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The Impact of Developmental Nicotine on Hippocampus-Dependent Memory: Evidence for a Critical Role of alpha2-Containing Nicotinic Acetylcholine Receptors

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The impact of developmental nicotine exposure on hippocampus-dependent memory:  
evidence for a critical role of alpha2-containing nicotinic acetylcholine receptors

DISSERTATION

submitted in partial satisfaction of the requirements  
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Elise Kleeman

Dissertation Committee:  
Professor Katumi Sumikawa, Chair  
Professor Raju Metherate  
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2015



# DEDICATION

To

my family and friends, for supporting me on this journey  
and, especially,  
to ManKin

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1. Nakauchi, S., Su, H., Malvaez, M., **Kleeman, E.**, Dang, R., Sumikawa, K., Early postnatal nicotine exposure causes hippocampus-dependent memory impairments in adolescent mice: Association with altered nicotinic cholinergic modulation of LTP, but not impaired LTP, *Neurobiology of Learning and Memory*, 2015. 118: 178-188.

2. **Kleeman, E.**, Nakauchi, S., Su, H., Lopez, A., Dang, R., Wood, M.A., Sumikawa, K., Impaired function of  $\alpha 2^*$  nicotinic acetylcholine receptors on oriens-lacunosum moleculare cells causes hippocampus-dependent memory impairments (in preparation)

## **AUTHOR'S NOTE**

This thesis contains only a portion of the research I have completed in satisfaction of the requirements for my doctorate degree. Other data, collected to address a separate research question, is not included but was presented in my advancement.

In order to explain the significance of my findings described this document, this thesis also includes data collected by my collaborators. The behavior and molecular work is my own, conducted either alone or while working with others, but electrophysiology and voltage sensitive dye experiments were run by Sakura Nakauchi and Hailing Su.

## ABSTRACT OF THE DISSERTATION

The impact of developmental nicotine exposure on hippocampus-dependent memory:  
evidence for a critical role of alpha2-containing nAChRs in OLM cells

By

Elise Kleeman

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2015

Professor Katumi Sumikawa, Chair

Approximately 10 percent of the children born in the United States each year are exposed to cigarette smoke in utero, putting them at heightened risk for many physical and cognitive problems. Here we used mouse and rat models of early developmental nicotine exposure in order to elucidate the hippocampal changes that underlie nicotine-induced memory impairment. We observed that mice exposed to nicotine during their first two postnatal weeks showed significantly impaired long-term hippocampus-dependent spatial memory, but normal short-term spatial memory and long-term hippocampus-independent recognition memory. We also observed several changes in CA1 hippocampal function in these nicotine-treated mice, including facilitated LTP, heightened network activity, and dysfunctional nicotinic modulation of network activity and LTP. Thus, we next used an  $\alpha 2$  knockout mouse line to explore the hypothesis that early life nicotine exposure, by downregulating  $\alpha 2$ -containing nicotinic acetylcholine receptors (nAChRs), causes this spatial memory deficit and these changes in hippocampal function. We found that  $\alpha 2$  knockout mice showed a similar pattern of

memory impairments as did those treated with early postnatal nicotine. Additionally, our findings suggest that abnormal activation of  $\alpha 2$ -containing nAChRs by early life nicotine is necessary to bring about the facilitation of LTP and heightened CA1 network activity we observed in nicotine-exposed mice. Because  $\alpha 2$ -containing nAChRs are only found in the hippocampus on oriens-lacunosum moleculare (OLM) interneurons, which modulate inputs to the CA1 region of the hippocampus from the CA3 and entorhinal cortex, these findings add to the recently emerging body of evidence that OLM cells play a critical role in learning and memory. Furthermore, our work suggests that  $\alpha 2$ -containing nAChRs are important mediators of the effect of developmental nicotine on the hippocampus, and may therefore be a valuable target for the development of therapeutics to treat the cognitive impairments induced by in utero nicotine exposure.

## INTRODUCTION

### 1. Effects on learning and memory of nicotine exposure during brain development

An estimated 10 percent of expectant mothers in the United States – approximately 400,000 women each year – report smoking during the third trimester of their pregnancy (Centers for Disease Control and Prevention, 2014), a crucial time of brain development in which cell differentiation and synaptogenesis shape and refine brain circuitry (Thompson et al., 2009). Children of mothers who smoked during pregnancy face a significantly greater risk for numerous health and cognitive problems, including long-lasting learning and memory deficits (Fried et al., 2003; Batstra et al., 2003; Rogers, 2008; Thompson et al., 2009; Bruin et al., 2010; however, see: Lambe et al., 2006). Although cigarette smoke contains more than 7,000 chemicals, nicotine – which accumulates to even higher concentrations in the fetus than in the mother’s blood (Jauniaux et al., 1999) – is thought to be the leading cause of these impairments (Pauly and Slotkin, 2008). Indeed, rodent models have shown that early perinatal exposure to nicotine alone results in persistent deficits in learning and memory, including long-term, hippocampus-dependent spatial memory (Sorenson et al., 1991; Yanai et al., 1992; Ankarberg et al., 2001; Vagelnova et al., 2004; Eppolito and Smith, 2006). However, nicotine’s effects on the brain are incredibly complex, and it remains to be understood exactly how transient early life exposure to this drug causes long-lasting cognitive dysfunction.

Significantly, the damaging effect of nicotine on memory is unique to developmental exposure, and does not occur following nicotine use in adulthood. On the contrary, in adult rodents and humans, both acute and chronic nicotine facilitate

cognition (Heishman et al., 1994; Rezvani and Levin, 2001; Kenney and Gould, 2008; Heishman et al., 2010). In particular, many rodent studies have shown that chronic adult nicotine exposure causes long-lasting improvement in performance on hippocampus-dependent spatial memory tasks (including: Poincheval-Fuhrman and Sara, 1993; Arendash et al., 1995; Socci et al., 1995; Levin et al., 1996a; Levin and Torry, 1996). Nicotine has even been investigated as a treatment for mild cognitive impairment associated with aging or Alzheimer's disease, though in those cases it resulted in only small improvements in cognitive function (Wilson et al., 1995; White and Levin, 1999; White and Levin, 2004; Newhouse et al., 2012). Interestingly, nicotine exposure during adolescence has an intermediate effect. Whereas chronic nicotine causes adult-like improvements in memory for passive avoidance (Trauth et al., 2000b), during this time in which the limbic and prefrontal regions are still undergoing developmental changes (reviewed in Casey et al., 2008), nicotine exposure impairs contextual fear conditioning (Spaeth et al., 2010; Portugal et al., 2012; however, see: Smith et al., 2006), and serial pattern learning (Fountain et al., 2008), a measure of executive function. Despite these opposing effects of nicotine based on the timing of exposure, it is possible that nicotine-induced impairment and facilitation are the result of alterations to the same molecular or cellular pathways critical for memory formation, and it is therefore beneficial to consider work done both in perinatal and adult animals as a foundation for further inquiries about the impact of developmental nicotine. However, if the same pathways do prove critical to these effects, it raises the further question of what developmental change drives this dramatic reversal of the effect of nicotine.



## **2. Nicotinic acetylcholine receptors, and their role in hippocampal development**

Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels that are abundantly located throughout the brain. They are found in interneurons and projection neurons, both presynaptically, where they regulate neurotransmitter release (reviewed in: Vizi and Lendvai, 1999), and postsynaptically. nAChRs in the central nervous system are pentameric polypeptide assemblies that are either homomers of  $\alpha 7$ ,  $\alpha 8$  or  $\alpha 9$  subunits, or heteromers of  $\alpha 2-6$  and  $\beta 2-4$  subunits. The combination of subunits present in an nAChR determine the properties of that receptor, including its affinity for acetylcholine or nicotine, its kinetics, and whether or not it desensitizes in response to nicotine (Papke and Heinemann, 1991; Papke, 1993; Sargent, 1993; McGehee and Role, 1995). Whereas the most common nicotinic receptor type in the brain, the  $\alpha 4\beta 2$  subtype, has a high affinity for acetylcholine and nicotine but is slow to respond after ligand binding (Whiting et al., 1991; Flores et al., 1992), the  $\alpha 7$  subtype, the most common nicotinic receptor in the hippocampus, has a low affinity for acetylcholine and nicotine, a fast response rate, and is more  $\text{Ca}^{2+}$  permeable (Seguela et al., 1993; Fusile, 2004). Like most nAChRs,  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs desensitize and are rendered unresponsive in the presence of nicotine, including at nicotine concentrations found in smokers (Galzi and Changeux, 1995; Fenster et al., 1997; Alkondon et al., 2000). However, there is at least one type of nAChR, thought to contain the  $\alpha 2$  subunit, that does not desensitize, and is thus continually activate for as long as the drug is present (Jia et al., 2009).

In humans, significant hippocampal development occurs during the third trimester of pregnancy, whereas roughly equivalent development in rodents happens during the

first two postnatal weeks (Dobbing and Sands, 1973; Seress et al., 2001; de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007). This period of axon sprouting, dendritic arborization and robust synaptogenesis (Zancanaro et al., 2001; Danglot et al., 2006; de Graaf-Peters and Hadders-Algra, 2006; Dwyer et al., 2009) is also, importantly, a time of rapid development of the cholinergic system, and coincides with a sharp spike in nAChR subunit upregulation (Shacka and Robinson, 1998; Adams et al., 2002; Son and Winzer-Serhan, 2006; Winzer-Serhan and Leslie, 2005). nAChRs have been shown to play an important role in modulating the strength of newly forming excitatory synapses and mediating the switch in the role of the neurotransmitter  $\gamma$ -aminobutyric acid (GABA) from being excitatory to inhibitory (Liu et al., 2006). It is therefore not surprising that some studies found these first two postnatal weeks of major hippocampal development in rodents encompasses a “critical period” during which nicotine exposure causes long-lasting molecular and cognitive effects (Miao et al., 1998; Eriksson et al., 2000). Given the many roles of nAChRs during development, prolonged nicotine exposure during this time could alter multiple aspects of hippocampal function.

### **3. Cellular and molecular effects of developmental nicotine exposure**

One of the challenges of trying to understand the impact of prolonged nicotine exposure is that the effects of acute, intermediate and chronic nicotine treatment can vary greatly (for example: Hsieh et al., 2001; Yamazaki et al., 2006a; Portugal, 2012; Leach et al., 2013). However, only prolonged chronic exposure appears to have a long-lasting impact on memory, persisting or emerging in rodents several weeks or months after nicotine treatment has ended. In order to understand the mechanisms underlying

the long-term memory impairments caused by early life nicotine exposure, we therefore focus specifically on the effects of chronic nicotine.

