GluR2, GluR5, and GluR6, a glutamine codon in the domain 2 can be edited to an arginine at the Q/R site. The arginine in edited versions of GluR2 causes low calcium permeability (Hume et al., 1991), low single channel conductance (Swanson et al., 1997), and an approximately linear current-voltage relation even in heteromeric receptors (Hume et al., 1991; Verdoorn et al., 1991; Egebjerg and Heinemann, 1993; Washburn et al., 1997). Both AMPA and NMDA receptors and metabotropic glutamate receptors are believed to play vital roles in various forms of synaptic plasticity, a process resulting in long lasting changes in synaptic efficacy (Fung and Lau, 1989; Malinow and Malenka, 2002; MacDonald et al., 2006; Genoux and Montgomery, 2007).

It is widely believed that a long-lasting change in synaptic efficacy is the cellular basis of learning and memory (Alkon and Nelson, 1990; Kandel, 1997). The most extensively characterized and analyzed forms of synaptic plasticity in the mammalian nervous system are long-term potentiation (LTP) and long-term depression (LTD). A remarkable feature of LTP and LTD is that a short period of synaptic activity (either high- or low-frequency stimulation) can trigger persistent changes of synaptic

transmission lasting at least several hours and often longer. This single property initially led investigators to suggest that these forms of plasticity are the cellular correlate of learning (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Work over the past 25 years that has elucidated many properties of LTP and LTD reinforces this view as well as suggests their involvement in various other physiological as well as pathological processes (Zoghbi et al., 2000; Martin et al., 2003).

Early studies provided support for postsynaptic exocytosis playing a role in synaptic plasticity. In hippocampal slices loading postsynaptic cells with toxins that specifically perturb membrane fusion could block LTP (Lledo et al., 1998). Another study in dissociated cultured neurons identified a form of dendritic exocytosis that was mediated by activation of CaMKII (Maletic-Savatic et al., 1998), an enzyme that play a critical role in LTP (Lisman et al., 1997). Thus, dendritic exocytosis was further linked to synaptic plasticity. These studies along with the demonstration of the role of silent synapses in LTP provided strong evidence that support trafficking of AMPA receptors to synapses.

It is widely accepted that during long term depression, an opposing process to LTP in which synaptic efficacy is diminished, AMPA receptors internalized from the postsynaptic membranes. The first experimental support for this idea came from the use of immunocytochemical techniques to examine the distribution of AMPA receptors following the generation of NMDA receptor dependent LTD in hippocampal cultures (Carroll et al., 1999). LTD caused a decrease in the proportion of synapses containing

detectable surface AMPA receptors while having no effect on the distribution of synaptic NMDARs. Generation of LTD in the hippocampus in vivo subsequently was found to cause a decrease in the number of AMPA receptors in synaptoneurosomes, providing further evidence for the role of AMPA receptor endocytosis (Heynen et al., 2000). That the loss of synaptic AMPA receptors during LTD involves their clathrin-mediated endocytosis is further supported by experiments in which LTD was blocked by loading CA1 pyramidal neurons or cerebellar Purkinje cells with a peptide that disrupts dynamin function (Luscher et al., 1999; Wang et al., 2002). Importantly, these results were the first demonstration that two forms of LTD that previously were thought to be mechanistically distinct, cerebellar LTD and NMDA receptor dependent LTD in the hippocampus, appear to share a common mechanism of expression. Inhibition of endocytosis also blocked the actions of insulin, which can cause a depression of synaptic currents that occludes LTD (Ebrahim et al., 2000; Lin et al., 2000).

In summary this section provides literature support for harmful effects of prenatal nicotine exposure and discusses the importance of mammalian hippocampus in learning and memory processes, and the important role of glutamate receptors in these processes.

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3. PRENATAL NICOTINE EXPOSURE RESULTS IN NEUROCHEMICAL AND BEHAVIORAL ALTERATIONS IN YOUNG RATS.

Abstract

Research has shown that prenatal nicotine exposure in the form of tobacco use during pregnancy can result in increased mortality and morbidity during fetal and neonatal life. The current study used a rodent model to assess behavioral and neurochemical consequences of prenatal nicotine exposure in offspring. In this rodent model throughout gestation pregnant dams were infused with nicotine (6 mg/kg/day) subcutaneously via osmotic mini pumps. Results of this study show that prenatal nicotine exposure results in decreased birth weight and weight gain. Prenatal nicotine exposed rats also showed decreased performance in negative geotaxis test, forced swimming and open field exploration tasks. In addition a modest decline in surface righting reflex task was also observed on PND 2. Results of neurochemical studies showed high quantity of reactive oxygen species and increased lipid peroxidation in hippocampal tissue without any aberrant protein oxidation. Hippocampal based spatial memory, when assessed with Y maze task, was deficient in prenatal nicotine rats. These results suggest that maternal smoking could result in reduced weight, weight gain and motor function and aberrant neurochemical reactions and memory processing in young offspring.

Introduction

Tobacco smoking during pregnancy remains to be a significant public health issue in spite of awareness programs and affects a large number of people in the US and worldwide. The proportion of pregnant women who smoke is estimated to be 20 to 25 %, resulting in birth of about 800,000 babies exposed to prenatal tobacco smoke annually in the US (Martin et al., 2003). Prenatal exposure to tobacco smoke results in the developing fetus being exposed to several harmful toxic substances. The adverse effects caused by these toxic substances range from reduced placental function to possible long term neurobehavioral alterations in the offspring (Ernst et al., 2001; Batty et al., 2006). While the reduced placental function and associated harmful effects may be due to more than one toxic substances in the tobacco smoke, neurobehavioral alterations are mainly due to nicotine, the major toxic compound in the tobacco smoke with psychoactive properties. Nicotine acts on the nAChRs by increasing their activity in small concentrations (Hogg et al., 2003). Nicotinic acetylcholine receptors (nAChRs) are ionotropic receptors that form ligand gated ion channels and their opening is triggered by the neurotransmitter acetylcholine (ACh), but they are also opened by their potent agonist nicotine (Itier and Bertrand, 2001). The nAChRs play important roles during development and plasticity. Therefore premature activation of nAChRs, as in the case of maternal smoking could result in alterations in neurodevelopmetal processes regulated by these receptors.

Activation of nicotinic acetylcholine receptors promotes synaptic contacts and the wiring during a critical period of postnatal development in hippocampus (Maggi et al.,

2003). It also has a major role in the control motor tone and movement (Cooper et al., 2003; Johnston and Silverstein, 1998). Interestingly the cholinergic innervation in the mammalian brain commences early, in fetal development. In the cortex cholinergic innervation begins about E19 in the mouse and the rat and around week 20 in the human fetus (Berger-Sweeney and Hohmann, 1997). Prenatal nicotine exposure therefore would interrupt the normal developmental regulation of cholinergic system. Chronic developmental nicotine administration has been reported to result in changes in nicotine binding in the brain (Schwartz and Kellar, 1983; van de Kamp and Collins, 1994; Narayanan et al., 2002; Staley et al., 2006). Apart from the molecular changes prenatal nicotine is believed to cause neuronal death and morphological changes in specific brain regions (Roy and Sabherwal, 1994; Roy et al., 1998; Roy and Sabherwal, 1998; Roy et al., 2002). In addition maternal smoking is strongly related to low birth weight, neonatal morbidity and mortality, hyperactivity and cognitive deficits in human (Hardy and Mellits, 1972; Conter et al., 1995; Cornelius et al., 2001; Wang et al., 2002; Batstra et al., 2003; Linnet et al., 2003; Linnet et al., 2005).

Several epidemiological studies and animal studies have come up with variable results with developmental nicotine induced neurobehavioral alterations. Chronic nicotine exposure in prenatal models has been found to either decrease birth weight (Cutler et al., 1996) or in no change in birth weight (Levin et al., 1993; Abreu-Villaca et al., 2004; Chen and Kelly, 2005). In addition, though majority of the studies have reported learning and memory deficits in prenatal nicotine exposed offspring (Sorenson et al., 1991; Yanai et al., 1992), there are conflicting reports and considerable variation in

learning and memory outcomes (Levin et al., 1993; Cutler et al., 1996). In addition there have only been few reports on changes in neurodevelopmental reflexes induced by prenatal nicotine (Ajarem and Ahmad, 1998). In the present study we used a rat model of prenatal nicotine exposure, subcutaneous nicotine infusion via osmotic mini pumps in pregnant dams (Murrin et al., 1987), to evaluate physical and neurobehavioral outcomes in young offspring. Results of this study show that prenatal nicotine exposure results in reduction in body weight, developmental reflexes and hippocampal based memory.

Materials and methods

Animals and Chemicals: Time pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and osmotic mini pumps (Alzet, Cupertino, CA) were implanted, under isoflurane anesthesia, beneath shoulder skin to deliver subcutaneous dose of nicotine at a rate of 6 mg/kg/day. Osmotic mini pumps were removed once the pups were delivered so that nicotine exposure was limited from ~ day3 of pregnancy to birth. Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Surface righting reflex: This test was done in young pups to measure motor function and coordination at the early stage of development. The pup was held on its back on a padded flat surface and then released. The righting reflex was defined as the number of seconds required for a pup lying on its back to right itself on all four limbs and was considered fully formed if the rat pup did so within 30 s.

Negative geotaxis: Negative geotaxis is an automatic, stimulus-bound orientation movement considered diagnostic of vestibular and/or proprioceptive function (de Castro et al., 2007). In this test, the pup was placed head down, on an inclined plane (30º) and the time taken to turn (by 180°) toward the high end of the plane was calculated.

Grip strength response: In this test, the pup was made to hang by its forepaws on a horizontal rod 30 cm above a soft pad. Gripping ability was considered developed when the pup was able to hang from the rod. The duration the animal was able to hang itself was recorded.

Rotorod test: Rats were trained to maintain their balance on a rotating rod with a speed of 20 rpm for 10 min. Fallen rats were repositioned on the rod. The training was followed by three experimental trials to assess sensorimotor coordination. Each trial was of 10 min duration and the speed was set at 20 rpm reached within the first min, 80 rpm reached at a rate of 8 rpm/min, and 80 rpm reached at a rate of 20 rpm/min for the 1^{st}, 2^{nd} and 3^{rd} trials respectively. Time taken for each rat to fall was calculated.

Forced swim test: Pups were forced to swim for 10 min period in a vessel containing 40 cm water. The time taken (latency) to reach the first floating state (immobile state except for small limb movements to keep afloat) and the total time spent on floating state was calculated.

Open field test: Each rat was placed gently into the center of the field (18X16 inches) and allowed to explore the arena for 10 min, and exploration and arousal were assessed as

number of grid crosses and rearing using a photobeam activity system (San Diego Instruments, San Diego, CA). Reduced exploratory (locomotor) activity is an index of increased levels of anxiety (Burt et al., 2007).

Biochemical assays: Lipid peroxidation was determined by using the thiobarbituric acid (TBA) assay. This assay involves the reaction of malondialdehde (MDA) with TBA to yield a pink complex, thiobarbituric acid reacting substances (TBARS). Hippocampal homogenate (10% w/v in PBS) was mixed with TCA (20%) and TBA (0.5%) and the mixture was heated for 15 min at 80 °C in a water bath. After cooling, the sample was centrifuged at 14,000 g for 10 min and the absorbance of the supernatant was read at 532 nm (Dhanasekaran et al 2007). Protein carbonyl assay: Hippocampal homogenate was mixed with Trichloroacetic acid (TCA) and centrifuged for 5 min at 12000 g. After discarding the supernatant the pellet was resuspended with TCA and centrifuged for 2 min at 12000 g. The supernatant was discarded and the pellets were dispersed with the addition of 2,4-dinitrophenylhydrazine (DNPH) and incubated in an orbital shaker at 37ºC for 30 min. The mixture was centrifuged for 10 min at 10,000 and equal voume of ethanol/ethyl acetate (1:1) was added to the pellet and mixed in a shaker for 1 min and centrifuged again at 10,000 g for 3 min. The pellet was washed again in ethanol/ethyl acetate. The pellet was sonicated with 6 M guanidine solution and the mixture was incubated for 30 min at 37ºC and subsequently centrifuged at 10, 000 g for 10 min. The supernatant was measure spectrophotmetically at 360 nm. Reactive oxygen species (ROS) was assayed by incubating the hippocampal homogenate 10 % w/v in PBS with dichlorodihydrofluorescein diacetate (DCFDA) for 1 hour. Reactive oxygen species

formed react with DCFDA to form a fluorescence product and was measured at 460/528 nm (Dhanasekaran et al., 2007).

Y maze test: Hippocampal based spatial memory was examined using a Y maze apparatus. Each animal was allowed to explore (3 min) two arms freely while entry into one arm (novel arm) was prevented by an opaque gate. After 3 hours from the exploration session each animal was allowed to explore all three arms for 5 min. Total number of entries and the time spent in the novel arm was calculated.

Results

In the current study we did not observe any maternal mortality or any significant offspring mortality. Only one offspring mortality was observed in the current study. We observed a significant reduction in birth weight in nicotine exposed pups (Fig. 1A; n=30, $p<0.001$, one way ANOVA followed by Fisher LSD *post hoc* analysis). Significant variation in body weight was also observed at 2 weeks of age (Fig. 1B; n=30, $p<0.001$, one way ANOVA followed by Fisher LSD *post hoc* analysis). Indeed low birth weight is regarded as a hallmark of maternal smoking and clinical studies have correlated maternal smoking with reduced weight in the offspring (Mochizuki et al., 1984; Mochizuki et al., 1985; Vogt Isaksen, 2004). Results of this study suggest that nicotine alone can cause reductions in birth weight and this reduction could be persistent even after withdrawal of nicotine exposure.