To identify some possible changes induced by developmental nicotine exposure, we can begin by considering the cellular and molecular pathways altered following chronic nicotine treatment in adults, about which more is known. One of the most well-documented molecular alterations that chronic nicotine administration causes in adult rodents is an increase in the binding of nicotine or other nAChR agonists to high-affinity nAChRs like  $\alpha 4\beta 2$ , and to a lesser degree, to low-affinity nAChRs like  $\alpha 7$  (Marks et al., 1983, 1992; Schwartz and Kellar, 1983; Flores et al., 1997; Pauly et al., 1991). This is thought to reflect an increase in the number of functional nAChRs in the brain (for opposing view, see: Vallejo et al., 2005), possibly as a compensatory effect in response to the desensitization of most nAChRs by nicotine. These findings mirror the general upregulation of nAChRs seen in the brains of adult human smokers (Schwartz and Kellar, 1985; Benwell et al., 1988; Nybäck et al., 1989; Breese et al., 1997; Teaktong et al., 2003). The strength of this upregulation varies from region to region (Pauly et al., 1991). However, it is particularly strong in the hippocampus in both humans (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999) and rats (Marks et al., 1983), suggesting that the hippocampus may be especially sensitive to chronic nicotine. Similarly, a few studies of early postnatal nicotine treatment have indicated that it leads to increased binding of epibatidine, an nAChR ligand that binds strongly to  $\alpha 4\beta 2$  (Sullivan and Bannon, 1996), in the hippocampus in rats (Huang and Winzer-Serhan, 2006; Huang et al., 2007a). Binding at low-affinity nAChRs, by contrast, was decreased (Nordberg et al., 1991; Huang and Winzer-Serhan, 2006). However, one challenge of

interpreting these results, as with those from studies of adult brains, is that ligands such as epibatidine bind (with varying affinities) to several different nAChR subtypes, so these findings only give a general idea of the molecular changes that have occurred. Subtle differences in the abundance of individual nAChR subtypes, each of which can have very different roles based on factors like their presence within different cell types, their location in a neuron, and their properties (such as  $\text{Ca}^{2+}$  permeability), could therefore have major significance in terms of cognitive function. However, these findings collectively suggest that one important change that may underlie nicotine's effect is in the nicotinic modulation of hippocampal function. Because nAChRs in the hippocampus play an important role in modulating synaptic plasticity (reviewed in: Placzek et al., 2009), any long-term compensatory changes in the number of nicotinic receptors following early life nicotine exposure could have an equally long-lasting effect on hippocampus-dependent forms of memory.

Another change in hippocampal function that has been observed following chronic nicotine exposure in late-adolescent to early-adult rats is a resultant facilitation of the induction of hippocampal long-term potentiation (LTP) – the strengthening of synaptic signaling thought to underlie some forms of memory – as well as an increase in the persistence of LTP (Fujii et al., 1999; Yamazaki et al., 2006b, c). This suggests that prolonged nicotine exposure, in addition to causing the nAChR changes discussed above, may also affect N-methyl-D-aspartate receptors (NMDARs), which are required for this form of LTP. Indeed, chronic nicotine treatment in rats results in an increase in excitatory postsynaptic current mediated by NMDARs, perhaps because of increased phosphorylation of their NR2B subunits (Yamazaki et al., 2006a, b). This change may

be triggered by a pathway that starts with the activation of nACh receptors on cholinergic neurons, causing the release of acetylcholine onto pyramidal cells in the hippocampus, activating M1 muscarinic acetylcholine receptors, and setting off a cascade of kinase activity that ends with NR2B phosphorylation (Yamazaki et al., 2006b). Other studies have also suggested a link between chronic nicotine effects and NMDA receptors. NMDA receptor antagonists, given concurrently with chronic nicotine, block nicotine-induced sensitization of both locomotor activity and nucleus accumbens dopamine release (Shoaib and Stolerman, 1992; Shoaib et al., 1994), and interrupt chronic-nicotine-induced upregulation of nACh receptors (Shoaib et al., 1997). Chronic nicotine exposure increases the activity of NMDA receptors mediating noradrenergic release in the hippocampus (Risso et al., 2004), and upregulates NMDAR binding in the hippocampus (Levin et al., 2005) and the prefrontal cortex (Wang et al., 2007). Early postnatal nicotine exposure has also been shown to affect NMDARs in the auditory system of rats, increasing NMDAR excitatory postsynaptic potentials and mRNA expression for the NR2A subunit in the auditory cortex, and decreasing NR2B mRNA levels in the thalamus (Aramarkis and Metherate, 1998; Aramarkis et al., 2000; Hseih et al., 2002). Because NMDARs play a critical role in hippocampal learning and memory (reviewed in: Nakazawa et al., 2004), it is therefore also important to consider whether changes in the expression or function of these receptors may underlie the memory impairments induced by early life nicotine exposure.

Thus, there are many possibilities for which changes may underlie the hippocampal memory impairments caused by early life nicotine: unusual nAChR activity at a time when these receptors are involved in shaping hippocampal circuitry, long-

lasting alterations in nAChR or NMDAR number or function, or any number of other factors, including decreased neuronal survival, spine density, and dendritic arbor size (Roy and Sabherwal, 1998; Huang et al., 2007a). Adding to the complication, there are many models of early life nicotine exposure being used to conduct research in this field, with wide variations in nicotine concentration, patterns of administration, and developmental age during treatment, and not all of these models result in behavioral changes. Therefore, it is not clear that the cellular or molecular transformations described above are actually associated with (and, therefore, may underlie) nicotine-induced memory impairments. Here we set out to identify a model of early postnatal nicotine exposure in mice and rats with demonstrated hippocampal memory impairments, which we then used to identify specific molecular or cellular changes that may be responsible for the cognitive damage caused by early life nicotine exposure.

# CHAPTER 1. IMPACTS OF A MODEL OF DEVELOPMENTAL NICOTINE EXPOSURE ON HIPPOCAMPAL FUNCTION AND MEMORY IN MICE

## 1. Rationale

In order to identify long-lasting cellular, molecular and circuitry changes in the hippocampus that may underlie nicotine-induced cognitive impairments in children whose mothers smoked during pregnancy, here we used a mouse model of early developmental nicotine exposure from postnatal day 1–15 (P1–P15). This age range, roughly corresponding to the third trimester of human pregnancy (Dobbing and Sands, 1973; Seress et al., 2001; de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007), and at an important time of hippocampal development in which cholinergic activity likely modulates the strength of newly forming synapses and mediates the switch in the role of GABA (Liu et al., 2006), seems to encompass a “critical period” during which nicotine exposure causes long-lasting molecular and cognitive effects in the hippocampus (Miao et al., 1998; Eriksson et al., 2000). In order to ensure that any function changes we observed were actually associated with behavioral changes, we first tested our mouse model to determine whether it resulted in impaired hippocampus-dependent memory during adolescence. We then used electrophysiological, pharmacological and voltage-sensitive dye imaging techniques to identify nicotine-induced changes in hippocampal long-term potentiation (LTP), which is thought to be the cellular substrate of learning and memory, as well as hippocampal network activity and the nicotinic modulation of hippocampal function – each possible causes of memory deficits. Though this work cannot conclusively identify which cellular or molecular

changes underlie the memory deficits induced by early life exposure to nicotine, it will help identify targets for future, more detailed study.

## **2. Methods**

### *2.1. Animals and nicotine treatment*

All animal procedures were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. C57BL/6 mouse litters were adjusted to five or six male and female pups, and were exposed to nicotine through maternal milk during postnatal days 1–15 by subcutaneously implanting nursing dams with Alzet osmotic minipumps (approximate nicotine output: 21 mg/kg/day). Here, we refer to these pups as maternal-nicotine-exposed mice. Naïve mice and mouse pups from dams implanted with saline-containing minipumps were used as controls. Both gave similar electrophysiological and behavioral results, thus data obtained from those groups were combined for statistical analysis. Also, because electrophysiological recordings from male and female mice yielded equivalent results, their data were combined for statistical analysis. Behavioral studies were performed using male adolescent mice.

### *2.2. Object location and object recognition memory tasks*

Training and testing for object location and object recognition memory were conducted on P45 and P46, respectively, and were carried out as previously described (Barrett et al., 2011, McQuown et al., 2011 and Roozendaal et al., 2010). Briefly, before



training, mice were handled 1–2 min daily for 5 days and then habituated to the experimental arena (white rectangular open field, 30 × 23 × 21.5 cm) 5 min a day for 6 days in the absence of objects. During training, mice were placed into the experimental arena with two identical objects (100 mL beakers, lightbulbs or vases) and were allowed to explore for 10 min (Stefanko et al., 2009). During the retention test (90 min later for short-term memory, or 24 h later for long-term memory), mice explored the experimental apparatus for 5 min. For the object location task, one familiar object was placed in a novel location, and another familiar object was placed in the same location as during training. For the object recognition task, a familiar and a novel object were placed in the same locations as used during training. All combinations of locations and objects were balanced across trials to eliminate bias. Training and testing trials were videotaped and analyzed by individuals blind to the treatment condition. A mouse was scored as exploring an object when its head was oriented toward the object and within a distance of 1 cm, or when its nose was touching the object. The relative exploration time was recorded and expressed by a discrimination index ( $DI = (t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}}) \times 100\%$ ).

### *2.3. Elevated-plus maze*

The elevated plus-maze task was performed as previously described (Vogel-Ciernia et al., 2013). The maze consisted of two open arms and two enclosed arms extending from a central platform, raised to a height of 40 cm above the floor. The light level in the testing room was adjusted to 15 lux. Testing consisted of placing a mouse onto the central platform of the maze facing an open arm, and recording its locomotion

for 5 min. The percentage of time spent in the closed and open arms was scored using EthoVision 3.1 (Noldus Information Technology). Between subjects, the maze was cleaned with 70% ethanol.

#### *2.4. Slice preparation*

Transverse hippocampal slices (300–400  $\mu\text{m}$ ) were prepared from mice (age 4–6 weeks) anesthetized with urethane. Slices were maintained at 30 °C for at least 1 h to recover in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 5;  $\text{NaH}_2\text{PO}_4$ , 1.25;  $\text{MgSO}_4$ , 2;  $\text{CaCl}_2$ , 2.5;  $\text{NaHCO}_3$ , 22; glucose, 10; and oxygenated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

#### *2.5. Extracellular field recordings*

Slices were submerged in a recording chamber and continually superfused at 2–3 ml/min with oxygenated ACSF at 30 °C. A bipolar stimulating electrode was placed at the Schaffer collateral (SC) pathway, and the slice stimulated with short current pulses (200 ms duration) every 20 s. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum of the CA1 region using glass electrodes filled with ACSF (3–8 M $\Omega$ ). At the beginning of each experiment, a stimulus response curve was established by measuring the slope of fEPSPs. The strength of the stimulus was adjusted to elicit fEPSPs that were 30–50% of the maximum response (requiring stimulus intensities of 40–80  $\mu\text{A}$ ). The intensity and duration of each stimulus pulse remained invariant thereafter for each experiment. Baseline responses were recorded to establish the stability of the slice. LTP was induced by theta burst stimulation (TBS; 10

theta bursts, with each burst containing 4 pulses at 100 Hz and individual bursts separated by 200 ms), weak TBS (two theta bursts of four pulses at 100 Hz), or by four trains of tetanus (100 pulses at 100 Hz, in 5 min intervals), as indicated. To evaluate the magnitude of early-LTP, the mean values of the slopes of fEPSPs from 40–50 min after TBS stimulation were calculated and expressed as a percentage of the mean baseline fEPSPs slopes. Late-LTP magnitude was evaluated by comparing baseline slopes to those from 176 to 180 min after tetanus. Paired-pulse facilitation was determined using the stimulus intensity required to induce a half-maximum response with interpulse intervals of 25–200 ms. Recorded signals were amplified (A-M Systems) and digitized, and analyzed using NAC 2.0 software (Theta Burst Corp.).