We performed surface righting reflex and negative geotaxis tests to assess motor coordination and orientation movement. Surface righting reflex was fully developed in

control as well as prenatal nicotine rats as all the animals tested met the discrimination criteria of 30 s. However, we observed a modest decline in prenatal nicotine animals only in PND2 suggesting a weak transient impairment in motor coordination which recovers very early in postnatal life (Fig. 3.1 C; n=10, $p<0.1$, one way ANOVA followed by Fisher LSD *post hoc* analysis). Interestingly prenatal nicotine rats showed much stronger deficits in negative geotaxis task (Fig. 3.1D; n=10, $p<0.01$, one way ANOVA followed by Fisher LSD *post hoc* analysis) suggesting that automatic, stimulus bound reflective response that results in directional movement against gravitational force is impaired in these animals (Fraenkel and Gunn, 1961).

Muscular strength and coordination in limbs was assessed by wirehanging test and rotorod test (Ikegami et al., 2000; Lalonde et al., 2002). Results of these tests did not show any significant difference between prenatal nicotine and control rats implying that there was no depreciation of muscular strength in limbs as a result of prenatal nicotine exposure (Fig. 3.2 A and B; n=10, $p>0.05$, one way ANOVA followed by Fisher LSD *post hoc* analysis). We performed forced swim test to assess whether muscular strength prevails for longer period of more challenging activity. Results of forced swim test study show that prenatal nicotine animals reached immobile state significantly sooner and spent significantly more time in immobile state than controls (Fig. 3.2 C and D; n=10, $p<0.05$, one way ANOVA followed by Fisher LSD *post hoc* analysis). These results suggest prenatal nicotine exposure results in accelerated muscular fatigue in tasks that require robust and prolonged muscular activity.

Open field test was performed to evaluate the locomotor activity of prenatal nicotine exposed animals since reports indicate maternal smoking results in high incidence of attention deficits and hyperactivity. Results of our open field tests show that nicotine exposed animals showed reduced locomotor activity ($p<0.05$) and rearing ($p<0.01$) without any significant dependence on the animal's sex (Fig. 3.3 A and B; n=25, two way ANOVA followed by Newman–Keul's comparison test). These results are in agreement with that of a recent study (LeSage et al., 2006) in which gestational intravenous administration of nicotine resulted in open field hypoactivity in rat offspring.

Aberrant oxidative degradation of lipids was evaluated by lipid peroxidase assay and our results show significantly increased lipid peroxidase activity in the hippocampus of prenatal nicotine exposed animals (Fig. 3.4 A; n=7, $p<0.05$, ANOVA). There was a concomitant modest increase in ROS levels (Fig. 3.4 C; n=7, $p<0.1$, ANOVA), but there was no significant difference in protein oxidation (protein carbonyl) levels (Fig 3.4 B; n=7, $p>0.05$, ANOVA). Despite the well established injurious effects caused by prenatal nicotine on the developing brain there is a lack of research focus on possible oxidative stress. One study reported lack of lipid peroxide activity in cortical regions of rhesus monkeys exposed to prenatal nicotine (Slotkin et al., 2005). Our results suggest a moderate effect of oxidative stress in the rat hippocampus subjected to prenatal nicotine.

Clinical and animal studies have indicated cognitive deficits in offsprings with prenatal nicotine exposure. Since mammalian hippocampus is a primary region involved in learning and memory processes we tested the hippocampal based spatial memory performance using a Y maze apparatus. Results of this study showed that number of

visits to the novel arm (Fig. 3.5 A) and the total duration of time spent in the novel arm (Fig. 3.5 B) were significantly reduced in both sexes of prenatal nicotine exposed rats without any significant variations within sex (n=10, $p<0.05$, two way ANOVA followed by Newman–Keul's comparison test). Though some studies showed sexual variability in cognitive deficits (Vaglenova et al., 2004; Eppolito and Smith, 2006), our results are similar to a previous report (Sorenson et al., 1991) that suggested similar cognitive impairments in both sexes.

Discussion

In the present investigation, we examined the physical and neurobehavioral outcomes of prenatal nicotine exposure in rats. Maternal tobacco smoking has a strong correlation with reduced birth weight and adverse neurobehavioral outcomes in the children (Winzer-Serhan, 2008). We have found a significant reduction in birth weights in nicotine exposed pups which indicate that nicotine in the tobacco smoke is sufficient and potent enough to cause fetal growth retardation resulting in low birth weight. Similar results were reported by some previous studies in which prenatal nicotine exposure significantly reduced the birth weight of pups (Vaglenova et al., 2004; LeSage et al., 2006). The results with animal studies are supported by epidemiological/clinical reports which indicate a link between maternal smoking or smoke exposure and low birth weight (Kramer, 1987; Ernst et al., 2001; Jaakkola et al., 2001; Jauniaux and Burton, 2007). Though some reports suggest there is no significant low birth weight in nicotine exposed offspring (Levin et al., 1993; Sheng et al., 2001; Chen and Kelly, 2005) the dose of

exposure was much lower than that used in this and other studies. Furthermore tobacco smoke and nicotine in particular affects placental morphology and function (Zdravkovic et al., 2006; Jauniaux and Burton, 2007) which potentially compromise fetal growth. In addition alterations in protein metabolism and enzyme activity have also been observed in fetuses exposed to tobacco smoke (Jauniaux and Burton, 2007). Results from this study and these reports suggest prenatal nicotine exposure can result in reduced birth weight. Results from this study also showed significantly reduced body weight in nicotine exposed animals compared to the coeval controls suggesting that at the age of 2 weeks prenatal nicotine induced changes in body weight and possibly changes in metabolism may persist long after cessation of nicotine exposure. Interestingly some reports have shown sexual variability in body weights (Peters and Tang, 1982; Vaglenova et al., 2004) and currently it is not clear how sexual maturation could differentially modify the compromised metabolic processes.

Prenatal nicotine exposure may not affect certain tasks that require motor coordination and muscle strength as evidenced by results of righting reflex, wire hanging and rotorod tests of our study. Similar results were reported by other reports showing righting reflex and motor coordination were unaffected (Fung and Lau, 1989; Matta and Elberger, 2007). It is believed that maternal smoking is strongly related to altered activity and conduct problems in the children (Winzer-Serhan, 2008). However, research reports on locomotor activity present conflicting results (LeSage et al., 2006; Winzer-Serhan, 2008). Though some studies reports hyperactivity (Peters et al., 1979; Fung and Lau, 1989; Ajarem and Ahmad, 1998; Vaglenova et al., 2004) others suggest hyperactivity is

limited to only a subset of animals with no overall effect (Richardson and Tizabi, 1994; Tizabi et al., 1997) and inconsistent effects across different measures of activity (Tizabi et al., 2000) or postnatal time points (Schlumpf et al., 1988; Paulson et al., 1994). Our study shows that prenatal nicotine exposure potently reduces locomotor activity in open field in young rats without any sex dependent variations. Similar results were also reported by other studies (Peters and Tang, 1982; Romero and Chen, 2004) suggesting that prenatal nicotine exposure could reduce locomotor activity. The variable results in the literature can be attributed to the dose, route of dose, formulation of nicotine, duration of exposure and stage of life at which locomotor activity was tested in the offspring. The reduced locomotor activity observed in our studies could be due to anxiety in new environments. Clinical studies and animal studies suggest that prenatal nicotine exposure might result in anxiety in the offspring (Vaglenova et al., 2004; LeSage et al., 2006; Winzer-Serhan, 2008).

Many clinical reports have suggest that maternal smoking is a risk factor for deficient cognitive function in the children (Breslau et al., 1991; Cornelius et al., 2001; Batstra et al., 2003; Batty et al., 2006; Jacobsen et al., 2006). In particular one study showed that in human adolescent tobacco smokers, prenatal exposure to active maternal smoking is associated with alterations in medial temporal lobe function and concomitant deficits in visuospatial memory (Jacobsen et al., 2006). We performed Y maze experiments to evaluate hippocampal based memory (Conrad et al., 1996; Conrad et al., 1997) and our results show that nicotine exposed rats had significantly lesser number of entries and spent lesser time in the novel arm. These results indicate an impairment in

hippocampal based memory. Impairments in spatial and working memory have been reported by other studies that reported prenatal and pre and postnatal nicotine effects on memory (Levin et al., 1996; Vaglenova et al., 2004; Eppolito and Smith, 2006). Taken together with the observations that prenatal nicotine causes morphological disruption in hippocampal cellular layers (Roy et al., 2002) our results strongly suggest a decline in hippocampal memory in offspring exposed to prenatal nicotine.

In conclusion the current study used a sensitive rodent model for prenatal nicotine exposure and the results of this study show parallel striking similarities with neurobehavioral alterations observed in children exposed to tobacco smoke *in utero*. The most important finding of this study is the establishment of memory deficits particularly that is dependent on hippocampus. An assessment of key physiological processes, in prenatal nicotine exposed hippocampus, that are believed to govern hippocampal memory formation and the changes that may impair memory processing would be of great interest as nicotinic cholinergic physiology is critical for memory processes.

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Figure Legends

Figure 3.1. Prenatal nicotine administration reduces body weights and developmental reflexes. (**A**) Reduction in birth weight (**B**) Reduction in body weight at 2 weeks of age (**C**) Modest increase in mean latency time in righting reflex in PND 2 (**D**) Increase in mean latency time in negative geotaxis. Data presented as mean±SEM. n=7, $^{\#}p<0.1$, $^{**}p<0.01$, $^{***}p<0.001$, one way ANOVA.

Figure 3.2. Prenatal nicotine exposure impairs only tasks that require long period of strong locomotor activity. (**A**) There was no significant difference in mean latency time in wire hanging task (**B**) mean latency time in rotorod test did not show any significant difference (**C**) prenatal nicotine rats showed significantly lesser latency time to reach first immobile state in forced swim test (**D**) significantly higher duration of time spent in immobile state. Data presented as mean±SEM. n=7, $^{\#}p<0.1$, $^{*}p<0.05$, one way ANOVA.

Figure 3.3. Prenatal nicotine exposed rats showed reduced sensorimotor activity in open field test. (**A**) Reduced number of activities implicating reduced distance traveled (B) Reduced number of rearings. Data presented as mean±SEM. n=7, $^{*}p<0.05$, $^{**}p<0.01$, one way ANOVA.

Figure 3.4. Prenatal nicotine exposure promotes aberrant biochemical reactions. (**A**) Increased lipid peroxidation (**B**) No change in protein oxidation assessed by protein

carbonyls (**C**) Modest increase in reactive oxygen species. Data presented as mean±SEM. n=5, $^{\#}p<0.1$, $^{*}p<0.05$, one way ANOVA.

Figure 3.5. Prenatal nicotine exposure induced reduction in Y maze memory tasks. (**A**) Reduced number of entries into the novel arm (**B**) Reduced time spent in the novel arm. n=8, $^{*}p<0.05$, one way ANOVA.

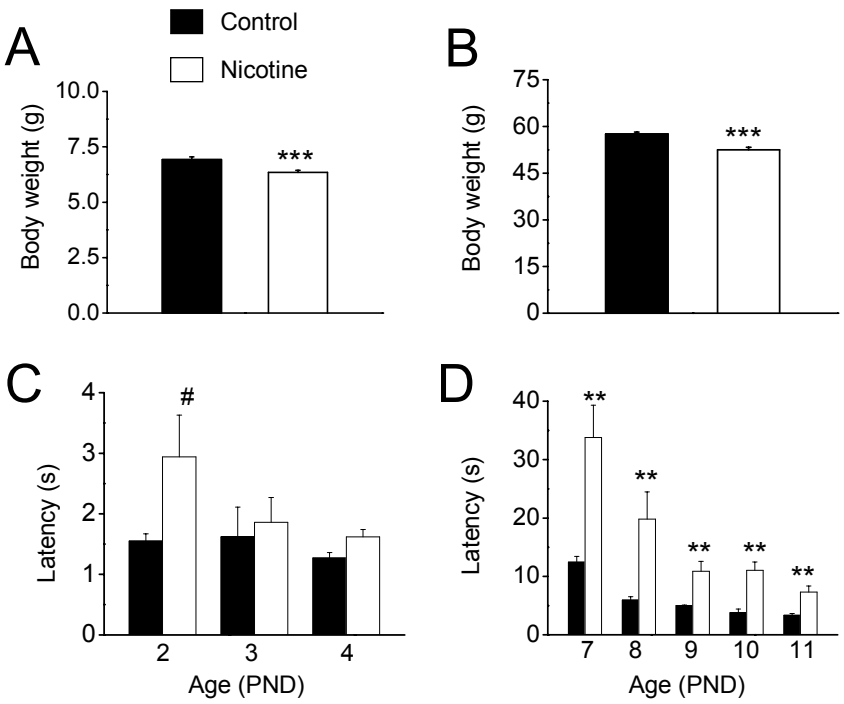
A
Control
Nicotine
Body weight (g)
10.0
7.5
5.0
2.5
0.0
***
B
Body weight (g)
75
60
45
30
15
0
***
C
Latency (s)
4
3
2
1
0
#
2
3
4
Age (PND)
D
Latency (s)
40
30
20
10
0
**
**
**
**
**
7
8
9
10
11
Age (PND)

Figure 3.1

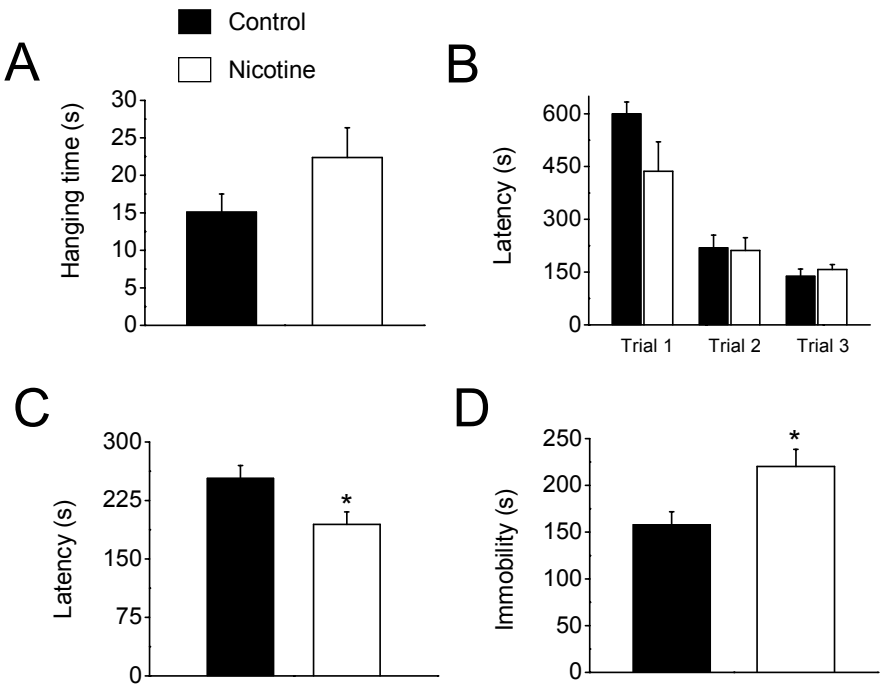
A
Control
Nicotine
Hanging time (s)
30
25
20
15
10
5
0
B
Latency (s)
600
450
300
150
0
Trial 1
Trial 2
Trial 3
C
Latency (s)
300
225
150
75
0
*
D
Immobility (s)
250
200
150
100
50
0
*

Figure 3.2

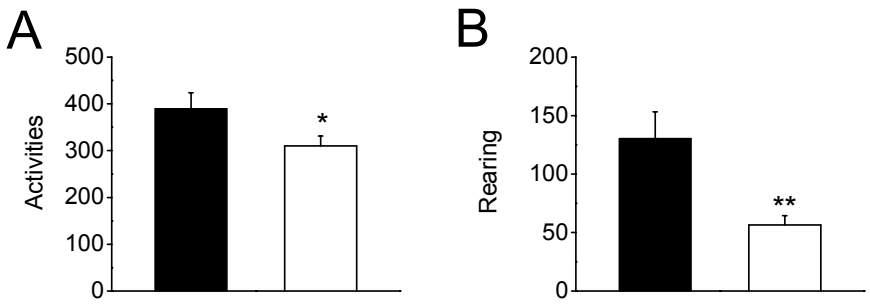

Figure 3.3

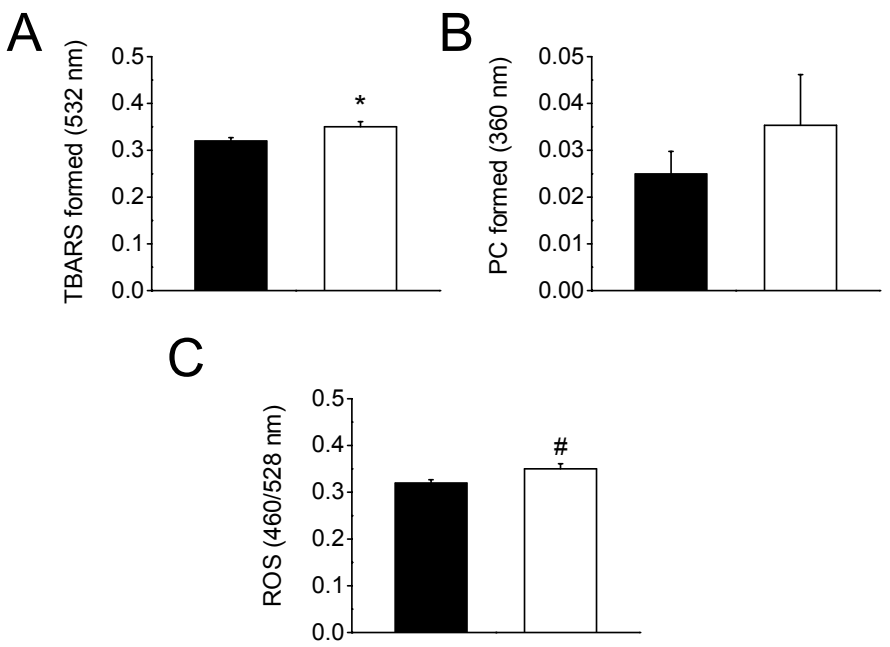
A
TBARS formed (532 nm)
0.5
0.4
0.3
0.2
0.1
0.0
*
B
PC formed (360 nm)
0.05
0.04
0.03
0.02
0.01
0.00
C
ROS (460/528 nm)
0.5
0.4
0.3
0.2
0.1
0.0
#

Figure 3.4

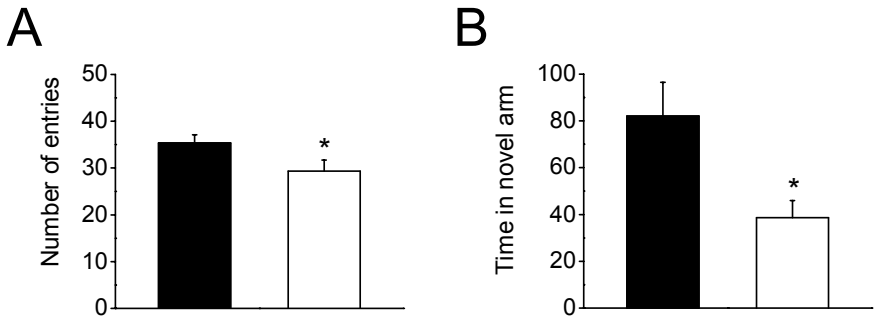
A
Number of entries
50
40
30
20
10
0
*
B
Time in novel arm
100
80
60
40
20
0
*

Figure 3.5

4. PRENATAL NICOTINE EXPOSURE RESULTS IN IMPAIRED SYNAPTIC PLASTICITY AND AMPA RECEPTOR SYNAPTIC CURRENTS IN HIPPOCAMPUS OF YOUNG RATS

Abstract

Maternal smoking is a well known risk factor for cognitive problems in young children. Animal studies have shown hippocampal memory forms are impaired due to prenatal nicotine exposure. In the mammalian hippocampus glutamate, in particular, AMPA receptors mediate most of the excitatory neurotransmission. In this study we analyzed long term potentiation (LTP) of hippocampal Shaffer collateral-CA1 synapses and the AMPA receptor mediated synaptic currents in 2-4 weeks old young rats that were exposed to prenatal nicotine. Nicotine (6 mg/kg/day) was administered to pregnant dams via osmotic mini pumps implanted subcutaneously throughout gestation. Results of this study show impaired LTP and AMPA receptor synaptic currents in prenatal nicotine exposed young rats suggesting impairments in these synaptic functions may underlie the cognitive deficits observed in children exposed to *in utero* tobacco smoke.

Introduction

Despite abundant adverse publicity smoking during pregnancy remains one of the major problem in maternal and offspring well being in US and many other countries. Epidemiological reports have indicated about half of the tobacco smoking women continue to smoke during their pregnancy which results in about half a million of births per annum with infants exposed to toxic substances from tobacco smoke during their fetal life (Ebrahim et al., 2000; Martin et al., 2003). Maternal smoking is strongly correlated with several birth complications (2004) including pre term birth (Nabet et al., 2007), still birth (Hogberg and Cnattingius, 2007), low birth weight (Comstock et al., 1971; Aronson et al., 1993; Horta et al., 1997), fetal growth retardation (Miller et al., 1976; Raymond et al., 1994; Horta et al., 1997) and risk of congenital anomaly (Chung et al., 2000; Man and Chang, 2006). In addition to these congenital problems neuro-behavioral anomalies are widespread in children who were exposed to *in utero* tobacco smoke. Maternal smoking is suspected to be associated with ADHD (Linnet et al., 2003) and results in deficient motor scores, verbal comprehension skills, design memory, problem solving and various other cognitive tasks (Gusella and Fried, 1984; Sexton et al., 1990; Cornelius et al., 2001; Huizink and Mulder, 2006). Thus it can be concluded maternal tobacco use results in many adverse effects to the offspring including cognitive impairments.

Though tobacco smoke contains several toxic substances the major psychoactive component is nicotine, which is an agonist of nicotinic acetylcholine receptors (nAChRs). These receptors are expressed at very early stages of fetal development in human and rodent brains (Naeff et al., 1992; Hellstrom-Lindahl et al., 1998) and studies have shown

that nAChRs in developing brain are functional and mediate critical developmental roles (Aramakis and Metherate, 1998; Gallardo and Leslie, 1998; Atluri et al., 2001; Barazangi and Role, 2001). Thus early and sustained modulation of nAChRs, as in the case of smoking during pregnancy, can result in profound nAChR desensitization (Hanganu and Luhmann, 2004), and long term disruption of neurodevelopmental processes (Metherate, 2004). In particular prenatal nicotine exposure has been shown to affect neurotransmitter systems (Oliff and Gallardo, 1999; Hsieh et al., 2002; Wang et al., 2002; Luo et al., 2007) and modifications in the neurotransmitter systems may explain the impaired neurobehavioral responses.

In mammalian brain hippocampus is the major region involved in memory processing and excitatory neurotransmission is largely mediated by glutamate receptors. In addition these receptors are critical for synaptic plasticity processes which are believed to underlie memory processing. Clinical studies and animal studies have shown prenatal nicotine can cause deficits in hippocampus dependent memory, and morphological changes in the hippocampus (Roy et al., 2002; Jacobsen et al., 2006). Studies from our lab also suggest that prenatal nicotine exposed animals are deficient in hippocampal memory based tasks in Y maze (unpublished data). In the mammalian hippocampus glutamatergic receptors are the major mediators of excitatory synaptic transmission and are critical for forms of synaptic plasticity that is believed to be the cellular correlates of memory formation. In particular (AMPA) subtypes of glutamate receptors mediate the fast component of the glutamatergic synaptic transmission and are critical for the expression phase of long term potentiation (LTP), a form of synaptic plasticity in which

excitatory synaptic transmission is strengthened. Impairments in AMPA receptor function could potentially lead to compromised excitatory synaptic transmission resulting in cognitive deficits. In this study we analyzed AMPA receptor mediated synaptic currents and LTP in young rats that were exposed to prenatal nicotine. We found prenatal nicotine exposure results in potent reductions in AMPA receptor synaptic currents and attenuations in LTP in critical hippocampal synapses suggesting that these impairments could underlie the cognitive deficits observed in children who were exposed to prenatal nicotine due to maternal smoking during pregnancy.

Materials and methods

Animals and Chemicals: Time pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and osmotic mini pumps (Alzet, Cupertino, CA) were implanted, under isoflurane anesthesia, beneath the skin to deliver subcutaneous dose of nicotine at a rate of 6 mg/kg/day. Osmotic mini pumps were removed once the pups were delivered so that prenatal nicotine exposure was limited from ~ day3 of pregnancy to birth. Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Preparation of Hippocampal slices and synaptosomes: Transverse hippocampal slices (400 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 $MgSO_4$, $CaCl_2$ 0.5, NaH_2PO_4

1.25, 25 $NaHCO_3$, 25 glucose, 75 sucrose 0.5 ascorbate and 2 kynurenic acid and was bubbled with 95%CO_2/5%O_2, bringing the solution to 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 and was bubbled with 95%CO_2/5%O_2. Synaptosomes were prepared by previously described (Johnson et al., 1997; Suppiramaniam et al., 2006) methods in which hippocampi were dissected out and homogenized in homogenizing buffer (mKRBS) using a Potter homogenizer with 10 strokes. 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 by bubbling with 95%O_2/5%CO_2. The buffer was also 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 synaptosomes was resuspended in 20 μl of mKRBS buffer.

Slice electrophysiology: Following incubation electrophysiological recordings were performed in recordings chambers with continuous 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 ~ 200 μm from the stimulating electrode. The frequency of the test stimulation was every 20 s. For stimulus response curves current intensity was increased from 0 to 300 μA at steps of 25 μA. For paired pulse ratio (PPR) and LTP experiments current intensity was set at 50% of maximal fEPSP and inter pulse intervals were set at 10, 20, 30, 40, 50, 75, 100, 150 and 200 ms in PPR experiments. In LTP experiments after at least 15 min of stable baseline recording 3 high frequency stimuli (HFS; 100 Hz) were delivered every 20 s. LTP was measured 50-60 min post HFS.

Whole cell current recordings were performed in CA1 pyramidal neurons voltage clamped at -65 mV. The patch pipette (6-10 MΩ) was filled in an internal solution comprising (in mM): 100 K-gluconate, 0.6 EGTA, 5 $MgCl_2$, 2 ATP-Na, 0.3 GTP-Na and 40 HEPES and the slices were perfused continuously with ACSF supplemented with picrotoxin, APV and tetradotoxin to isolate AMPA receptor mediated miniature excitatory postsynaptic currents (mEPSC).

Single channel electrophysiology: Incorporation of AMPA receptors 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 borosilicate glass pipette (100 MΩ). The synthetic phospholipids was 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 was 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 was 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). 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, 3-5 μl suspension of the synaptosomes was delivered to the ECF. After addition of AMPA (290 nM), 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 pClamp9 software (Molecular Devices) and saved in 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 all points-current amplitude histograms was constructed and fitted with 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 variable metric fitting method to identify distinct open and close times.

Results

Prenatal nicotine impairs basal excitatory synaptic transmission in Schaffer collateral synapses in hippocampus.

Previous studies have shown morphological alterations in the hippocampus and impaired hippocampal based behavioral tasks in rats exposed to prenatal nicotine. To investigate whether these alterations cause functional anomalies in excitatory synaptic transmission we recorded fEPSPs from Schaffer collateral-CA1 synapses to characterize for possible changes in basal synaptic function. Presynaptic fiber volley (FV) and slope of fEPSPs were analyzed in response to different stimulation intensities. In prenatal nicotine exposed rats the amplitude of FV was significantly reduced from that of controls ($p<0.05$) (Fig. 4.1A), suggesting that prenatal nicotine administration significantly affect the number of afferent axons. Input/Output (I/O) curves, measured by plotting stimulation intensities versus the corresponding fEPSP (Fig. 4.1B) and I/O curves determined by plotting the amplitude of the FV versus fEPSP slope (Fig. 4.1C) were significantly reduced ($p<0.05$) between prenatal nicotine exposed rats and controls, suggesting that prenatal nicotine exposure impairs basal synaptic transmission in Schaffer collateral-CA1 synapses.