## *2.6. Voltage-sensitive dye imaging*

Voltage-sensitive dye (VSD) imaging and recording was performed as previously described (Nakauchi et al., 2007 and Tominaga et al., 2000). Briefly, slices were submerged in a recording chamber mounted on the stage of a fluorescence microscope (BX51WI; Olympus). A 4× objective lens (0.28 NA; Olympus) focused the excitation light on the CA1 region of the hippocampus. VSD imaging was performed with a CCD camera (MiCAM02; BrainVision) which has a 6.4 × 4.4 mm<sup>2</sup> imaging area. To avoid bleaching of the dye, an electronically controlled shutter remained closed until 100 ms before the start of each recording. In each stimulation trial, frames were recorded at 250 Hz for 1024 ms. Eight or 16 trials were averaged to improve the signal-to-noise ratio. Extracellular potential recordings were performed simultaneously with the optical recordings to ensure that the optical response was consistent with the electrical

response. The fractional change in fluorescence intensity ( $\Delta F/F$ ) was used to normalize the difference in the amount of VSD in each slice, and signal gain and threshold levels were adjusted to optimize the signal-to-noise ratio of the response relative to background. Activated areas were smoothed by averaging images with spatial and cubic filters. Data were analyzed and displayed using BV-Analyzer (BrainVision). To quantitatively compare optical responses across different slices, the maximum optical responses to a single stimulus were sampled at  $3 \times 21$  grid points along the stratum oriens, stratum pyramidale and stratum radiatum layers of the hippocampal CA1, anchored to the stimulation site in the stratum radiatum. The 21 points of each layer were divided into two groups (proximal and distal to the site of stimulation), and the average optical responses in each group were calculated. As the outcomes were not substantially different between the proximal and distal groups, we have reported only the distal results below. Peak depolarization and hyperpolarization amplitudes were measured and compared. Measurement of the integrated negative area under the baseline was calculated for responses to a single stimulation with a cut-off time of 500 ms, a time point selected to minimize variability.

### *2.7. Drugs*

Nicotine, AP5, picrotoxin, methyllycaconitine (MLA), dihydro- $\beta$ -erythroidine (DH $\beta$ E), mecamylamine and DNQX were obtained from Sigma. All drugs were dissolved in ACSF and bath-applied for approximately 5–10 min.

### *2.8. Statistical analysis*

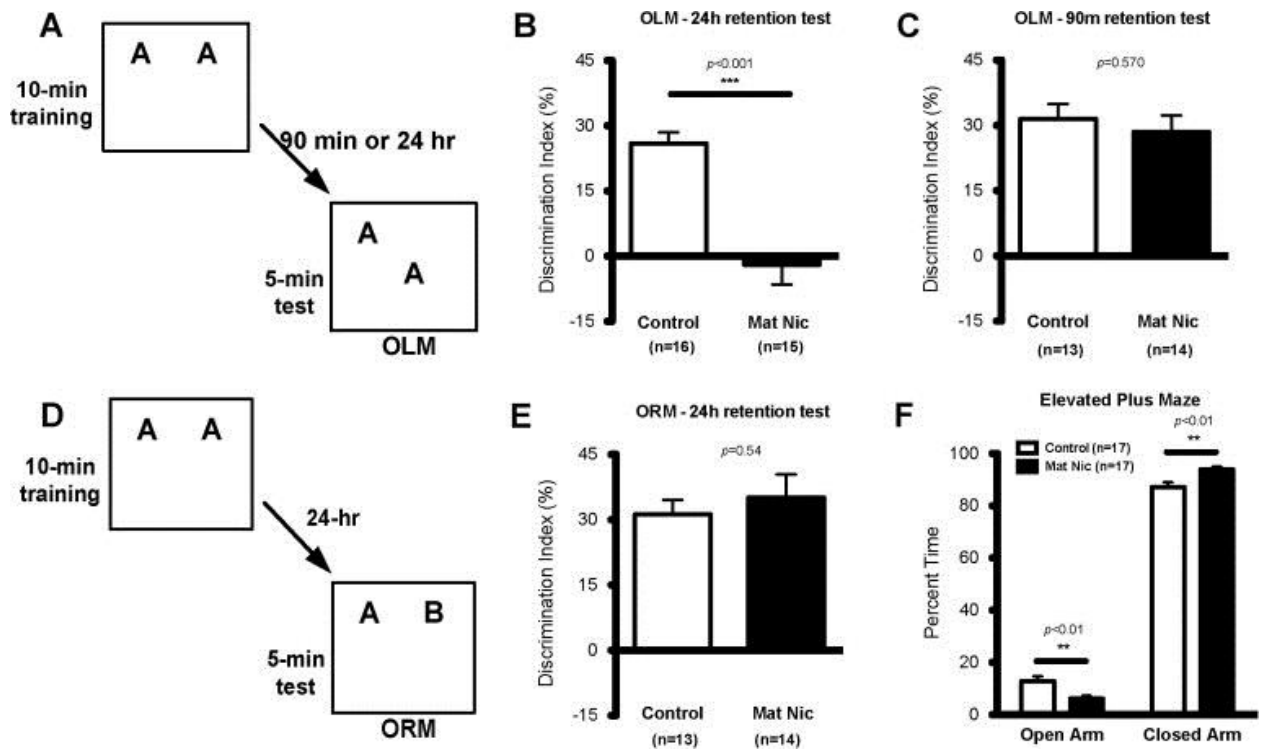
Behavior datasets were analyzed using Student's t-tests or one-way analysis of variance (ANOVA) with Bonferroni post hoc tests where appropriate. Alpha levels were set at 0.05. Electrophysiological data was normalized relative to baseline, expressed as mean  $\pm$  SEM, and analyzed for significance using ANOVAs and post hoc Tukey HSD tests. In all graphs, p values are depicted as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. Optical and physiological data were plotted and analyzed using Origin 8.1 (OriginLab).

### **3. Results**

#### *3.1. Early postnatal nicotine exposure disrupts hippocampus-dependent memory and increases anxiety*

The overall aim of this study was to examine the long-lasting impact of early postnatal nicotine exposure on hippocampal CA1 function. Therefore, we first tested maternal nicotine (MN)-exposed and control mice for long- and short-term object location memory (Fig. 1.1A-C), a CA1-dependent task (Assini et al., 2009, Barrett et al., 2011, Haettig et al., 2013; McQuown et al., 2011). MN-exposed mice exhibited significant deficits in long-term object location memory as compared to control mice (Fig. 1.1B; Control, n = 16, mean DI  $\pm$  SEM: 25.89  $\pm$  2.60; MN, n = 15, mean DI  $\pm$  SEM: -1.97  $\pm$  4.58; Student's t-test:  $t_{29} = 5.37$ , p < 0.0001). Importantly, there were no differences between groups with regard to total exploration time during training (Control, n = 16, mean DI  $\pm$  SEM: 30.14  $\pm$  2.07; MN, n = 15, mean DI  $\pm$  SEM: 30.55  $\pm$  2.03; t-test:  $t_{29} = 0.14$ , p = 0.88) and testing (Control, n = 16, mean DI  $\pm$  SEM: 6.25  $\pm$  0.59; MN, n = 15, mean DI  $\pm$  SEM: 5.59  $\pm$  0.63; t-test:  $t_{29} = 0.76$ , p = 0.46). We next examined short-

term object location memory in a different cohort of animals at 90 min after training. Short-term object location memory did not differ between MN mice and controls (Fig. 1.1C; Control,  $n = 13$ , mean DI  $\pm$  SEM:  $31.47 \pm 3.47$ ; MN,  $n = 14$ , mean DI  $\pm$  SEM:  $28.49 \pm 3.79$ ; Student's t-test:  $t_{25} = 0.58$ ,  $p = 0.57$ ). Again, there were no significant differences in total exploration time between groups (training: Control,  $n = 13$ , mean DI  $\pm$  SEM:  $17.46 \pm 1.49$ ; MN,  $n = 14$ , mean DI  $\pm$  SEM:  $16.17 \pm 1.06$ ; Student's t-test:  $t_{25} =$



**Figure 1.1.** Maternal nicotine-exposed mice have impaired long-term spatial memory and increased anxiety. (A) For the hippocampus-dependent object location memory (OLM) task, mice were trained for 10 min with two identical objects, and tested either 90 min or 24 h later with one object moved to a new location. (B) MN mice ( $n = 15$ ) showed significantly impaired 24-h long-term OLM compared to controls ( $n = 16$ ), and had a discrimination index not significantly different from zero. There were no significant differences between groups in total exploration time during training or testing. (C) In the OLM task, MN mice ( $n = 14$ ) did not show any difference in 90-min short-term memory from controls ( $n = 13$ ). There were no significant differences in total exploration time during training or testing. (D) For the hippocampus-independent object recognition memory (ORM) task, mice were trained for 10 min with two identical objects, and tested 24 h later after one object was replaced by a novel item. (E) In the ORM task, there was no difference in the 24-h long-term memory demonstrated by MN mice ( $n = 14$ ) or controls ( $n = 13$ ), and no significant differences between the groups in total exploration time during training or testing. (F) MN mice ( $n = 17$ ) spent significantly less time in the open arm of the elevated plus maze than did control mice ( $n = 17$ ), demonstrating increased anxiety.

0.72,  $p = 0.48$ ; test: Control,  $n = 13$ , mean DI  $\pm$  SEM:  $4.28 \pm 0.47$ ; MN,  $n = 14$ , mean DI  $\pm$  SEM:  $5.01 \pm 0.88$ ; t-test:  $t_{25} = 0.82$ ,  $p = 0.41$ ). Together, these results indicate that maternal nicotine-exposed mice exhibit specific long-term memory impairments for object location, a hippocampus-dependent task.

To examine whether the postnatal nicotine exposure induced more broad long-term memory impairments in MN mice, we used the hippocampus-independent object recognition memory task (Fig. 1.1D). MN mice showed similar long-term memory for object recognition to control mice (Fig. 1.1E; MN,  $n = 14$ , mean DI  $\pm$  SEM:  $36.91 \pm 5.79$ ; Control,  $n = 13$ , mean DI  $\pm$  SEM:  $30.38 \pm 3.52$ ; Student's t-test:  $t_{25} = 0.94$ ,  $p = 0.35$ ). Additionally, both groups showed similar total exploration times during training (Control,  $n = 16$ , mean DI  $\pm$  SEM:  $32.61 \pm 1.44$ ; MN,  $n = 15$ , mean DI  $\pm$  SEM:  $31.46 \pm 1.43$ ; t-test:  $t_{29} = 0.57$ ,  $p = 0.57$ ) and testing (Control,  $n = 16$ , mean DI  $\pm$  SEM:  $10.82 \pm 0.91$ ; MN,  $n = 15$ , mean DI  $\pm$  SEM:  $8.33 \pm 1.03$ ; t-test:  $t_{29} = 1.82$ ,  $p = 0.08$ ). Thus, MN mice exhibit normal long-term memory for object recognition, a hippocampus-independent task.