Altered presynaptic function in prenatal nicotine exposed rats.

Impaired basal synaptic transmission observed in the prenatal nicotine exposed rats suggests that normal function of the hippocampal synapses is altered by abnormal and consistent surge of nicotinic activity. This concept is further studied by analyzing

PPR that measures transient enhancement of neurotransmitter release induced by two closely spaced stimuli. PPR was significantly lower in prenatal nicotine exposed rats than controls given an interstimulus interval (ISI) of 10 to 200 ms (Fig. 4.1D). This suggests that prenatal nicotine does affect transient enhancement of neurotransmitter release. However there was no significant difference ($p>0.05$) of postsynaptic potentiation (PTP) in prenatal nicotine exposed and control rats (Fig. 4.2B). This suggests that prenatal nicotine exposure does not impair neurotransmitter release for a short period of time following HFS because PTP relies on presynaptic function and may represent transient increases in neurotransmitter release caused by loading of presynaptic terminals with calcium ions after tetanic conditioning.

Prenatal nicotine causes diminished AMPA receptor synaptic currents

In Shaffer collateral-CA1 synapses AMPA receptors contribute to most of the excitatory synaptic transmission. Therefore we compared AMPA receptor mediated whole cell synaptic currents (in the form of mEPSCs) in CA1 pyramidal cells and single channel currents from synaptosomes from prenatal nicotine exposed and control rats. In prenatal nicotine exposed animals AMPA receptor mediated mEPSCs showed significant reductions in both amplitude and frequency ($p<0.05$). A rightward shift in inter-event interval cumulative fraction plot and a leftward shift in amplitude cumulative fraction plot (Fig. 4.3B and D) indicate reductions in frequency and amplitude of mEPSCs in prenatal nicotine rats. While there was no significant difference in rise time (τ_r) between prenatal nicotine and controls (data not shown) decay time (τ_d) showed significant

differences. Decay times of controls were best fitted with two terms resulting in two different decay times, τ_{d1} and τ_{d2} (Fig. 4.4A). However decay times of mEPSCs from prenatal nicotine exposed rats were best fitted with one term τ_{d2} which was significantly lesser ($p<0.05$) than that of the controls and the shorter decay time τ_{d1} was missing (Fig. 4.4B). Plots of rise time versus amplitude and decay time versus amplitude were constructed to assess dendritic filtering. Plots for prenatal nicotine and controls groups did not show any strong correlation (Fig. 4.4C and D) implying dendritic filtering did not affect the mEPSCs. In summary, these results suggest reduced presynaptic glutamate release and reduced number of AMPA receptors in the synapses. Analysis of single channel currents revealed that in prenatal nicotine rats open probability of single channel AMPA receptors were significantly reduced ($p<0.05$) compared to the controls (Fig. 4.5). When open and close dwell times of single channel were analyzed data from prenatal nicotine rats showed significantly higher close time and smaller open times (Fig. 4.6). Single channel conductance, however did not show any significant difference (not shown). These results suggest that prenatal nicotine exposure could alter the intrinsic functional properties of single channel synaptic AMPA receptors.

Impaired LTP in prenatal nicotine exposed rats.

Since basal synaptic transmission and AMPA receptor mediated synaptic currents were diminished in prenatal nicotine exposed rats we next investigated whether these alterations would contribute to alterations in LTP. LTP was measured 50 -60 min after conditioning with 3 HFS interspaced at 20 s. The average fEPSP slope, as a percentage of

the baseline over the 50-60 min interval post HFS, demonstrated impaired maintenance of LTP in prenatal nicotine exposed rats (106±5.63%; n=6 rats) compared to control rats (132.69±6.32%; n=5 rats) (Fig. 4.2). This data show that prenatal nicotine exposure is associated with impaired synaptic plasticity, suggesting that impaired LTP may account for the cognitive deficits observed in children exposed to prenatal nicotine in the form of maternal smoking.

Discussion

Prenatal nicotine exposure results in varied adverse neurobehavioral outcomes in the offspring and one of the major effect is impaired cognition. We therefore hypothesized that prenatal nicotine exposed rats may also have compromised synaptic plasticity and excitatory neurotransmission in the hippocampus. In this study we showed that prenatal nicotine exposed rats have deficits in basal synaptic transmission and plasticity. In parallel we have shown that prenatal nicotine exposed rats have deficits in memory when tested in Y maze task (unpublished data). To begin addressing the neurophysiological alterations in prenatal nicotine exposed rats we showed that these animals exhibit decreased basal synaptic transmission in Schaffer collateral-CA1 synapses, altered presynaptic neurotransmitter release, deficient AMPA receptor synaptic current and diminished LTP. These studies implicate prenatal nicotine exposure potently disrupts function in excitatory synapses in the hippocampus that could contribute to the observed cognitive deficits.

The predominant anomalies in AMPA receptor mediated synaptic currents could have contributed to the deficits in basal synaptic transmission and LTP. At basal levels glutamate release is also altered as revealed by mEPSC recordings. Thus compromised functions in both presynaptic postsynaptic compartments could contribute to the deficits in LTP since both postsynaptic and presynaptic mechanisms are critical for normal Schaffer collateral LTP (Sanes and Lichtman, 1999; Luscher et al., 2000; Choi et al., 2003; Lisman, 2003). We observed potent reductions in synaptic AMPA receptor single channel function and single channel properties are believed to be important for LTP properties (Ambros-Ingerson and Lynch, 1993). Therefore changes in single channel properties could have also contributed to the deficits in LTP. Since normal hippocampal synaptic function and plasticity are important for learning and memory (Bliss and Collingridge, 1993; Milner et al., 1998), it could be speculated that these physiological alterations could contribute to the cognitive impairments in children who were subjected to prenatal nicotine exposure due to maternal smoking.

Excitatory synaptic transmission in the CA1 region is regulated by inhibitory inputs by GABAergic interneurons (McBain and Fisahn, 2001). GABAergic input to CA1 pyramidal neurons are in turn controlled by nAChRs in the hippocampus (Potier et al., 2006). During development GABA receptors play excitatory function which is modified to inhibitory by spontaneous nicotinic cholinergic activity (Cornelius et al., 2001). However, prenatal nicotine exposure might cause early alterations in this process resulting in increased and persistent inhibitory input to the primary excitatory neurons in the hippocampus. In addition nicotine exposure to immature rat hippocampus has been

shown to result in persistent decrease of synaptic efficacy in Schaffer collateral-CA1 synapses (Maggi et al., 2003). In particular this study demonstrated that at high probability synapses nicotine induced long term depression of AMPA mediated synaptic currents. These reports support the results of this study that prenatal nicotine exposure can induce depression of AMPA receptor synaptic currents, excitatory transmission and plasticity in the hippocampus of young rats.

The complete pathway of molecular mechanisms by which both presynaptic and postsynaptic function are impaired is unknown. However persistent nicotinic cholinergic activity might facilitate increased GABAregic toning over a period of postnatal life resulting in a depression of presynaptic function. In addition prenatal nicotine also causes metabolic aberrations (Holloway et al., 2005) that could contribute to decreased neuronal function. Therefore, in conclusion, it is possible that the complex interplay among nicotinic cholinergic, GABAergic and glutamatergic transmission during development is modified by nicotine exposure and possible alterations in biochemical signaling induced by metabolic alterations might contribute to the modifications in neurotransmission. Putative changes in biochemical signaling and comprehensive mechanisms underlying changes in inhibitory-excitatory neurotransmission are areas of future research.

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Figure legends

Figure 4.1. Impaired basal synaptic transmission in prenatal nicotine exposed rats. (A-C) Effects of prenatal nicotine exposure on excitatory synaptic transmission in hippocampal Schaffer collateral-CA1 synapses in hippocampal slices from 2-4 weeks old rats. The data (mean±SEM) from multiple recordings of prenatal nicotine exposed rats (○) and controls (●) were pooled. (**A**) Plot of presynaptic fiber volley amplitude (FV) versus stimulus intensity. FV of prenatal nicotine rats were significantly reduced than that of controls. (**B**) Input/output plot of fEPSP slope versus stimulus intensity. Representative traces of fEPSPs depicting differences in I/O responses of prenatal nicotine and control groups. (**C**). Input/output plot of fEPSP slope versus FV. Significant difference was observed between prenatal nicotine and control groups. Lines (black-control, gray-nicotine) represent best fit linear regression (**D**). Paired pulse ratio plot showing significant difference between prenatal nicotine. n=6. Calibration bars equal 1 mV and 10 ms.

Figure 4.2. Impaired LTP in prenatal nicotine exposed rats. LTP and PTP in prenatal nicotine and control rats were measured in hippocampal slices at Schaffer collateral/CA1 synapses. (**A**) LTP was induced with three 1s HFS at 100 Hz and at an intensity that evoked 50% of maximal fEPSP. The fEPSPs slopes show significant differences between prenatal nicotine and controls. Representative traces at baseline and at 60 min after HFS are shown. Calibration bars equal 0.8 mV and 7 ms. (**B**) PTP was measured at 1–

7 min post HFS. At 1–7 min PTP did not show significant difference. (**C**) At 50–60 min LTP was significantly lower in prenatal nicotine rats compared to controls. Control data are represented by shaded circles and bars and prenatal nicotine data by open circles and bars. Data represent mean ± SEM, n=6, ANOVA.

Figure 4.3. AMPA receptor mediated synaptic currents are diminished in prenatal nicotine exposed rats. Data of controls are shown as solid circles and solid bars and that of prenatal nicotine group are shown as open circles and open bars. (**A**) Sample segments of voltage clamp whole cell currents recordings at -80 mV show AMPA receptor mEPSCs are reduced in amplitude and frequency in prenatal nicotine rats compared to the controls. Calibration bar equals 15pA and 150 ms. (**B**) Cumulative frequency plot for mEPSC amplitudes show that amplitude of prenatal nicotine rats is significantly lesser than that of controls. (**C**) Bar plot depicting the significantly lesser amplitude of prenatal nicotine group than that of controls. (**D**) Cumulative frequency plot interevent intervals show mEPSC frequency of prenatal group is shifted right and significantly lesser than that of controls. n=9, Kolmonogrov-Smirnov test.

Figure 4.4. Temporal properties of mEPSCs show decreased decay time in prenatal nicotine exposed rats. (A) Average trace of mEPSCs from a control rat showing two decay times and τ_{d1} and τ_{d2}. (B) Average trace of mEPSCs from a prenatal nicotine exposed rat showing one decay time τ_{d2} which was significantly lower the corresponding τ_{d2} of the controls (* $p<0.05$). (C) Amplitude versus decay time plot fitted with best

linear regression show dendritic filtering did not affect mEPSCs. There was no correlation between decay time and amplitude of mEPSCs in control (●; black fit line) and prenatal nicotine (○; gray fit line) rats. (D) Amplitude versus rise time plot for control (●; black fit line) and prenatal nicotine (○; gray fit line) rats also did not show any correlation. n=9, ANOVA.

Figure 4.5. Single channel open probability of hippocampal synaptic AMPA receptors is reduced in prenatal nicotine exposed rats. (**A**) Amplitude histogram of control rats shows two distinct peaks for close and open states. Representative trace show channel activity by downward deflections. (B) Amplitude histogram and representative trace of prenatal nicotine rats show a reduction in channel open probability (Po). The traces represent recordings of AMPA elicited currents at a holding potential of -80 mV. Calibration bars represent 5 pA and 50 ms.

Figure 4.6. Prenatal nicotine exposure resulted in inhibition of hippocampal synaptic AMPA receptor single channel kinetics by altering dwell time distributions. Log transformed open and closed time histograms were fitted best with 2 terms by exponential log probability variable metric method. (**A**) Close time histogram of synaptosomal AMPA receptor single channels from control and (**B**) from prenatal nicotine rats. (**C**) Open time histogram from control and (**D**) from prenatal nicotine rats. (**E**) Bar plots for open time and (**F**) close time showing significant decreases in open times and increases in close times. Shaded bars represent control data and blank bars

represent prenatal nicotine data. Data represent mean ± SEM, n=5, *$p < 0.05$, **$p<0.001$; ANOVA.

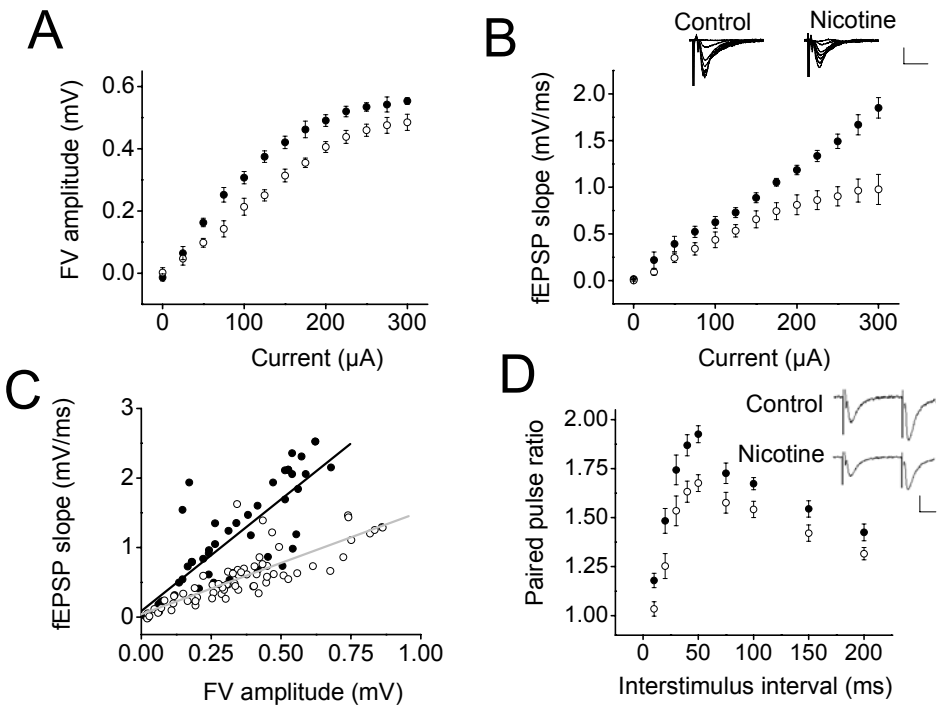
A
FV amplitude (mV)
0.6
0.4
0.2
0.0
0
100
200
300
Current (μA)
B
Control
Nicotine
fEPSP slope (mV/ms)
2.0
1.5
1.0
0.5
0.0
0
100
200
300
Current (μA)
C
fEPSP slope (mV/ms)
3
2
1
0
0.00
0.25
0.50
0.75
1.00
FV amplitude (mV)
D
Paired pulse ratio
2.00
1.75
1.50
1.25
1.00
Control
Nicotine
0
50
100
150
200
Interstimulus interval (ms)

Figure 4.1

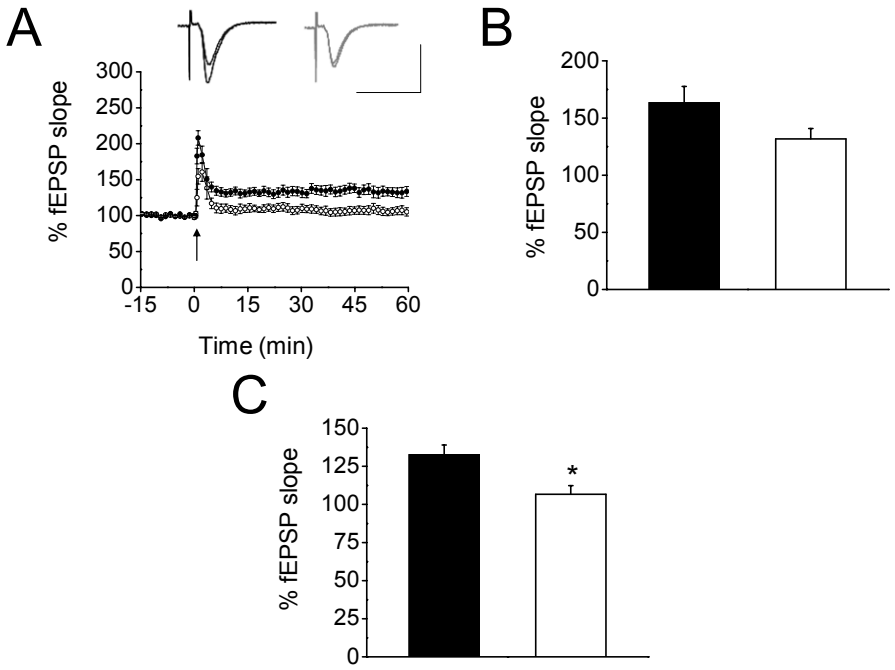
A
% fEPSP slope
300
250
200
150
100
50
0
-15
0
15
30
45
60
Time (min)
B
% fEPSP slope
200
150
100
50
0
C
% fEPSP slope
150
125
100
75
50
25
0
*

Figure 4.2

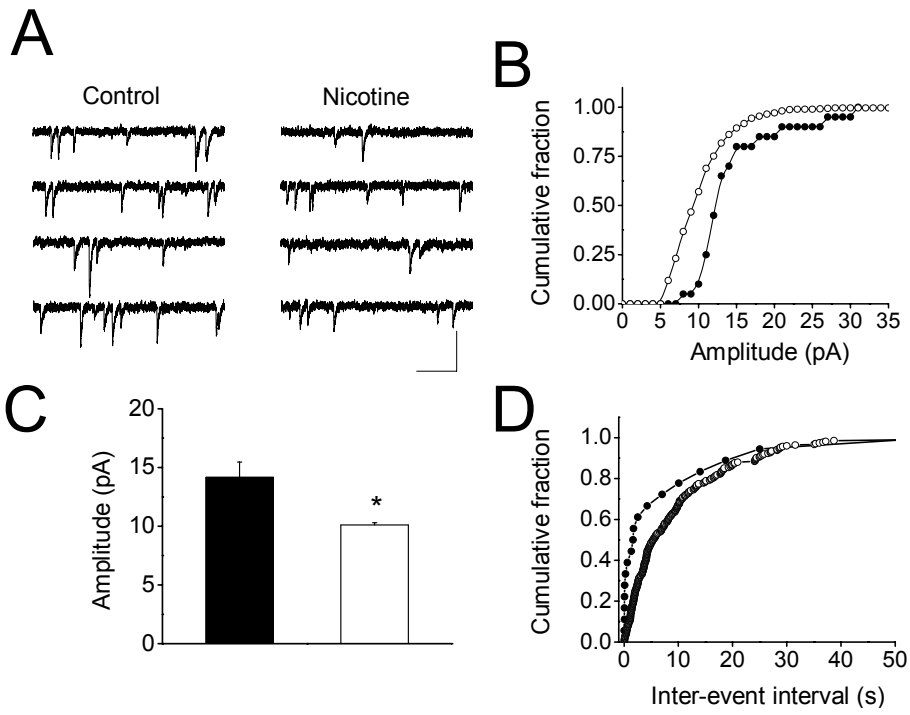
A
Control
Nicotine
B
Cumulative fraction
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
35
Amplitude (pA)
C
Amplitude (pA)
20
15
10
5
0
*
D
Cumulative fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Inter-event interval (s)

Figure 4.3

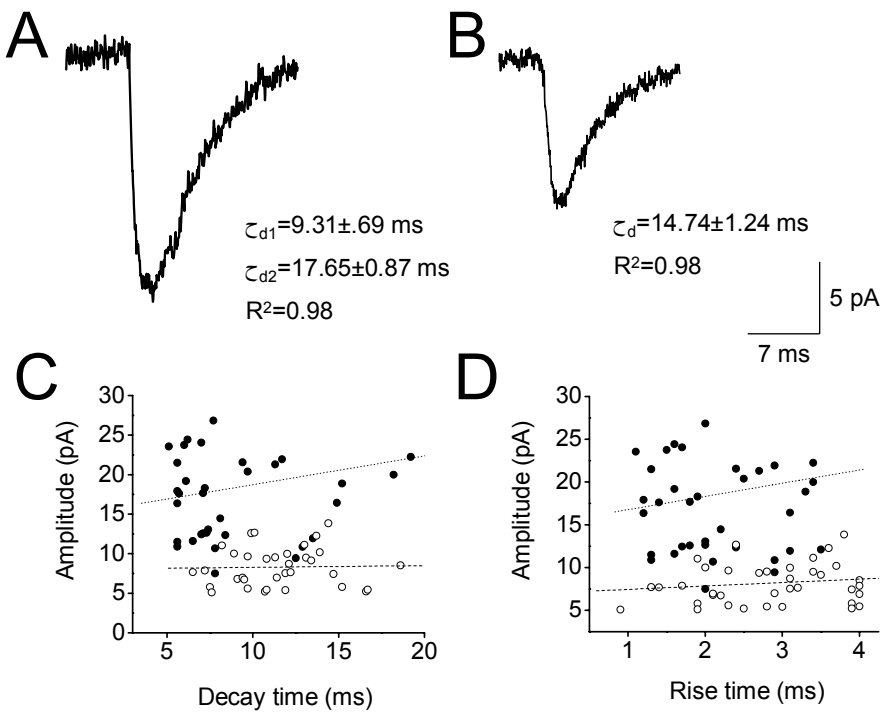
A
B
τ_{d1}=9.31±.69 ms
τ_{d2}=17.65±0.87 ms
R^2=0.98
τ_d=14.74±1.24 ms
R^2=0.98
5 pA
7 ms
C
D
Amplitude (pA)
Decay time (ms)
Amplitude (pA)
Rise time (ms)

Figure 4.4

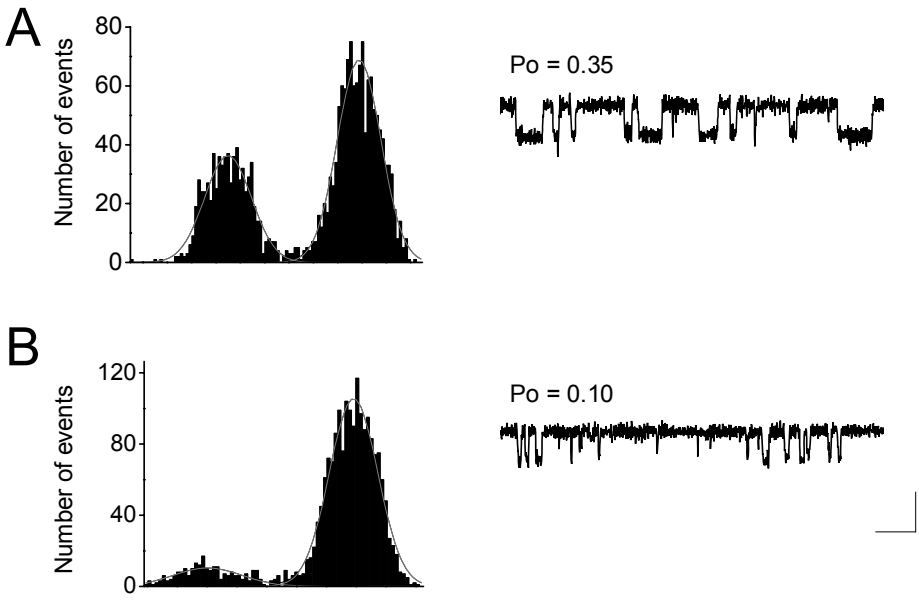
A
Number of events
80
60
40
20
0
Po = 0.35
B
Number of events
120
80
40
0
Po = 0.10

Figure 4.5

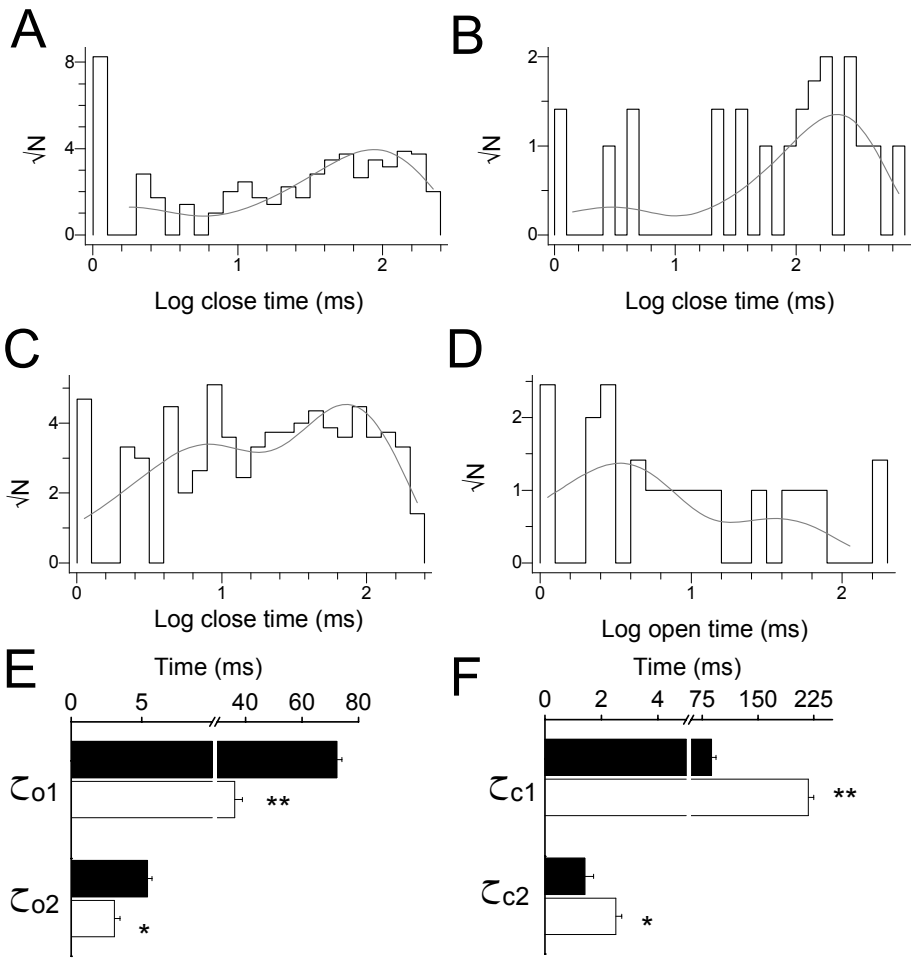
A
B
C
D
E
F
√N
Log close time (ms)
Log open time (ms)
Time (ms)
τo1
τo2
τc1
τc2
**
*

Figure 4.6

5. LONG LASTING IMPAIRMENTS IN SYNAPTIC PLASTICITY AND AMPA RECEPTOR SYNAPTIC CURRENTS IN RATS EXPOSED TO PRENATAL NICOTINE

Abstract

Maternal smoking is associated with cognitive deficits in children. Some studies indicate such cognitive deficits may extend to older ages and thus may be long lasting. Since mammalian hippocampus is a major region in learning and memory processing this study analyzed long term potentiation (LTP) and AMPA receptor mediated synaptic currents, which are critical cognitive functions, in the hippocampus of 2 months old young adult rats. Results of this study reveal that LTP in Shaffer collateral-CA1 synapses is impaired and the AMPA receptor mediated synaptic currents are attenuated with parallel deficits in both presynaptic and postsynaptic functions. These results suggest that prenatal nicotine exposure induces changes in hippocampal function that might extend beyond young age.