Although we observed no differences in total exploration time during the object location and object recognition experiments that would confound interpretation of the performance measures on memory, we did observe that MN mice appeared to be more anxious. Thus, we examined anxiety more directly using an elevated plus maze. MN mice ( $n = 17$ ) exhibited a modest yet significant increase in anxiety, spending less time in the open arms than control mice ( $n = 17$ ; Fig. 1.1F; two-way ANOVA, main effect of Arm  $F_{(1,62)} = 97.52$ ,  $p < 0.0001$ , no effect of Treatment  $F_{(1,62)} = 0.00$ ,  $p = 1.00$ , significant interaction  $F_{(1,62)} = 0.67$ ,  $p < 0.0001$ ; Bonferroni post hoc test: Control vs MN: open arm,  $p < 0.01$ ; closed arm,  $p < 0.01$ ). It is unlikely that the increased anxiety exhibited by MN

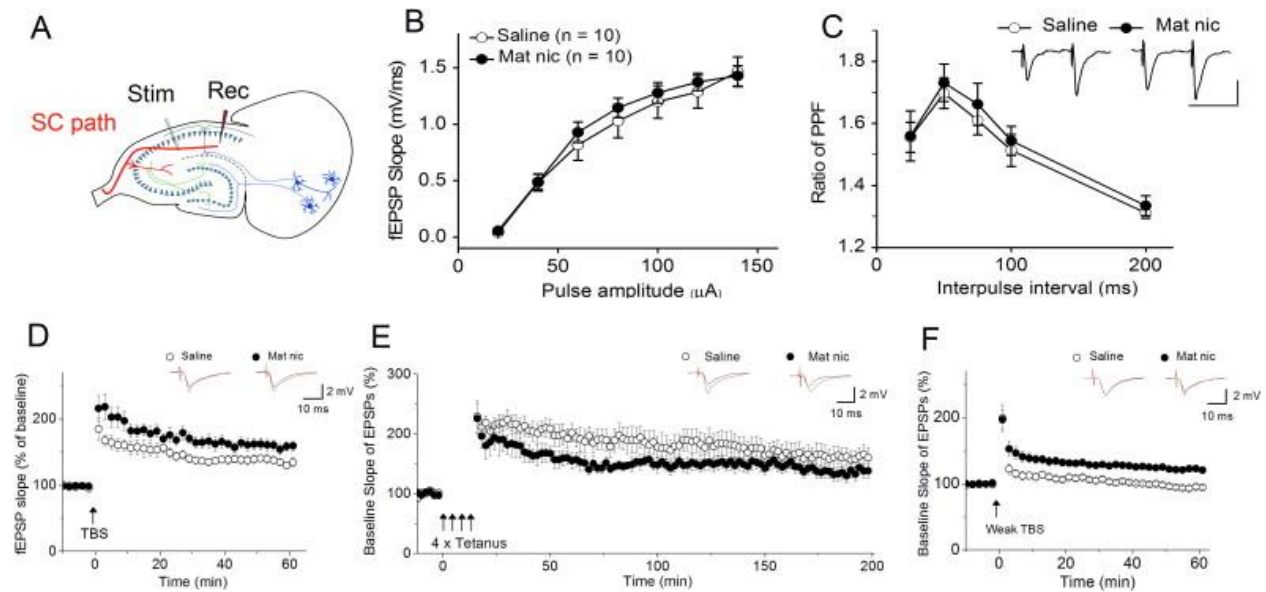
mice affected the memory experiments (Fig. 1.1B, C and E) because MN mice had normal total exploration times, normal short-term memory for object location, and normal long-term memory for object recognition. Together, these results suggest that MN mice have an impairment in hippocampal function that gives rise to the specific hippocampus-dependent long-term memory impairment for object location.

### *3.2. Early postnatal nicotine exposure lowers the LTP induction threshold*

To gain insight into the mechanism underlying the observed deficit in hippocampal memory, we first looked for MN-induced changes in synaptic transmission at the SC pathway by recording fEPSPs in hippocampal slices (Fig. 1.2A). We found no significant differences between slices from control or MN mice in either the stimulus–response relationships (Fig. 1.2B;  $F_{(1,134)} = 1.07$ ,  $p = 0.30$ ) or in paired-pulse facilitation (Fig. 1.2C;  $F_{(1,69)} = 0.69$ ,  $p = 0.41$ ), suggesting that early postnatal nicotine exposure significantly alters neither the basal synaptic transmission nor the probability of transmitter release.

Because LTP at the SC pathway has been strongly implicated as one of the cellular mechanisms of hippocampal learning and memory, we next examined whether MN impaired LTP at SC synapses. Theta burst stimulation (TBS) was used to induce early-LTP in hippocampal slices from nicotine-treated and control mice. Contrary to our expectations, postnatal nicotine exposure resulted in a strong, but not statistically significant, trend for a small increase in LTP magnitude (Fig. 1.2D; saline,  $139 \pm 6\%$ ,  $n = 6$ , vs. MN,  $161 \pm 8\%$ ,  $n = 8$ ,  $F_{(1,13)} = 4.19$ ,  $p = 0.06$ ). We also used four bursts of tetanus stimulation to induce long-lasting, protein-synthesis and dopamine-dependent late-LTP.





**Figure 1.2.** Early postnatal nicotine exposure facilitates the induction of LTP in the SC pathway of adolescent mice. (A) The placement of the stimulating and recording electrodes used to measure fEPSPs in the CA1. There was no significant difference between slices from control and MN mice (B) in the stimulus–response relationship (control:  $n = 10$ , MN:  $n = 10$ ) or (C) in paired-pulse facilitation (control:  $n = 6$ , MN:  $n = 8$ ), as shown by the ratio of the second fEPSP slope to the first fEPSP slope at different interpulse intervals (insert: representative traces for the 50 ms interpulse interval; horizontal calibration bar: 50 ms; vertical calibration bar: 1 mV). (D) MN slices ( $n = 8$ ) showed a trend for a small increase in the magnitude of early-LTP induced by TBS. (E) There was no difference between MN ( $n = 6$ ) and control ( $n = 7$ ) hippocampal slices in the magnitude of late-LTP induced by four bursts of high frequency stimulation. However, (F) weak TBS, which does not induce LTP in control hippocampal slices ( $n = 8$ ), induced LTP in MN slices ( $n = 6$ ). (D–F) Traces above each graph are representative waveforms recorded before and 40–50 min (D and F) or 176–180 min (E) after LTP-inducing stimulation.

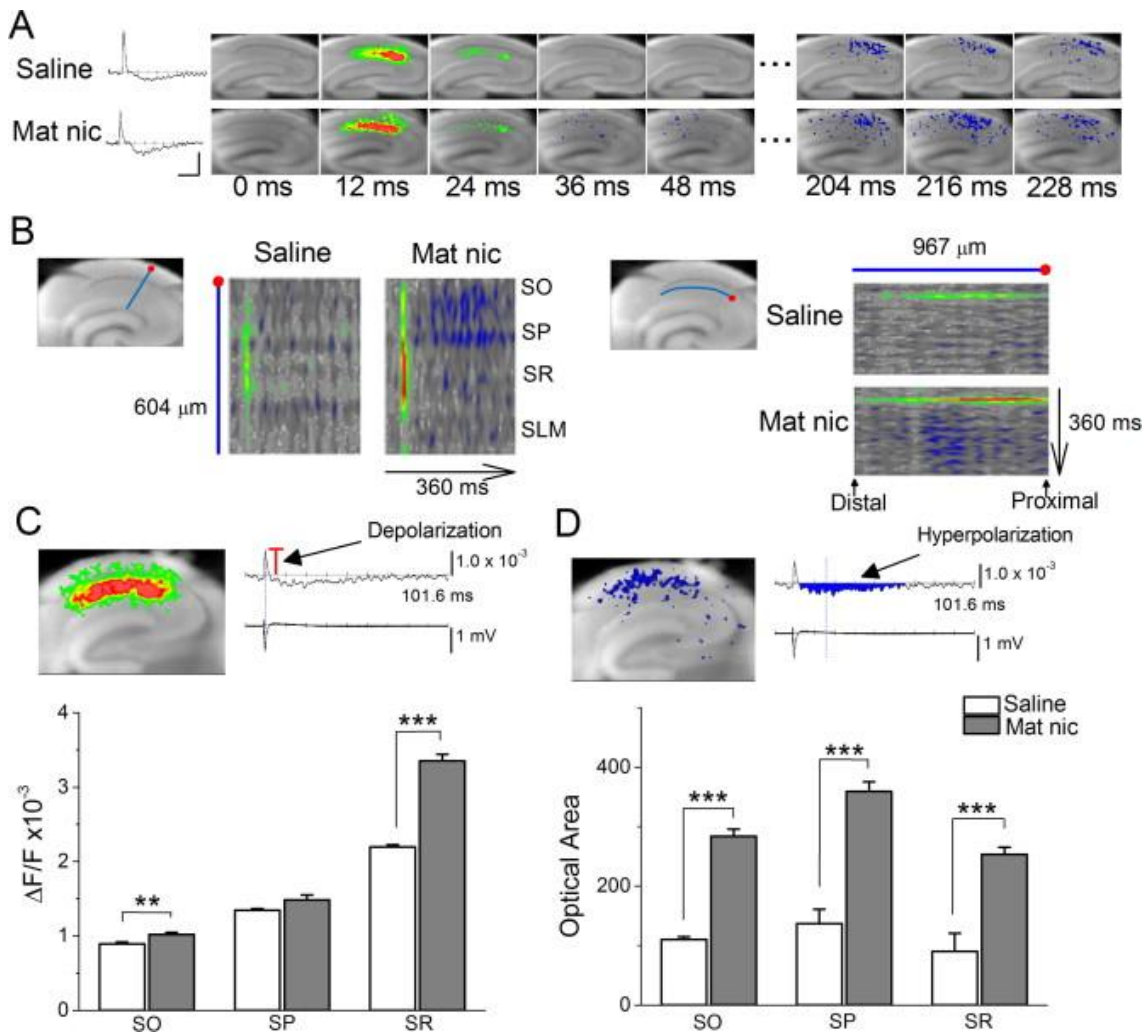
Hippocampal slices from MN mice showed no difference in late-LTP from control slices (Fig. 1.2E; control,  $160 \pm 14\%$ ,  $n = 7$ , vs. MN,  $138 \pm 11\%$ ,  $n = 6$ ,  $F_{(1,12)} = 1.39$ ,  $p = 0.23$ ).

Because these results suggested that, if anything, postnatal nicotine exposure enhances LTP at the SC pathway, we also explored whether it altered the threshold for LTP induction. We found that weak TBS, which is sub-threshold for LTP induction in hippocampal slices from control animals, is able to induce LTP in MN slices (Fig. 1.2F; saline,  $96 \pm 3\%$ ,  $n = 8$ , vs. MN,  $122 \pm 3\%$ ,  $n = 6$ ,  $F_{(1,13)} = 44.91$ ,  $p < 0.001$ ). Thus early postnatal nicotine exposure, which causes CA1-dependent memory impairments, unexpectedly facilitates LTP at SC synapses. The observation of increased TBS-

induced LTP following maternal nicotine has been previously reported in the dentate gyrus of rats (Mahar et al., 2012).