Introduction

Drug use during pregnancy can result in harmful teratogenic effects in the children. Nicotine is the most commonly abused drug in the form of tobacco smoking and chewing. About 11% of pregnant women in the United States report smoking at some point during, or throughout their pregnancies (Martin et al., 2003). Cigarette use during pregnancy has been shown to be a high risk factor for abortion, fetal mortality, sudden infant death syndrome, decreased IQ scores, behavioral disorders like hyperactivity and conduct disorder (Ernst et al., 2001), and deficits in fine motor skills, attention, and auditory processing (Cutler et al., 1996). In particular deficits on tasks that require learning, memory, and problem solving skills were impaired in children up to age 10 following exposure to gestational smoke exposure (Cornelius et al., 2001; DiFranza et al., 2004). Collectively these reports suggest high risk of cognitive deficits in young offspring exposed to tobacco smoke during gestation.

Though tobacco smoke contain several harmful compounds (Stedman, 1968), nicotine is the main psychoactive compound with deleterious effects on developing brain (Schlumpf et al., 1988). Nicotine is a potent agonist of nicotinic acetylcholine receptors (nAChRs), which are expressed during fetal brain development (Naeff et al., 1992; Hellstrom-Lindahl et al., 1998) and play important roles in brain development (Levin et al., 1996). Alterations in the onset of nicotinic cholinergic activity could result in potent aberrations in the developmental roles. One characteristic molecular change due to chronic nicotine exposure in developing animals, human smokers and human fetuses exposed to smoking *in utero* is the increased expression of α4/β2-containing nAChRs,

which form most of the high affinity nicotine binding sites in the brain (van de Kamp and Collins, 1994; Nguyen et al., 2004; Staley et al., 2006). Furthermore nicotine has been shown to affect neuronal cell replication, leading to a decrease in total cell count, and subsequent deficits in synaptic connectivity and neurochemical activity (Wang et al., 2002).

In support of clinical and epidemiological reports several animal studies have shown cognitive deficits in prenatal nicotine exposed rodents. Attentional and spatial memory deficits have been noted in the Morris water maze and radial-arm maze tasks in prepubertal, adolescent, and adult rats prenatally exposed to nicotine (Sorenson et al., 1991; Levin et al., 1993; Cutler et al., 1996; Eppolito and Smith, 2006). Studies from this lab also show that young rats that received prenatal nicotine exposure show deficits in Y maze memory tasks (unpublished data). Many of the behavioral memory tasks tested in these studies rely on hippocampal function and deficits are suggestive of impaired function of hippocampus. Changes in neuronal size, distribution, ultrastucture, and subcellular structure in hippocampus, including CA1 subfield, were observed in prenatal nicotine exposed rats (Roy and Sabherwal, 1998; Roy et al., 2002). In addition concurrent physiological changes like astrogliosis along with structural changes in CA1 subfield has also been reported (Abdel-Rahman et al., 2005). Results of these studies implicate changes in hippocampal structure and function playing a role in cognitive deficits.

Excitatory synaptic transmission and plasticity are believed to play critical role in memory acquisition in the hippocampus. Studies from our lab show that excitatory

synaptic transmission, AMPA receptor synaptic currents and synaptic plasticity in Schaffer collateral-CA1 synapses were strongly reduced in young rats that were exposed to nicotine prenatally (unpublished data). Though several studies support that cognitive deficits exist in young children and animals some studies suggest that the cognitive deficits resulting from prenatal nicotine could last throughout a considerable period up to young adult stage (Yanai et al., 1992; Vaglenova et al., 2004; Eppolito and Smith, 2006). However some reports suggest no cognitive defects at adult animals as there was no effect on acquisition of choice accuracy performance in the radial-arm maze in 60 day old animals (Levin et al., 1993). In this context the current study was performed to analyze hippocampal synaptic plasticity, excitatory transmission and AMPA receptor synaptic currents in Schaffer collateral-CA1 synapses to assess for any deficits in adult age. Our results show that deficits in these functional paradigms are present in adult rats, suggesting prenatal nicotine induced changes in hippocampus could be long lasting.

Materials and methods

Animals and Chemicals: Time pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and osmotic mini pumps (Alzet, Cupertino, CA) were implanted, under isoflurane anesthesia, beneath the skin to deliver subcutaneous dose of nicotine at a rate of 6 mg/kg/day. Osmotic mini pumps were removed once the pups were delivered so that prenatal nicotine exposure was limited from ~ day3 of pregnancy to birth. Unless specified, all the chemicals were purchased from Sigma (St. Louis, MO).

Preparation of Hippocampal slices and synaptosomes: Transverse hippocampal slices (400 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 $MgSO_4$, $CaCl_2$ 0.5, NaH_2PO_4 1.25, 25 $NaHCO_3$, 25 glucose, 75 sucrose 0.5 ascorbate and 2 kynurenic acid and was bubbled with 95%CO_2/5%O_2, bringing the solution to 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 and was bubbled with 95%CO_2/5%O_2. Synaptosomes were prepared by previously described (Johnson et al., 1997; Suppiramaniam et al., 2006) methods in which hippocampi were dissected out and homogenized in homogenizing buffer (mKRBS) using a Potter homogenizer with 10 strokes. 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 by bubbling with 95%O_2/5%CO_2. The buffer was also 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 low protein binding filter followed by spinning at 1000 x g for 15 min in a microfuge at 4°C. The supernatant was removed, and

the pellet which contained synaptosomes was resuspended in 20 µl of mKRBS buffer.

Slice electrophysiology: Following incubation electrophysiological recordings were performed in recordings chambers with continuous 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 ~ 200 µm from the stimulating electrode. The frequency of the test stimulation was every 20 s. For stimulus response curves current intensity was increased from 0 to 300 µA at steps of 25 µA. For paired pulse ratio (PPR) and LTP experiments current intensity was set at 50% of maximal fEPSP and inter pulse intervals were set at 10, 20, 30, 40, 50, 75, 100, 150 and 200 ms in PPR experiments. In LTP experiments after at least 15 min of stable baseline recording 3 high frequency stimuli (HFS; 100 Hz) were delivered every 20 s. LTP was measured 50-60 min post HFS.

Whole cell current recordings were performed in CA1 pyramidal neurons voltage clamped at -65 mV. The patch pipette (6-10 MΩ) was filled in an internal solution comprising (in mM): 100 K-gluconate, 0.6 EGTA, 5 $MgCl_2$, 2 ATP-Na, 0.3 GTP-Na and 40 HEPES and the slices were perfused continuously with ACSF supplemented with picrotoxin, APV and tetradotoxin to isolate AMPA receptor mediated miniature excitatory postsynaptic currents (mEPSC).

Single channel electrophysiology: Incorporation of AMPA receptors from synaptosomal fractions in artificial lipid bilayers was carried out using 'tip-dip" method (Suppiramaniam et al., 2006). In brief, a phospholipid bilayer was formed at the tip of a polished borosilicate glass pipette (100 MΩ). The synthetic phospholipids was prepared by dissolving 1,2 –diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids Inc., Alabaster, AL) in anhydrous at hexane (Aldrich Chemical Co., Milwaukee, WI) to obtain a concentration of 1 mg/ml. Approximately 3-5 μl of synthetic phospholipids was 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 was 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). 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, 3-5 μl suspension of the synaptosomes was delivered to the ECF. After addition of AMPA (290 nM), 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 pClamp9 software (Molecular Devices) and saved in a computer hard disk. Only the data exhibiting long stretches of single channel current transition without base line drifts was chosen for quantitative analysis. All points-current amplitude histograms were constructed and fitted with 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 variable metric fitting method to identify distinct open and close times.

Results

Prenatal nicotine impairs basal excitatory synaptic transmission in Schaffer collateral synapses in adult hippocampus.

Previous studies have shown morphological alterations in the hippocampus and impaired hippocampal based behavioral tasks in rats exposed to prenatal nicotine. In addition results from younger (2-4 weeks) animals show deficits in basal excitatory synaptic transmission. To investigate whether these alterations are persistent to a young adult stage and cause functional anomalies in excitatory synaptic transmission we recorded fEPSPs from Schaffer collateral-CA1 synapses, in 2-3 months old rats, to characterize for possible changes in basal synaptic function. Presynaptic fiber volley (FV) and slope of fEPSPs were analyzed in response to different stimulation intensities. In prenatal nicotine exposed rats the amplitude of FV was not significantly reduced from that of controls ($p>0.05$) (Fig. 5.1A), suggesting that prenatal nicotine administration did not persistently affect the number of afferent axons. However, input/Output (I/O) curves, measured by plotting stimulation intensities versus the corresponding fEPSP (Fig. 5.1B) and (I/O) curves determined by plotting the amplitude of the FV versus fEPSP slope (Fig. 5.1C) were significantly ($p<0.05$) reduced in prenatal nicotine exposed rats compared to controls, suggesting that prenatal nicotine exposure might cause lasting

impairments in basal synaptic transmission in Schaffer collateral-CA1 synapses.

Altered presynaptic function in prenatal nicotine exposed rats.

Impaired basal synaptic transmission observed in the young adult prenatal nicotine exposed rats suggests that normal function of the hippocampal synapses is altered. To further assess whether there is a concomitant alterations in presynaptic release PPR was analyzed. PPR was significantly lower in prenatal nicotine exposed rats than controls given an interstimulus interval (ISI) of 20 to 200 ms (Fig. 1D; $p<0.05$). This suggests that prenatal nicotine affects transient enhancement of neurotransmitter release. In addition there was a significant difference ($p<0.05$) of postsynaptic potentiation (PTP) in prenatal nicotine exposed and control rats (Fig. 5.2B). This suggests that prenatal nicotine exposure impairs neurotransmitter release for a short period of time following HFS because PTP relies on presynaptic function and may represent transient increases in neurotransmitter release caused by loading of presynaptic terminals with calcium ions after tetanic conditioning.

Impaired LTP in prenatal nicotine exposed rats.

LTP experiments were conducted to analyze whether LTP impairments in younger ages persists to adulthood. LTP was measured 50-60 min after conditioning with 3 HFS interspaced at 20 s. The average fEPSP slope, as a percentage of the baseline over the 50-60 min interval post HFS, demonstrated impaired maintenance of LTP in prenatal nicotine exposed rats (105.02±8.90%;) compared to control rats (132.44±12.91%) (Fig.

5.2A and C). This data show that prenatal nicotine exposure is associated with impaired synaptic plasticity lasting through adult age and suggests that impaired LTP may account for the cognitive deficits observed in children exposed to prenatal nicotine in the form of maternal smoking.

Prenatal nicotine causes diminished AMPA receptor synaptic currents

In the previous studies we observed AMPA receptor mediated synaptic currents were diminished in young animals exposed to prenatal nicotine. AMPA receptor synaptic currents are critical for basal synaptic transmission and LTP because in Shaffer collateral-CA1 synapses and other major synapses these receptors contribute to most of the excitatory synaptic transmission. Therefore, next we compared AMPA receptor mediated whole cell synaptic currents (in the form of mEPSCs) in CA1 pyramidal cells and single channel currents from synaptosomes from young adult prenatal nicotine exposed rats and age matched control rats. In prenatal nicotine exposed animals AMPA receptor mediated mEPSCs showed significant reductions in both amplitude and frequency (Fig. 5.3A and B; n=7, $p<0.05$). A leftward shift in amplitude cumulative fraction plot and a rightward shift in inter-event interval cumulative fraction plot and (Fig. 5.3B and D) indicate reductions in frequency and amplitude of mEPSCs in prenatal nicotine rats. Temporal parameters of mEPSC were analyzed and the decay phase was fitted with two terms (Mini Analysis Program) resulting in two distinct decay times (τ_d). The τ_ds of prenatal nicotine and controls showed significant differences (Fig. 5.4 A and B; $p<0.05$). There was no significant difference ($p<0.05$) in rise time (τ_r) between prenatal nicotine and

controls (not shown). Plots of rise time versus amplitude and decay time versus amplitude were constructed to assess dendritic filtering. Plots for prenatal nicotine and controls groups did not show any strong correlation (Fig. 5.4C and D) implying dendritic filtering did not affect the mEPSCs (Eppolito and Smith, 2006). In summary, these results suggest that even at young adult age prenatal nicotine induced deficits in AMPA receptor synaptic currents are present and might contribute to the cognitive deficits. The results also suggest reduced presynaptic glutamate release and reduced number of AMPA receptors in the synapses.

Because 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) we analyzed synaptic AMPA receptor single channel currents from synaptosomes reconstituted in lipid bilayers. Results show that channel open probability (Po) in prenatal nicotine rat were significantly reduced (Po=0.10±0.043) compared to the controls (0.30±0.018; Figure 5.5, n=5, $p<0.05$). In addition analyses of dwell times revealed open times (τ_o) were significantly reduced and close times were significantly increased (τ_c) in prental nicotine rats (Figure 5.6). These results suggest that alterations in single channel properties may play a role in deficient mEPSCs and LTP in prenatal nicotine exposed rats.

Discussion

Though prenatal nicotine exposure results in varied adverse neurobehavioral outcomes in the offspring, the long lasting effect is impaired cognition extending to

young school age children. This is supported by several animal studies reporting deficits in hippocampal based memories. In addition some studies have suggested cognitive deficits observed in young life may extend longer. Results from our lab show young animals have deficits in LTP and synaptic currents mediated by AMPA receptors in the key synapses of hippocampal Shaffer collateral-CA1 pathway. Taken together this led to our hypothesis that these deficits in synaptic function observed in young animals may also be observed in adult animals, implying that prenatal nicotine induced changes could be long lasting. In this study we showed that prenatal nicotine exposed rats have deficits in basal synaptic transmission and plasticity. To begin addressing the neurophysiological alterations in prenatal nicotine exposed rats we showed that these animals exhibit decreased basal synaptic transmission in Schaffer collateral-CA1 synapses, altered presynaptic neurotransmitter release, deficient AMPA receptor synaptic current and diminished LTP. These results implicate that, even long after cessation of exposure, prenatal nicotine exposure potently disrupts function in excitatory synapses in the hippocampus that could contribute to the observed cognitive deficits.