### *3.3. Early postnatal nicotine enhances depolarizing and hyperpolarizing neuronal activity in the CA1 region*

In the absence of clear impairments in SC-LTP that might underlie the observed deficit in hippocampal memory, we next used voltage-sensitive dye imaging as a more sensitive measure of network activity, because it allows for the visualization of changes in neuronal membrane potential, not just synaptic activity. This approach has the further benefit of quantifying neural activity over a much wider region of the CA1 than is possible with fEPSP recordings. Electrical stimulation of the SC pathway, the intensity of which was adjusted to evoke similar amplitudes of fEPSPs between different slices, caused the spread of optical signal in all anatomical layers of the CA1. Such signals can be presented as traces or as pseudocolor images of the fractional change in fluorescence intensity ( $\Delta F/F$ ; Fig. 1.3A). Depolarizing responses originating from the site of stimulation peaked at 12 ms in slices from both control and MN mice, but the peak signals in the stratum radiatum and stratum oriens were significantly stronger in the MN slices (Fig. 1.3A-C). Furthermore, in MN slices, we observed significantly stronger hyperpolarizing responses in all anatomical layers of the CA1 region at ~210 ms (Fig. 1.3A, B and D). The MN-induced changes are clearly visible in pseudocolor representations of line scans across the anatomical layers of the CA1 (Fig. 1.3B, left), and along the stratum radiatum (Fig. 1.3B, right) over time. Depolarizing activity was blocked with the addition of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid



**Figure 1.3.** Early postnatal nicotine exposure increases depolarization and hyperpolarization in the CA1 region after SC stimulation. (A–D) Voltage-sensitive dye imaging, which detects changes in neuronal activity not restricted to synapses, showed that MN exposure increased depolarization and hyperpolarization in the CA1 after SC stimulation. (A) Left, sample trace of optical response ( $\Delta F/F$ ) over time for a point in control and MN slices. Horizontal calibration scale: 82 ms, vertical scale:  $1.0 \times 10^{-3}$ . Right, sample pseudocolor representations of VSD signal after SC stimulation in control and MN slices. Red: depolarization, blue: hyperpolarization. (B) Left, pseudocolor representation of line scanning across CA1 layers (along the blue line beginning at the red dot, as shown in the slice image) over time in control and MN hippocampi. Scanning began 100 ms before stimulation and peaked at 8 ms after stimulation. Length of line is 604  $\mu\text{m}$ . Right, pseudocolor representation of line scanning along the stratum radiatum (along the blue line, starting from the red dot) over time in control and MN-treated hippocampi. Length of line is 967  $\mu\text{m}$ . (C) Top, sample pseudocolor image of maximum depolarizing responses (left), and simultaneous optical ( $\Delta F/F$ ) and fEPSP recordings (right). Bottom, averages of maximum optical responses (shown by the red bar in the sample optical trace) within CA1 layers in control ( $n = 6$ ) and MN ( $n = 10$ ) slices show that nicotine treatment enhanced depolarizing responses in the stratum oriens and radiatum. SO: Control;  $0.89 \pm 0.03$ ,  $n = 6$  vs. MN;  $1.02 \pm 0.03$ ,  $n = 10$ ;  $F_{(1,15)} = 10.22$ ,  $p < 0.01$ . SP: Control;  $1.35 \pm 0.02$ ,  $n = 6$  vs. MN;  $1.49 \pm 0.07$ ,  $n = 10$ ;  $F_{(1,15)} = 2.60$ ,  $p = 0.13$ . SR: Control;  $2.20 \pm 0.03$ ,  $n = 6$  vs. MN;  $3.35 \pm 0.09$ ,  $n = 10$ ;  $F_{(1,15)} = 92.16$ ,  $p < 0.001$ . (D, E) Continued on next page.

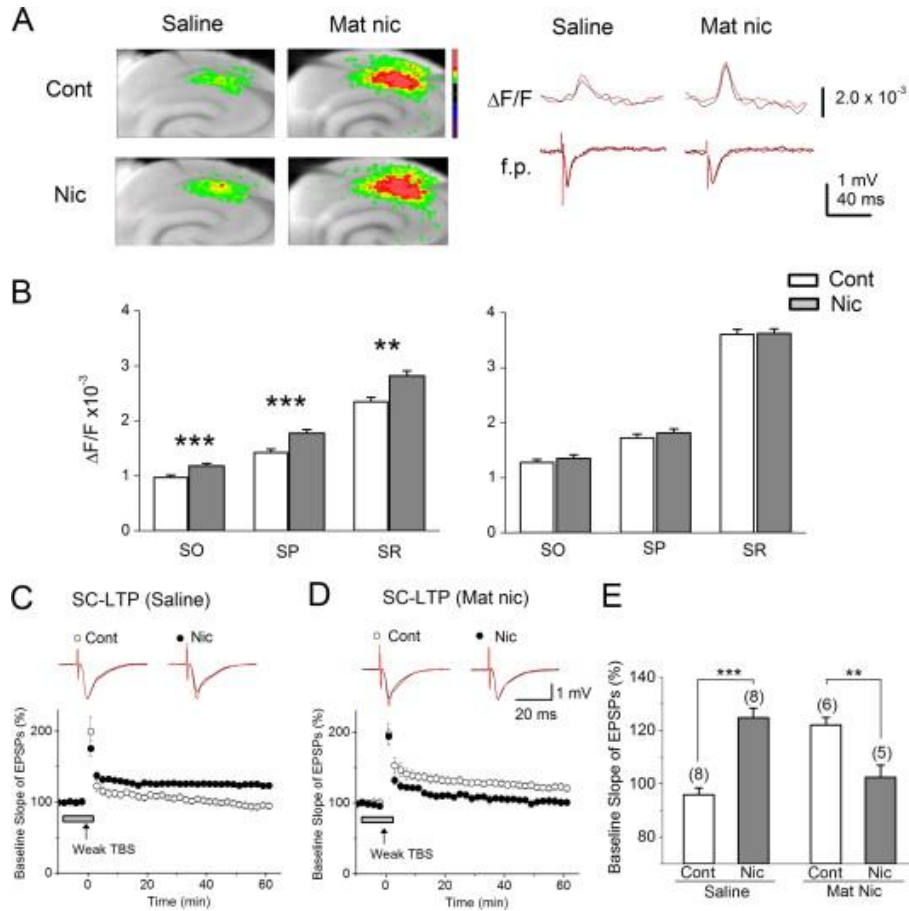
**Figure 1.3, continued.** (D) Top, sample pseudocolor image of inhibitory optical response (left), alongside simultaneous optical ( $\Delta F/F$ ) and fEPSP recordings (right). Bottom, averages of the areas of inhibitory optical responses (as shown by blue region in sample optical trace) within CA1 layers in control ( $n = 6$ ) and MN ( $n = 10$ ) slices show that nicotine treatment enhanced hyperpolarizing responses in the stratum oriens, pyramidale and radiatum. SO: Control,  $110.43 \pm 4.28$ ,  $n = 6$  vs. MN,  $283.95 \pm 11.92$ ,  $n = 10$ ,  $F_{(1,15)} = 118.59$ ,  $p < 0.001$ . SP: Control,  $137.14 \pm 24.01$ ,  $n = 6$  vs. MN,  $359.05 \pm 16.11$ ,  $n = 10$ ,  $F_{(1,15)} = 63.44$ ,  $p < 0.001$ . SR: Control,  $90.17 \pm 30.64$ ,  $n = 6$  vs. MN,  $253.54 \pm 11.97$ ,  $n = 10$ ,  $F_{(1,15)} = 34.13$ ,  $p < 0.001$ . SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum.  $**p < 0.01$ ,  $***p < 0.001$ .

receptor and N-methyl-D-aspartate receptor antagonists DNQX and AP5; hyperpolarizing activity was partially blocked with the GABA<sub>A</sub> antagonist picrotoxin, and completely blocked with the further addition of the GABA<sub>B</sub> antagonist CGP55845 (data not shown). These results indicate that MN treatment significantly increases both excitatory and inhibitory neuronal activity throughout the hippocampal CA1 after SC stimulation.

#### *3.4. Early postnatal nicotine exposure alters nicotinic modulation of depolarizing neuronal activity and LTP in the CA1*

nAChRs are key modulators of neuronal activity, and prolonged exposure to nicotine is known to alter the number and function of certain nAChR subtypes. In order to understand possible mechanisms for the MN-induced enhancement of depolarization and hyperpolarization in the CA1, we therefore investigated the possibility that early postnatal nicotine treatment alters nicotinic modulation of neuronal activity.

Using hippocampal slices from control and MN mice, we simultaneously recorded fEPSPs and VSD optical signal, both in the presence and absence of bath application of nicotine (1  $\mu$ M; Fig. 1.4A). In hippocampi from saline-treated controls, acute nicotine significantly increased depolarization in all anatomical regions measured, as determined by maximum optical signal (Fig. 1.4B, left graph). However, bath application of nicotine

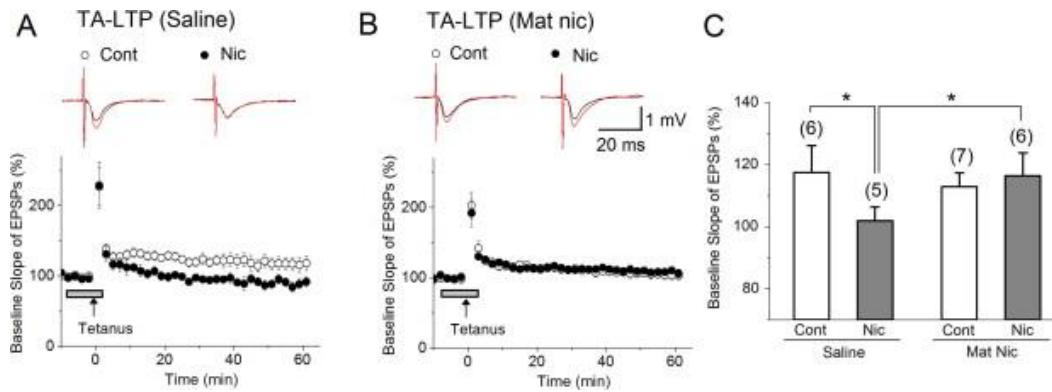


**Figure 1.4.** Early postnatal nicotine exposure alters acute nicotinic modulation of depolarizing neuronal activity and LTP at SC pathway. (A) Left, sample pseudocolor representations of maximum optical signal from voltage sensitive dye after SC stimulation, recorded first in the absence and then in the presence of nicotine, for control and MN slices. Right, sample simultaneous optical ( $\Delta F/F$ ) and fEPSP (f.p.) traces comparing responses to SC stimulation under baseline (black) and bath nicotine (red) conditions for saline and MN slices.  $\Delta F/F$  increased in the presence of nicotine in saline but not MN slices, while fEPSP amplitude showed no changes. (B) Averages of maximum optical responses within CA1 layers in saline (left graph,  $n = 9$ ) and MN (right graph,  $n = 13$ ) slices show that, although MN hippocampi show higher baseline activity than saline hippocampi, they show no change in response to bath application of nicotine, unlike control slices. Saline – SO: Control,  $0.97 \pm 0.03$ ,  $n = 9$  vs. Nicotine,  $1.18 \pm 0.03$ ,  $n = 9$ ,  $F_{(1,17)} = 23.3$ ,  $p < 0.001$ . SP: Control,  $1.42 \pm 0.05$ ,  $n = 9$  vs. Nicotine,  $1.77 \pm 0.05$ ,  $n = 9$ ,  $F_{(1,17)} = 25.22$ ,  $p < 0.001$ . SR: Control,  $2.35 \pm 0.10$ ,  $n = 9$  vs. Nicotine,  $2.93 \pm 0.11$ ,  $n = 9$ ,  $F_{(1,17)} = 15.37$ ,  $p < 0.01$ . MN – SO: Control,  $1.33 \pm 0.07$ ,  $n = 13$  vs. Nicotine,  $1.35 \pm 0.07$ ,  $n = 13$ ,  $F_{(1,25)} = 0.04$ ,  $p = 0.83$ . SP: Control,  $1.72 \pm 0.09$ ,  $n = 13$  vs. Nicotine,  $1.82 \pm 0.08$ ,  $n = 13$ ,  $F_{(1,25)} = 0.63$ ,  $p = 0.44$ . SR: Control,  $3.61 \pm 0.15$ ,  $n = 13$  vs. Nicotine,  $3.62 \pm 0.14$ ,  $n = 13$ ,  $F_{(1,25)} = 0.005$ ,  $p = 0.94$ . (C) Weak TBS induces LTP in slices from saline-treated control mice in the presence ( $n = 8$ ), but not in the absence ( $n = 8$ ), of acute nicotine. However, (D) in MN slices, weak TBS induces LTP in the absence of nicotine ( $n = 6$ ), but this LTP induction is blocked in the presence of nicotine ( $n = 5$ ). (E) MN treatment reverses the effect of nicotine on SC-LTP, as shown by the percent change in the slope of fEPSPs with and without bath nicotine, measured 50–55 min after weak TBS stimulation. (C and D) LTP-inducing stimulation was delivered at the time indicated by the arrow, and nicotine administration is indicated by the horizontal bar. Traces above each graph are representative waveforms recorded before (black) and 50 min after (red) LTP-inducing stimulation in control and bath-nicotine-treated slices. Cont: control; Nic: nicotine.  $**p < 0.01$ ,  $***p < 0.001$ .

did not cause detectable changes in the amplitude of fEPSPs (Fig. 1.4A, right, bottom traces), suggesting that in control slices, acute nicotine acts indirectly on pyramidal cells, as in wild-type mice (Nakauchi et al., 2007). By contrast, in slices from MN mice – which, in the absence of acute nicotine, show stronger optical signals than slices from saline-treated mice (Fig. 1.4A) – bath application of nicotine did not increase either fEPSP amplitude (Fig 1.4A, right, bottom traces) or excitatory optical signal (Fig. 1.4B, right graph) in any of the anatomical regions recorded in the CA1. These results indicate that early postnatal nicotine exposure causes long-lasting disruptions to the nicotinic modulation of excitatory activity in the CA1 region.

Similarly, we found alterations in the nicotinic cholinergic modulation of LTP after MN treatment. We have previously shown that weak TBS, which alone is sub-threshold for LTP induction, induces LTP at SC synapses of naïve mice with the addition of 1  $\mu$ M nicotine (Nakauchi et al., 2007; Nakauchi and Sumikawa, 2012). Here, we confirmed this nicotinic modulation in saline-treated control mice (Fig. 1.4C and E; Control,  $96 \pm 3\%$ ,  $n = 8$ , vs. Acute nicotine,  $125 \pm 3\%$ ,  $n = 8$ ,  $F_{(1,15)} = 41.00$ ,  $p < 0.001$ ). However, in hippocampal slices from MN mice, when weak TBS – which alone induces LTP – was administered in the presence of nicotine, the induction of LTP was unexpectedly blocked (Fig. 1.4D and E; Control,  $122 \pm 3\%$ ,  $n = 6$ , vs. Acute nicotine,  $102 \pm 5\%$ ,  $n = 5$ ,  $F_{(1,10)} = 14.12$ ,  $p < 0.01$ ).

We also observed a similar effect of MN exposure on nicotinic modulation when stimulating another excitatory input onto CA1 pyramidal neurons, the temporoammonic (TA) pathway. In hippocampal slices from saline-exposed mice, the induction of LTP by tetanic stimulation of the TA pathway (Fig. 1.5A and C;  $118 \pm 9\%$  of basal levels) is



**Figure 1.5.** Early postnatal nicotine exposure alters acute nicotinic modulation of LTP at TA pathway. (A) At TA synapses in hippocampal slices from saline-treated control mice ( $n = 6$ ), tetanus stimulation (100 pulses at 100 Hz) induced LTP; LTP induction was blocked in the presence of bath application of 1  $\mu$ M nicotine ( $n = 5$ ). (B) In hippocampal slices from MN mice, bath application of nicotine failed to block the TA-LTP ( $n = 6$ ) that was induced by tetanus stimulation in the absence of nicotine ( $n = 7$ ). (C) MN treatment blocks the effect of nicotine on TA-LTP, as shown by the percent change in the slope of fEPSPs with and without bath nicotine, measured 50–55 min after tetanus stimulation. (A and B) LTP-inducing stimulation was delivered at the time indicated by the arrow, and nicotine administration is indicated by the horizontal bar. Traces above each graph are representative waveforms recorded before (black) and 50 min after (red) LTP-inducing stimulation in control and bath-nicotine-treated slices. Cont: control; Nic: nicotine.  $*p < 0.05$ .

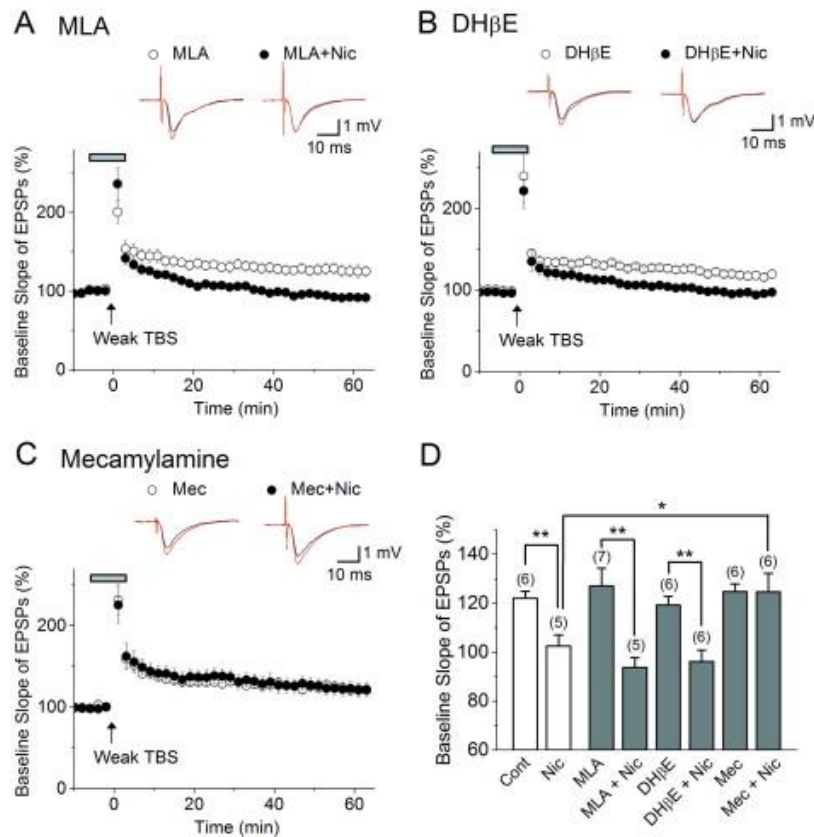
blocked by the addition of 1  $\mu$ M nicotine (Fig. 1.5A and C; Control,  $118 \pm 9\%$ ,  $n = 6$ , vs. Acute nicotine,  $90 \pm 5\%$ ,  $n = 5$ ,  $F_{(1,10)} = 6.48$ ,  $p < 0.05$ ), as with naïve mice (Nakauchi et al., 2007). However, in slices from MN mice, although tetanic stimulation induced similar LTP as in controls (Fig. 1.5A-C; Saline,  $118 \pm 9\%$ ,  $n = 6$ , vs. MN,  $113 \pm 5\%$ ,  $n = 7$ ,  $F_{(1,12)} = 0.27$ ,  $p = 0.61$ ), nicotine failed to block LTP induction (Fig. 1.5B and C; Control,  $113 \pm 5\%$ ,  $n = 7$ , vs. Acute nicotine,  $116 \pm 9\%$ ,  $n = 6$ ,  $F_{(1,12)} = 0.12$ ,  $p = 0.73$ ). Combined, these results suggest that early postnatal nicotine exposure cripples the ability of nAChRs to modulate hippocampal CA1 activity.

### 3.5. Nicotinic modulation of LTP in MN mice is driven by a newly recruited nAChR subtype

In order to begin identifying the alterations in molecular and cellular mechanisms driving these changes in network activity and synaptic plasticity, we next investigated