In hippocampal Shaffer collateral-CA1 synapses, presynaptic glutamate release contributes to the regulation of LTP (Stanton et al., 2005; Lisman and Raghavachari, 2006; Lauri et al., 2007). Therefore the impaired presynaptic release as observed in PTP and PPR could have contributed to the deficiencies in LTP. In addition frequency of mEPSC was reduced in prenatal nicotine animals indicating release rates may be compromised which could possibly impair enhancement in presynaptic function subsequent to tetanus conditioning. Basal synaptic transmission also shows deficiencies

indicating overall synaptic transmission remains compromised at young adult age. The predominant anomalies in AMPA receptor mediated synaptic currents could have contributed to the deficits in basal synaptic transmission and LTP. Thus compromised functions in both presynaptic postsynaptic compartments could have contributed to the deficits in LTP since both postsynaptic and presynaptic mechanisms are critical for normal Schaffer collateral LTP (Sanes and Lichtman, 1999; Luscher et al., 2000; Choi et al., 2003; Lisman and Raghavachari, 2006). Since normal hippocampal synaptic function and plasticity are important for learning and memory (Bliss and Collingridge, 1993; Milner et al., 1998), it could be speculated that these physiological alterations could contribute to the cognitive impairments in children who were subjected to prenatal nicotine exposure due to maternal smoking.

Presynaptic glutamate release in CA1 synapses is known to be regulated by several endogenous factors. In particular neurotrphins like brain derived neurotrophic factor (BDNF) has been shown to modulate presynaptic release (Gottschalk et al., 1998). Interestingly hippocampal BDNF expression is regulated by cholinergic system (da Penha Berzaghi et al., 1993) and a recent report suggest in human ventral tagmental area smoking affects genes involved with BDNF signaling and glutamatergic transmission (Flatscher-Bader et al., 2008)**.** In addition to the possible alterations in BDNF signaling, alterations in local-circuit, gamma-aminobutyric acid (GABA) releasing inhibitory interneurons might cause changes in presynaptic glutamate release because in the hippocampus these GABAergic interneurons have been known as the regulators of excitatory transmission of principal neurons (McBain and Fisahn, 2001). Interestingly

GABAergic input to CA1 pyramidal neurons are in turn controlled by nAChRs in the hippocampus (Potier et al., 2006). During postnatal development GABA receptors play excitatory function which is modified to inhibitory by spontaneous nicotinic cholinergic activity (Cornelius et al., 2001; Adams et al., 2002). Prenatal nicotine exposure might cause early alterations in this process resulting in increased and persistent inhibitory input to the primary excitatory neurons in the hippocampus. Thus these changes could contribute to alterations in presynaptic function in prenatal nicotine exposed animals.

As evidenced by the results of this study, prenatal nicotine exposure effects changes in postsynaptic mechanisms as well. The reduced basal synaptic transmission, LTP deficits and reduced AMPA receptor mEPSC amplitude and τ_d are suggestive of severe depression of AMPA receptor function and expression. Our results are supported by a previous study which showed nicotine exposure to immature rat hippocampus results in persistent decrease of synaptic efficacy in Schaffer collateral-CA1 synapses (Maggi et al., 2003). In particular this study demonstrated that at high probability synapses nicotine induced long term depression of AMPA mediated synaptic currents. These studies support the results of this study that prenatal nicotine exposure can induce depression of AMPA receptor synaptic currents, excitatory transmission and plasticity in the hippocampus of young rats. Our results show that synaptic single channel AMPA receptor currents are also diminished in prenatal nicotine rats which could contribute to the reduced whole cell currents and the overall deficiency in excitatory transmission and plasticity.

In conclusion, the current study provides a mechanistic support for the cognitive deficits observed in adult prenatal nicotine exposed rats. In addition the current study provides partial evidence that possible changes in the complex interplay among nicotinic cholinergic, GABAergic and glutamatergic transmission during development is modified by nicotine exposure and possible alterations in biochemical signaling induced by metabolic alterations might contribute to the modifications in neurotransmission.

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Figure legends

Figure 5.1. Impaired basal synaptic transmission in prenatal nicotine exposed rats. (**A**) The fEPSP plotted against the intensity of stimulus applied to the Schaffer collateral. Prenatal nicotine exposure had a significant effect on slope of the fEPSP (postsynaptic measurement) at increasing stimulus intensities. (**B**) No significant difference between prenatal nicotine and the control groups was observed in the amplitude of the fiber volley (FV) at varying stimulus intensities, indicating equivalent transfer and conversion of the electrical stimulus into axonal depolarization. (**C**) A significant decrease in overall synaptic transmission was detected as the slope of the fEPSP plotted against the amplitude of the FV at increasing stimulus intensities. Black regression line indicates control and gray line indicate prenatal nicotine data. (**D**) PPR in prenatal nicotine and controls. Prenatal nicotine exposure had a significant effect on PPR at many interpulse intervals. Control data are represented by filled circles and prenatal nicotine data are represented by open circles. For representative traces calibration bars equal 0.8 mV and 7 ms; $n = 6$; $p < 0.05$; ANOVA. Data represent mean ± SEM.

Figure 5.2. Impaired LTP in prenatal nicotine exposed rats. LTP and PTP in prenatal nicotine and control rats were measured in hippocampal slices at Schaffer collateral/CA1 synapses. (**A**) LTP was induced with three 1s HFS at 100 Hz and at an intensity that evoked 50% of maximal fEPSP. The fEPSPs slopes show significant differences between prenatal nicotine and controls. Representative traces at baseline and at 60 min after HFS are shown. Calibration bars equal 0.8 mV and 7 ms. (**B**) PTP was measured at 1–7 min

post HFS. At 1–7 min PTP was significantly lower in prenatal nicotine rats compared to controls. (**C**) At 50–60 min LTP was significantly lower in prenatal nicotine rats compared to controls. Control data are represented by shaded circles and bars and prenatal nicotine data by open circles and bars. Data represent mean ± SEM, n=6, $*p < 0.05$, $**p < 0.001$; ANOVA.

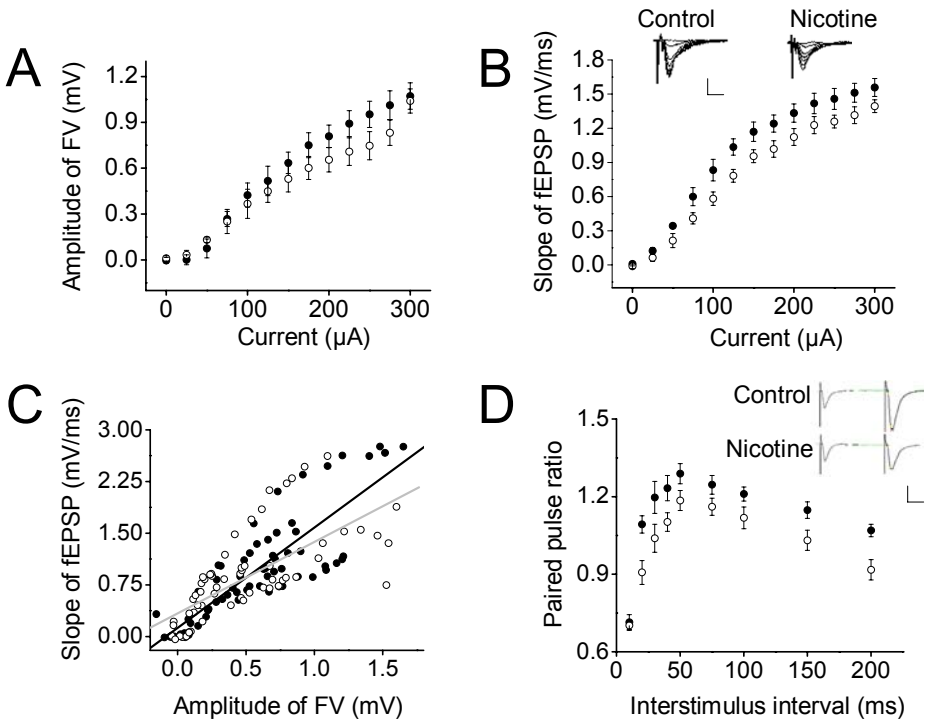
Figure 5.3. Prenatal nicotine exposure reduces whole cell AMPA receptor mEPSCs in hippocampal CA1 pyramidal neurons. (**A**) Representative traces showing reductions in amplitude and frequency of AMPA mEPSCs in controls and prenatal nicotine animals. Calibration bars equal 7 pA and 250 ms (**B**) Prenatal nicotine exposure resulted in significant reduction in peak amplitude. (**C**) Cumulative fraction plot of amplitude showing a shift of the curve to the right in prenatal nicotine data representing a decrease in the amplitude. (**D**) Cumulative fraction plot of interevent interval showing a shift of the curve to the left in prenatal nicotine data representing a decrease in the mEPSC frequency. Data represent mean ± SEM, n=8, $*p < 0.05$; ANOVA.

Figure 5.4. Prenatal nicotine exposure alters temporal properties of AMPA mEPSCs. (**A**) Average trace of mEPSCs of control and (**B**) average trace of mEPSCs of prenatal nicotine rats showing reduction in amplitude and reductions in decay times (τ_d). Calibration bars equal 4 pA and 15 ms. (**C**) Relationship between decay time and amplitudes of mEPSCs. There was no significant correlation between these two parameters for prenatal nicotine and control data. (**D**) Relationship between rise time and amplitudes of mEPSCs also did not show significant correlation for both prenatal nicotine

and control data. Black regression lines indicate control and gray line indicate prenatal nicotine data. Shaded circles indicate control data and open circles indicate prenatal nicotine data. Data represent mean ± SEM, n=8, *$p < 0.05$; ANOVA.

Figure 5.5. Prenatal nicotine exposure alters the single channel properties of hippocampal synaptic AMPA receptors. (**A**) Amplitude histogram of control rats shows two distinct peaks for close and open states. Representative trace show channel activity by downward deflections. (**B**) Amplitude histogram and representative trace of prenatal nicotine rats show a reduction in channel open probability (Po). The traces represent recordings of AMPA elicited currents at a holding potential of -96 mV.

Figure 5.6. Prenatal nicotine exposure resulted in inhibition of hippocampal synaptic AMPA receptor single channel kinetics by altering dwell time distributions. Log transformed open and closed time histograms were fitted best with 2 and 3 terms respectively by exponential log probability variable metric method. (**A**) Open time histogram of synaptosomal AMPA receptor single channels from control and (**B**) from prenatal nicotine rats. (**C**) Close time histogram from control and (**D**) from prenatal nicotine rats. (**E**) Bar plots for open time and (**F**) close time showing significant decreases in open times and increases in close times. Shaded bars represent control data and blank bars represent prenatal nicotine data. Data represent mean ± SEM, n=5, *$p < 0.05$, **$p<0.001$; ANOVA.


A
Amplitude of FV (mV)
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0.6
0.3
0.0
0
100
200
300
Current (μA)
B
Control
Nicotine
Slope of fEPSP (mV/ms)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0
100
200
300
Current (μA)
C
Slope of fEPSP (mV/ms)
3.00
2.25
1.50
0.75
0.00
0.0
0.5
1.0
1.5
Amplitude of FV (mV)
D
Control
Nicotine
Paired pulse ratio
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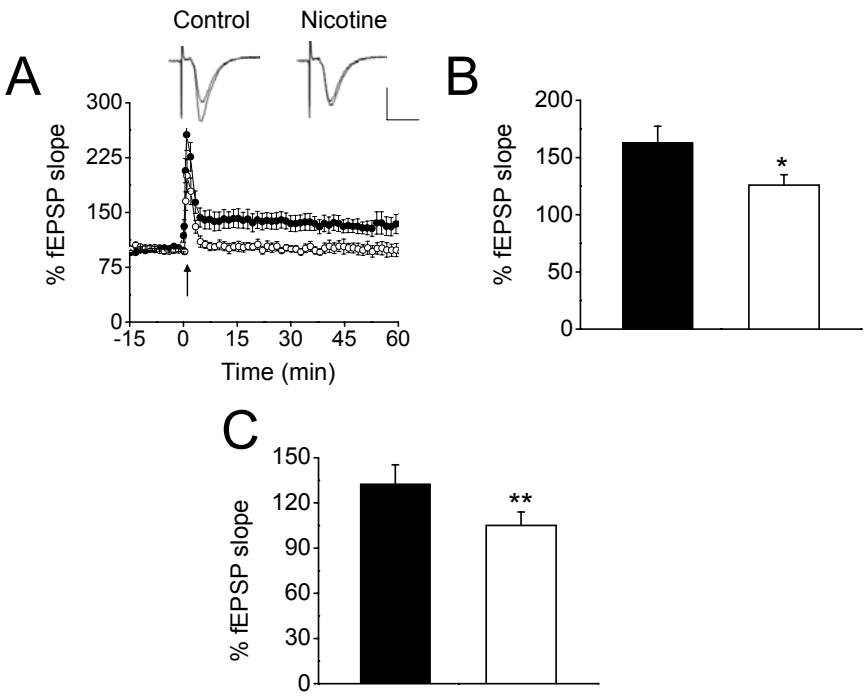
1.2
0.9
0.6
0
50
100
150
200
Interstimulus interval (ms)