which nAChRs mediated LTP induction at SC synapses in MN mice. To start, we bathed MN slices in MLA (20 nM), an antagonist selective for  $\alpha 7$  nAChRs, DH $\beta$ E (500 nM), an antagonist of  $\beta 2$ -containing nAChRs (e.g.,  $\alpha 2\beta 2$ ,  $\alpha 4\beta 2$ ), or the non-selective nAChR antagonist mecamylamine (3  $\mu$ M), and administered weak TBS. MN-facilitated LTP induction persisted with each of these antagonists (Fig. 1.6A-D; Control,  $122 \pm 3\%$ ,  $n = 6$ , vs. MLA,  $127 \pm 7\%$ ,  $n = 7$ ,  $F_{(1,12)} = 0.39$ ,  $p = 0.54$ ; Control,  $122 \pm 3\%$ ,  $n = 6$ , vs. DH $\beta$ E,  $119 \pm 3\%$ ,  $n = 6$ ,  $F_{(1,11)} = 0.39$ ,  $p = 0.55$ ; Control,  $122 \pm 3\%$ ,  $n = 6$ , vs. Mecamylamine,  $125 \pm 3\%$ ,  $n = 6$ ,  $F_{(1,11)} = 0.46$ ,  $p = 0.51$ ). This suggests that activation of nAChRs by TBS-induced ACh release is not driving the facilitation of LTP that results from early prenatal nicotine treatment. However, when these antagonists were applied in the presence of nicotine (1  $\mu$ M), we found that the suppressive effect of acute nicotine on LTP induction in MN slices was blocked by mecamylamine, but not MLA or DH $\beta$ E (Fig. 1.6A–D; Nicotine,  $102 \pm 5\%$ ,  $n = 5$ , vs. Nicotine + Mecamylamine,  $125 \pm 8\%$ ,  $n = 6$ ,  $F_{(1,10)} = 5.65$ ,  $p < 0.05$ ; Mecamylamine,  $125 \pm 3\%$ ,  $n = 6$ , vs. Nicotine + Mecamylamine,  $125 \pm 8\%$ ,  $n = 6$ ,  $F_{(1,11)} = 4.57$ ,  $p = 0.98$ ; Nicotine,  $102 \pm 5\%$ ,  $n = 5$ , vs. Nicotine + MLA,  $94 \pm 4\%$ ,  $n = 5$ ,  $F_{(1,9)} = 2.12$ ,  $p = 0.18$ ; MLA,  $127 \pm 7\%$ ,  $n = 7$ , vs. Nicotine + MLA,  $94 \pm 4\%$ ,  $n = 5$ ,  $F_{(1,11)} = 12.87$ ,  $p < 0.01$ ; Nicotine,  $102 \pm 5\%$ ,  $n = 5$ , vs. Nicotine + DH $\beta$ E,  $96 \pm 5\%$ ,  $n = 6$ ,  $F_{(1,10)} = 0.95$ ,  $p = 0.36$ ; DH $\beta$ E,  $119 \pm 3\%$ ,  $n = 6$ , vs. Nicotine + DH $\beta$ E,  $96 \pm 5\%$ ,  $n = 6$ ,  $F_{(1,11)} = 17.06$ ,  $p < 0.01$ ). We have previously shown that in naive mice, DH $\beta$ E inhibits the facilitation of LTP induced by acute nicotine (Nakauchi & Sumikawa, 2012). Early postnatal nicotine exposure, however, appears to recruit a different nAChR subtype, containing neither  $\beta 2$  nor  $\alpha 7$  subunits, for the nicotinic modulation of LTP.





**Figure 1.6.** Nicotinic modulation of LTP in maternal nicotine-exposed mice is not driven by  $\alpha 7$  or  $\alpha 4\beta 2$  nAChRs. In MN hippocampal slices, (A and D) the  $\alpha 7$  antagonist MLA (20 nM) alone has no effect on LTP induced by weak TBS ( $n = 7$ ) and did not block the suppressive effect of 1  $\mu$ M nicotine ( $n = 5$ ). Likewise, (B and D) Dh $\beta$ E (500 nM), an antagonist of certain heteromeric nAChRs including  $\alpha 2\beta 2$  and  $\alpha 4\beta 2$ , did not affect LTP in MN slices ( $n = 6$ ) and did not block the suppressive effect of 1  $\mu$ M nicotine ( $n = 6$ ). (C and D) Mecamylamine (3  $\mu$ M) also does not affect the induction of LTP by weak TBS in MN slices ( $n = 6$ ), but does block the effect of 1  $\mu$ M nicotine ( $n = 6$ ). (A–C) Traces above each graph are representative waveforms recorded before (black) and 50–55 min after (red) LTP-inducing stimulation. Weak TBS stimulation was delivered at the time indicated by the arrow. Administration of drugs is indicated by the horizontal bar. (D) Histograms show the percent change in the slope of fEPSPs, measured 50–55 min after weak TBS. Nic = nicotine; Mec = mecamylamine. \* $p < 0.05$ , \*\* $p < 0.01$ .

However, the identity and location of the nAChR subtype involved in this nicotinic suppression of LTP in MN mice remains to be determined.

#### 4. Discussion

Several studies have attempted to model the cognitive impact to offspring of smoking during pregnancy by characterizing the memory impairments in rodents

exposed to perinatal nicotine. Using different protocols of nicotine administration and testing, some found clear impairments in learning and memory (Sorenson et al., 1991, Vaglenova et al., 2004, Yanai et al., 1992 and Portugal et al., 2012), whereas others reported no nicotine effects (Cutler et al., 1996 and Huang et al., 2007), only subtle impairments (Levin et al., 1993b), or only dose- or sex-specific impairments (Ankarberg et al., 2001 and Eppolito and Smith, 2006). Combined, this body of work demonstrates that the effects of nicotine exposure during development are sensitive to a combination of factors including sex, dose and timing of exposure. It is therefore particularly important, if aiming to identify long-term physiological changes that might underlie nicotine-induced cognitive deficits, to validate that the model of nicotine treatment being studied affects behavior. To our knowledge, this is the first study that uses a nicotine model with demonstrated learning and memory impairments to identify specific functional changes that may be the cause of nicotine-induced cognitive impairments.

Our behavioral experiments were conducted in adolescent mice one month after the end of nicotine exposure from P1 to P15 via maternal milk. We found that this early postnatal nicotine treatment resulted in a long-lasting impairment in long-term memory for the hippocampus-dependent object location task, but no impairment in either short-term object location memory or in long-term object recognition memory, a task that is thought to be dependent on the perirhinal cortex (Moses et al., 2005; Norman and Eacott, 2004). This indicates that the cognitive impairments induced by early postnatal nicotine exposure are not global, but rather that there are certain learning and memory processes that are particularly sensitive to nicotine effects. Nearly all previous investigations of the effect of perinatal nicotine exposure on memory in rodents have

studied hippocampus-dependent tasks, with the exception of one study showing that prenatal nicotine exposure also impairs active avoidance (Vaglenova et al., 2004), a limbic-system- and prefrontal-cortex-dependent task (McNew and Thompson, 1966; Moscarello and LeDoux, 2013). However, studies of the effect of chronic nicotine exposure in adult rodents do suggest that the hippocampus is particularly sensitive to nicotine (Kenney & Gould, 2008). Similarly, human studies of the impact of smoking during pregnancy have found that it causes impairments in some aspects of learning and memory, but not others (Fried et al., 2003). We also observed increased anxiety in MN mice, which others have shown using different models of perinatal nicotine exposure (Huang et al., 2007 and Vaglenova et al., 2004). Interestingly, the ventral hippocampus is required for anxiety-like behavior (Bannerman et al., 2004; Kjelstrup et al., 2002), again suggesting the hippocampus's sensitivity to the effects of nicotine.

This study identified several significant changes in electrophysiological and network activity in the hippocampus that could underlie the learning and memory impairments we observed after early postnatal nicotine exposure. Because LTP is a leading candidate for many forms of memory, we had expected that we would see MN-induced impairments in LTP magnitude or induction. However, early nicotine exposure resulted in facilitated LTP induction and a trend for a small increase in the magnitude of LTP. This raises the possibility that facilitated LTP can induce behavioral impairments by strengthening synapses that compete with those required for object location memory, or that, the observed memory impairments are driven by a different mechanism or form of synaptic plasticity.

Among the changes we observed in MN mice during adolescence was increased depolarization and hyperpolarization in the CA1 region of the hippocampus after stimulation of the Schaffer collateral. One possibility is that this reflects a pervasive restructuring of CA1 connectivity, brought about by the prolonged, nicotine-induced activation of nAChRs during a period critical for hippocampal development. In rodents, nAChRs are present and functional in the brain during late gestation (Naeff et al., 1992, Tribollet et al., 2004 and Zoli et al., 1995), and the expression of nAChRs transiently increases during the first two postnatal weeks (Shacka and Robinson, 1998), with particular increases in  $\alpha 2$ ,  $\alpha 5$  and  $\alpha 7$  subunits (Adams et al., 2002; Son and Winzer-Serhan, 2006; Winzer-Serhan and Leslie, 2005). During this period, nicotinic receptors are important modulators of the strength of newly formed excitatory synapses (Maggi et al., 2004; Maggi et al., 2003). Additionally, in this two-week period, nicotinic receptors containing  $\alpha 7$  subunits drive the transition in GABAergic signaling from being excitatory to inhibitory (Liu et al., 2006), which further shapes hippocampal network development. Therefore, the presence of nicotine during this critical window could have caused long-term changes in hippocampal circuitry or synaptic connectivity. Indeed, voltage-sensitive dye imaging revealed significant changes in the network activity of hippocampal slices from MN mice. A similar increase in CA1 excitatory activity has also been observed in rats exposed to one week of postnatal nicotine (Damborsky et al., 2012). Interestingly, we did not observe any corresponding change in fEPSP stimulus response-curves or in paired pulse facilitation, suggesting that these changes in hippocampal physiology are not occurring solely at the SC synapse.

It is also possible that long-lasting compensatory changes in nAChR number or function – rather than altered network connectivity established during postnatal development – underlies the altered hippocampal activity that results from postnatal nicotine exposure. nAChRs continue to play an important role in modulating hippocampal activity throughout life, and anomalous nAChR expression is associated with cognitive impairment and memory disorders including Alzheimer's disease (reviewed in Posadas et al., 2013). Early life nicotine exposure in rodents, particularly during the second postnatal week, has been shown to cause persistent changes in nAChRs, upregulating expression of high-affinity nAChRs such as  $\alpha 4\beta 2$  heteromers (Huang and Winzer-Serhan, 2006, Miao et al., 1998 and Narayanan et al., 2002), while drastically decreasing low affinity nAChRs such as homomeric  $\alpha 7$ -containing receptors (Eriksson et al., 2000). Furthermore, prenatal nicotine exposure in rats has been shown to stifle the increase in nAChR-mediated current that normally occurs during adolescence (Britton et al., 2007). Likewise, our results suggest long-term, MN-induced alterations in the normal nicotinic modulation of CA1 activity. We showed that early postnatal nicotine exposure blocked the effect of acute nicotine on CA1 network activity and on TA-LTP, and reversed the facilitating effect of acute nicotine on LTP induced at the SC pathway. It is therefore possible that MN-induced memory impairments are caused by a lack of nicotinic modulation crucial for the synaptic plasticity mechanisms of learning and memory.

This study has identified several significant changes in hippocampal cellular and network activity following early postnatal nicotine exposure, but it still remains to be determined what underlies these changes. We have previously shown that in naïve

mice, acute nicotine enhances hippocampal network activity and LTP by driving the inhibition of feedforward inhibition in the SC pathway (Nakauchi and Sumikawa, 2012 and Yamazaki et al., 2005). Our results here demonstrate that early postnatal nicotine exposure enhances hippocampal network activity and LTP, and impairs the ability of acute nicotine to enhance hippocampal network activity and LTP. Together, one possible mechanism of enhanced excitatory optical signal and LTP observed in MN mice is the persistent inhibition of feedforward inhibition, occluding the effect of acute nicotine.