Figure 5.1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Control
Nicotine
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225
150
75
0
-15
0
15
30
45
60
Time (min)
B
% fEPSP slope
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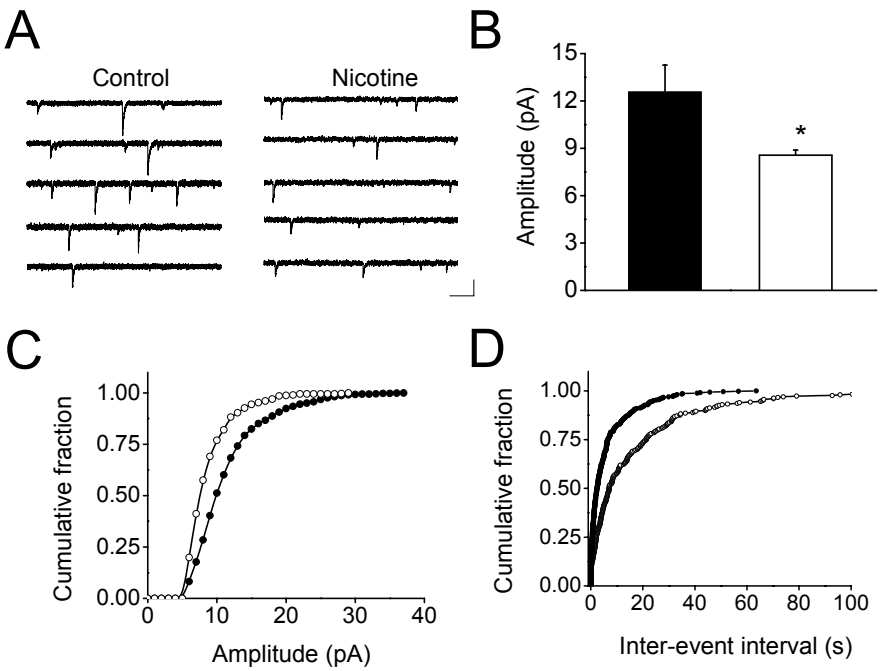
150
100
50
0
*
C
% fEPSP slope
150
120
90
60
30
0
**

Figure 5.2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Control
Nicotine
B
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9
6
3
0
*
C
Cumulative fraction
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0.75
0.50
0.25
0.00
0
10
20
30
40
Amplitude (pA)
D
Cumulative fraction
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0.00
0
20
40
60
80
100
Inter-event interval (s)

Figure 5.3

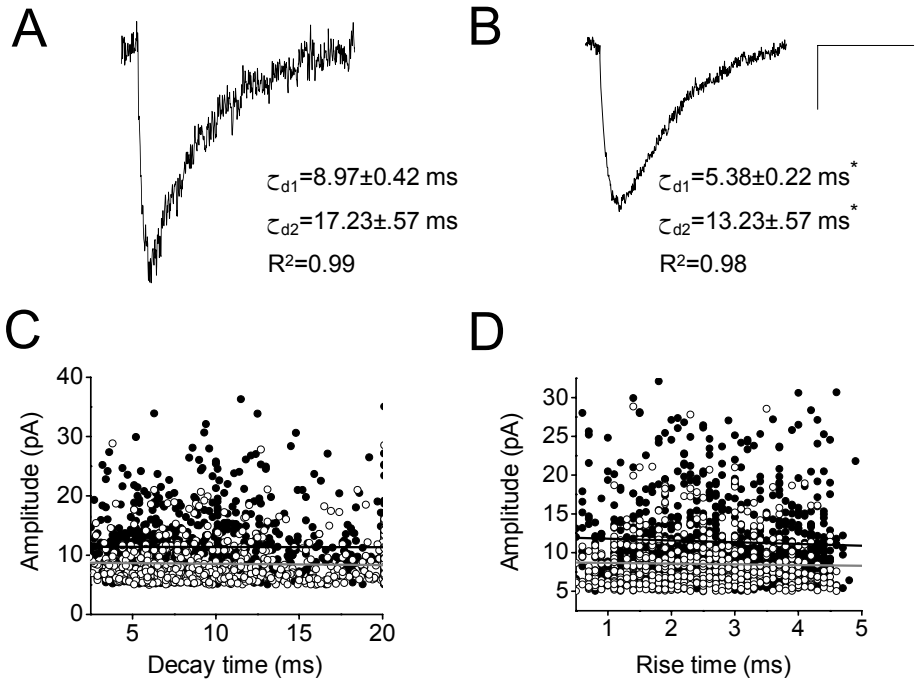
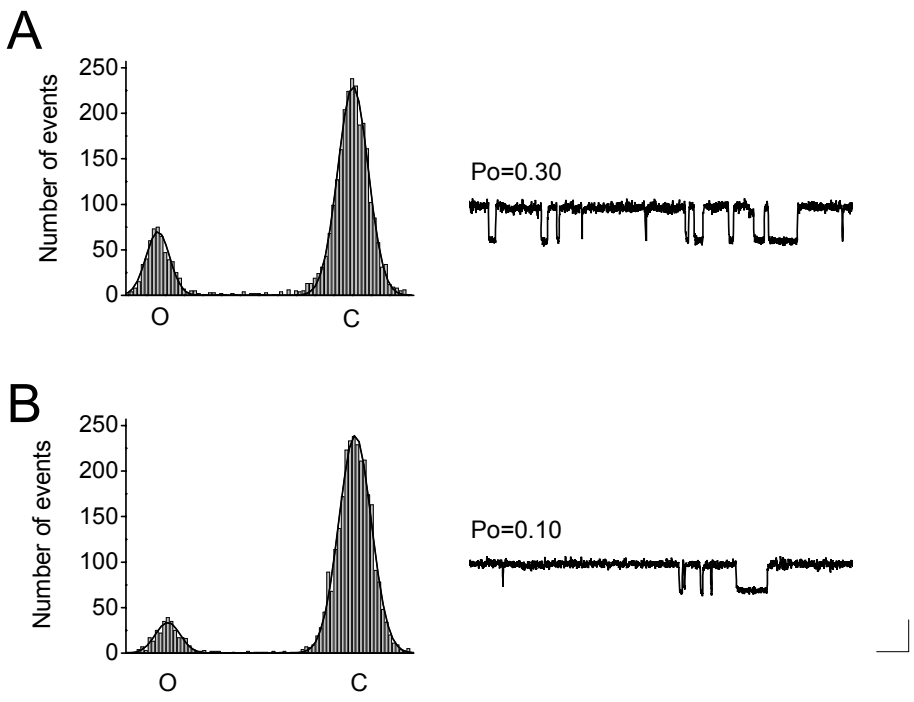

A
B
τ_{d1}=8.97±0.42 ms
τ_{d2}=17.23±.57 ms
R^2=0.99
τ_{d1}=5.38±0.22 ms*
τ_{d2}=13.23±.57 ms*
R^2=0.98
C
D
Amplitude (pA)
Decay time (ms)
Amplitude (pA)
Rise time (ms)

Figure 5.4


A
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200
150
100
50
0
O
C
Po=0.30
B
Number of events
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200
150
100
50
0
O
C
Po=0.10

Figure 5.5

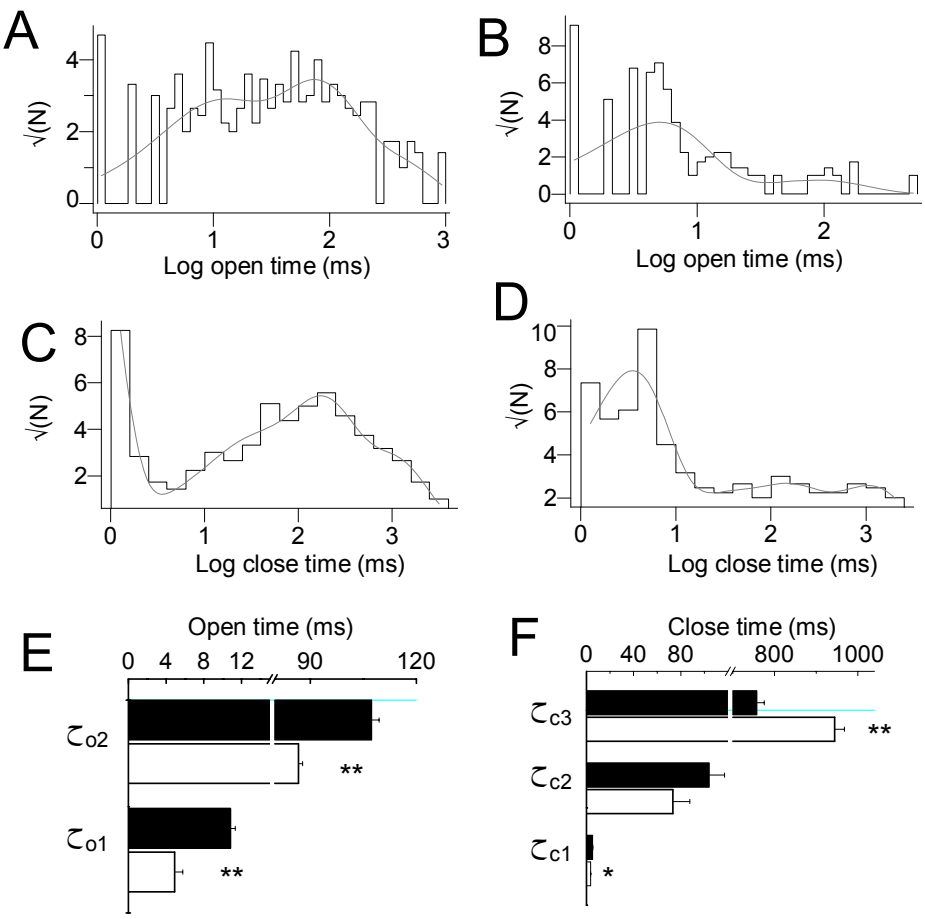
A
√(N)
Log open time (ms)
B
√(N)
Log open time (ms)
C
√(N)
Log close time (ms)
D
√(N)
Log close time (ms)
E
Open time (ms)
0 4 8 12 90 120
τo2
**
τo1
**
F
Close time (ms)
0 40 80 800 1000
τc3
**
τc2
τc1
*

Figure 5.6

6. SUMMARY AND CONCLUSIONS

Maternal smoking has been long recognized as a harmful conduct affecting both mother and the fetus. Despite public awareness programs an alarming proportion of pregnant women smoke during pregnancy. Exposure to tobacco smoke results in low oxygen and high carbon monoxide blood levels, reduced placental function and increased vasoconstriction. These adverse effects deprive the fetus of oxygen, and several chemical compounds, including nicotine, that are in tobacco smoke reach the fetus. In addition, maternal smoking is associated with an increased risk of perinatal morbidity such as low birth weight, perinatal mortality and Sudden Infant Death Syndrome. Though tobacco smoke contains several harmful substances, nicotine is the major psychoactive component in tobacco and believed to be causative compound for tobacco use and addiction.

One major harmful outcome of prenatal nicotine exposure, in the form of maternal tobacco smoking, in children is the learning and memory deficits, along with certain neurobehavioral impairments. Several animal studies provided empirical supports for these impairments and in particular the learning and memory deficits were shown to be dependent on hippocampal function. Mammalian hippocampus is one of the primary regions of memory processing and in this brain region fast excitatory neurotransmission is largely mediated by AMPA receptors. AMPA receptors are also critical for

expression of LTP, a form of synaptic plasticity that is believed to be a cellular correlate of memory processing. The current study contains detailed analyses of neurobehavioral outcomes, neurochemical changes in the hippocampus, excitatory synaptic transmission and plasticity (LTP) in Schaffer collateral-CA3 synapses in the hippocampus, AMPA receptor whole cell synaptic currents in pyramidal neurons in CA region and single channel currents from AMPA receptors from hippocampal synapses. These analyses were performed to understand the changes in hippocampus, especially those that could be underlying mechanisms for cognitive deficits associated with prenatal nicotine exposure.

In the first part of the study prenatal nicotine induced neurobehavioral alterations, developmental reflexes, neurochemical changes in hippocampus and alterations in Y maze memory tasks were analyzed. Results of this study showed that prenatal nicotine exposure causes impairments in motor coordination, enhanced levels of reactive oxygen species and elevated lipid peroxidation. In addition rats that were exposed to prenatal nicotine showed decreased muscular strength when challenged with prolonged and demanding forced swim task and showed decreased performance in hippocampus dependent Y maze memory task. Prenatal nicotine administration was achieved by subcutaneous implantation of osmotic mini pumps that delivered nicotine (6 mg/kg/day) throughout gestation. Thus the results of this first part of study revealed that the rat model of prenatal nicotine exposure used in this study showed some of the harmful outcomes observed in children who were exposed to tobacco smoke *in utero* and also the results suggest that impaired hippocampal function could underlie the cognitive deficits.

Based on the results of the first part of study, the second part of the study analyzed whether basal synaptic transmission and LTP are altered in the key Shaffer collateral-CA1 synapses of the hippocampus in young rats that were exposed to prenatal nicotine. The results of this study showed that basal synaptic transmission and presynaptic glutamate release were compromised in prenatal nicotine rats. When AMPA receptor mediated mEPSCs were assessed they showed deficiencies in both presynaptic and postsynaptic components and the LTP in the Schaffer collateral-CA1 synapses was also impaired. In addition single channel synaptic AMPA receptor current properties were also diminished. These finding suggest that LTP impairment could be caused by deficiencies in basal synaptic transmission and deficiencies in synaptic currents mediated by AMPA receptors. The observed deficiencies also suggest that both presynaptic and postsynaptic function are impaired in young rats exposed to prenatal nicotine. Overall the results suggest that deficiencies in synaptic plasticity and reduced AMPA receptor function might underlie the cognitive deficits observed in children exposed to tobacco smoke *in utero*.

The third part of this study focused on whether the impairments in hippocampal synaptic function observed in young rats are long lasting up to young adult age. Results of this study showed impairments in hippocampal function observed in young animals were also observed in the young adult age suggesting that prenatal nicotine exposure induced changes in hippocampus could be long lasting. Overall this study has provided valuable and novel findings that reveals the mechanisms that could underlie cognitive deficits in children with *in utero* tobacco smoke exposure. In addition this study provides

a basis for future research to address the intrinsic mechanisms responsible for reduced synaptic plasticity and synaptic currents.