The facilitation of LTP by acute nicotine is absent in  $\alpha 2$  and  $\beta 2$  knockout mice and interrupted by DH $\beta$ E, an antagonist of  $\beta 2$ -containing nAChRs, in wild-type mice (Nakauchi & Sumikawa, 2012). However, in MN-treated mice, the suppressive effect of acute nicotine on LTP was blocked by the non-specific nAChR antagonist mecamylamine, but not by DH $\beta$ E or by MLA, an antagonist of  $\alpha 7$ -containing nAChRs. This suggests that early postnatal nicotine exposure alters nAChR or circuit function to such a degree that a different nAChR subtype now plays the dominant role in LTP modulation, with completely opposite effect. Interestingly,  $\alpha 2$ -containing nAChRs, which are located on stratum oriens/alveus (O/A) interneurons (Ishii et al., 2005 and Wada et al., 1989), drive the inhibition of feedforward inhibition via circuitry-dependent mechanism, and hippocampal slices from  $\alpha 2$  knockout mice have impaired responses to acute nicotine at both SC and TA pathways (Nakauchi et al., 2007) that are very similar to what we observed in MN slices. Thus, it is possible that the lack of acute nicotine's effect in MN mice is due to altered function of  $\alpha 2$ -containing nAChR-expressing O/A interneuron. The expression of  $\alpha 2$  mRNA in O/A interneurons is upregulated during

early postnatal period (Son & Winzer-Serhan, 2006). It is possible that early postnatal nicotine exposure continuously excites these interneurons via activation of  $\alpha 2$ -containing nAChRs to cause a long-lasting disturbance of GABAergic inhibition and its nicotinic control, affecting nicotinic modulation of LTP and hippocampal-dependent memory in adolescent mice.

This study shows that early postnatal nicotine exposure results in long-lasting and pervasive changes to the CA1 region of the mouse hippocampus, including impairments in long-term spatial memory, and significant changes in CA1 network activity and nicotinic control of synaptic plasticity. Thus, these findings demonstrate the significance of nAChR activity during early brain development, and indicate the critical role of timing-dependent cholinergic induction of synaptic plasticity (Gu et al., 2012; Gu and Yakel, 2011; Ji et al., 2001) and other nicotinic cholinergic-dependent mechanisms of synaptic plasticity (Halff et al., 2014; Ishibashi et al., 2014; Nakauchi and Sumikawa, 2012; Yamazaki et al., 2006a) in spatial memory.

## CHAPTER 2. THE ROLE OF $\alpha$ 2-CONTAINING nAChRs IN MEMORY IMPAIRMENTS INDUCED BY DEVELOPMENTAL NICOTINE EXPOSURE

### 1. Rationale

We have previously shown that nicotine exposure in mice during the first two postnatal weeks – a time of significant hippocampal development roughly equivalent to the third trimester of human pregnancy (Seress et al., 2001; de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007) – causes a persistent impairment to long-term hippocampus-dependent memory (Nakauchi et al., 2015). This memory deficit was associated with several changes in hippocampal function, including facilitated long-term potentiation (LTP) and hyperactivity of CA1 circuits in response to Schaffer collateral stimulation (Nakauchi et al., 2015). Similar changes were also observed with a rat model of early postnatal nicotine exposure (Chen et al., submitted). However, it remains to be determined which, if any, of these functional changes could cause associated spatial memory impairments.

Additionally, in our rodent models we observed several changes to the way in which nAChR activation modulated CA1 activity. In particular, we found that early postnatal nicotine exposure impaired the function of oriens-lacunosum moleculare (OLM) interneurons, which are located in the stratum oriens and have projections into the stratum lacunosum-moleculare (Freund and Buzsaki, 1996). These interneurons receive cholinergic inputs from the medial septum, and can facilitate LTP at Schaffer collateral synapses and block LTP at temporoammonic pathway synapses (Nakauchi et al., 2007; Leao et al., 2012; Chen et al., submitted). They can therefore affect the



relative strength of inputs to CA1 pyramidal cells from the entorhinal cortex, which conveys sensory information, and from the CA3, which conveys internal representations of the multisensory context (Kesner, 2007; Gilbert and Brushfield, 2009). Thus, these OLM cells are thought to be critical mediators of the formation of spatial memories (Leao et al., 2012; Lovett-Barron et al., 2014), and early-postnatal-nicotine-induced decreases in their responsiveness to nicotinic stimulation and in their signaling to pyramidal cells (Chen et al., submitted) may critically impair hippocampal memory formation.

OLM interneurons are unique among CA1 hippocampal cells in that they express the uncommon  $\alpha 2$ -containing nAChR subtype (Wada et al., 1989; Ishii et al., 2005; Jia et al., 2009; Leao et al., 2012), which, unlike most, if not all other nAChR subtypes, is thought to be continually active in the presence of nicotine, and does not desensitize (Jia et al., 2009). Thus, early postnatal nicotine exposure, by acting on these receptors, may cause long-lasting alterations to the normal development of the hippocampus. Importantly, we found that after two weeks of maternal nicotine treatment in rats, the number of  $\alpha 2$ -mRNA-expressing OLM cells was significantly decreased (Chen et al., submitted). Combined, our previous findings suggest the possibility that early life nicotine exposure downregulates  $\alpha 2$ -containing nAChRs, and disrupts the normal function of OLM cells to cause profound changes in CA1 function and CA1-dependent behavior.

Therefore, here we used an  $\alpha 2$  knockout mouse line to investigate whether the downregulation of  $\alpha 2$ -containing nAChRs may underlie the memory impairments observed following early postnatal nicotine, and we also address whether some of the

nicotine-induced changes in hippocampal function that we previously observed could be contributing to these memory deficits.

## **2. Materials and methods**

### *2.1. Animals and nicotine treatment*

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. Efforts were made to minimize the number of animals used.

Alpha2-null mutant ( $\alpha$ 2KO) mice were generated as described (Loftipour et al., 2013), and were compared against wild-type C57BL/6 control mice. In order to avoid any behavioral changes caused by differences in maternal care, for the behavior experiments the wild-type and  $\alpha$ 2KO pups were fostered with CD1 dams immediately after birth. Mouse litters were adjusted to five male or female pups.

For experiments involving early postnatal nicotine exposure, pups were exposed to nicotine through maternal milk during postnatal days 1-15 by subcutaneously implanting nursing dams with osmotic minipumps (Alzert; approximate nicotine output: 21 mg/kg/day). Here, we refer to these pups as maternal-nicotine-exposed mice. Mouse pups from dams implanted with saline minipumps were used as controls. As electrophysiological recordings and behavior from male and female mice yielded equivalent results, their data were combined for statistical analysis.

### *2.2 Object location and object recognition memory tasks*

Training and testing for object location and object recognition memory were conducted between P44 and P67, and were carried out as previously described (Vogel-Ciernia and Wood, 2014) by experimenters blind to the treatment group. Briefly, before training the mice were handled 2 minutes daily for 5 days and then habituated to the experimental arena (white rectangular open field, 30 x 23 x 21.5 cm) 5 min a day for 6 days in the absence of objects. During training, mice were placed into the experimental arena with two identical objects (100 mL beakers or metal tins) and were allowed to explore for 10 minutes. During the retention test 24 hrs later to test long-term memory, for the object location task, one of the familiar objects was placed in a novel location, and the other was placed in one of the locations used during training. For the object recognition task, a familiar and a novel object were placed in the same locations as were used during training. All combinations of locations and objects were balanced across trials to eliminate bias. Training and testing trials were videotaped and analyzed by individuals blind to the treatment condition. A mouse was scored as exploring an object when its head was oriented toward the object and within a distance of 1 cm, or when its nose was touching the object. The relative exploration time was recorded and expressed by a discrimination index ( $DI = (t_{\text{novel}} - t_{\text{familiar}}) / (t_{\text{novel}} + t_{\text{familiar}}) \times 100\%$ ).

### *2.3. Elevated plus-maze*

The elevated plus-maze task was performed as previously described (Nakauchi et al., 2015). The maze consisted of two open arms and two enclosed arms extending from a central platform, raised to a height of 40 cm above the floor. The light level in the testing room was adjusted to 15 lux. Testing consisted of placing a mouse onto the

central platform of the maze facing an open arm, and recording its locomotion within the arms of the platform for 5 min. The percentage of time spent in the closed and open arms was scored using ANY-maze version 4.99b (Stoelting). Between subjects, the maze was cleaned with 10% ethanol.

#### *2.4 Slice preparation*

Transverse hippocampal slices (300-400  $\mu\text{m}$ ) were prepared from mice (age 4-6 weeks) anesthetized with urethane. Slices were maintained at 30°C for at least 1 h to recover in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 4;  $\text{NaH}_2\text{PO}_4$ , 1.25;  $\text{MgSO}_4$ , 2;  $\text{CaCl}_2$ , 2.5;  $\text{NaHCO}_3$ , 22; glucose, 10; and oxygenated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ .

#### *2.5 Extracellular field recordings*

Slices were submerged in a recording chamber and continually superfused at 2-3 mL/min with oxygenated ACSF at 30°C. A bipolar stimulating electrode was placed at the Schaffer collateral (SC) pathway, and the slice stimulated with short current pulses (200 ms duration) every 20 s. Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum of the CA1 region using glass electrodes filled with ACSF (3-8 M $\Omega$ ). At the beginning of each experiment, a stimulus response curve was established by measuring the slope of fEPSPs. The strength of the stimulus was adjusted to elicit fEPSPs that were 30-50% of the maximum response (requiring stimulus intensities of 40-80  $\mu\text{A}$ ). The intensity and duration of each stimulus pulse remained invariant thereafter for each experiment. Baseline responses were recorded to

establish the stability of the slice. Weak TBS (two theta bursts of four pulses at 100 Hz) was used to try to induce LTP. To evaluate LTP magnitude, the mean values of the slopes of fEPSPs from 40-50 min after TBS stimulation were calculated and expressed as a percentage of the mean baseline fEPSPs slopes.

## *2.6 Voltage-sensitive dye imaging*

Voltage-sensitive dye (VSD) imaging and recording was performed as previously described (Tominaga et al., 2000; Nakauchi et al., 2007). Briefly, slices were submerged in a recording chamber mounted on the stage of a fluorescence microscope (BX51WI; Olympus). A 4x objective lens (0.28 NA; Olympus) focused the excitation light on the CA1 region of the hippocampus. VSD imaging was performed with a CCD camera (MiCAM02; BrainVision) which has a 6.4 x 4.4 mm<sup>2</sup> imaging area. To avoid bleaching of the dye, an electronically controlled shutter remained closed until 100 ms before the start of each recording. In each stimulation trial, frames were recorded at 250 Hz for 1024 ms. Eight or 16 trials were averaged to improve the signal-to-noise ratio. Extracellular potential recordings were performed simultaneously with the optical recordings to ensure that the optical response was consistent with the electrical response. The fractional change in fluorescence intensity ( $\Delta F/F$ ) was used to normalize the difference in the amount of VSD in each slice, and signal gain and threshold levels were adjusted to optimize the signal-to-noise ratio of the response relative to background. Activated areas were smoothed by averaging images with spatial and cubic filters. Data was analyzed and displayed using BV-Analyser (BrainVision). To quantitatively compare optical responses across different slices, the maximum optical

responses were measured and compared. The magnitude of voltage changes was illustrated using pseudocolor.

## 2.7 Statistical analysis

Behavior datasets were analyzed with Student's *t*-tests. *p* values of less than 0.05 were considered statistically significant. Electrophysiological data was normalized relative to baseline, expressed as mean  $\pm$  SEM, and analyzed for significance using ANOVAs and *post hoc* Tukey HSD tests. In all graphs, *p* values are depicted as follows: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. Optical and physiological data were plotted and analyzed using Origin 8.1 (OriginLab).

## 3. Results

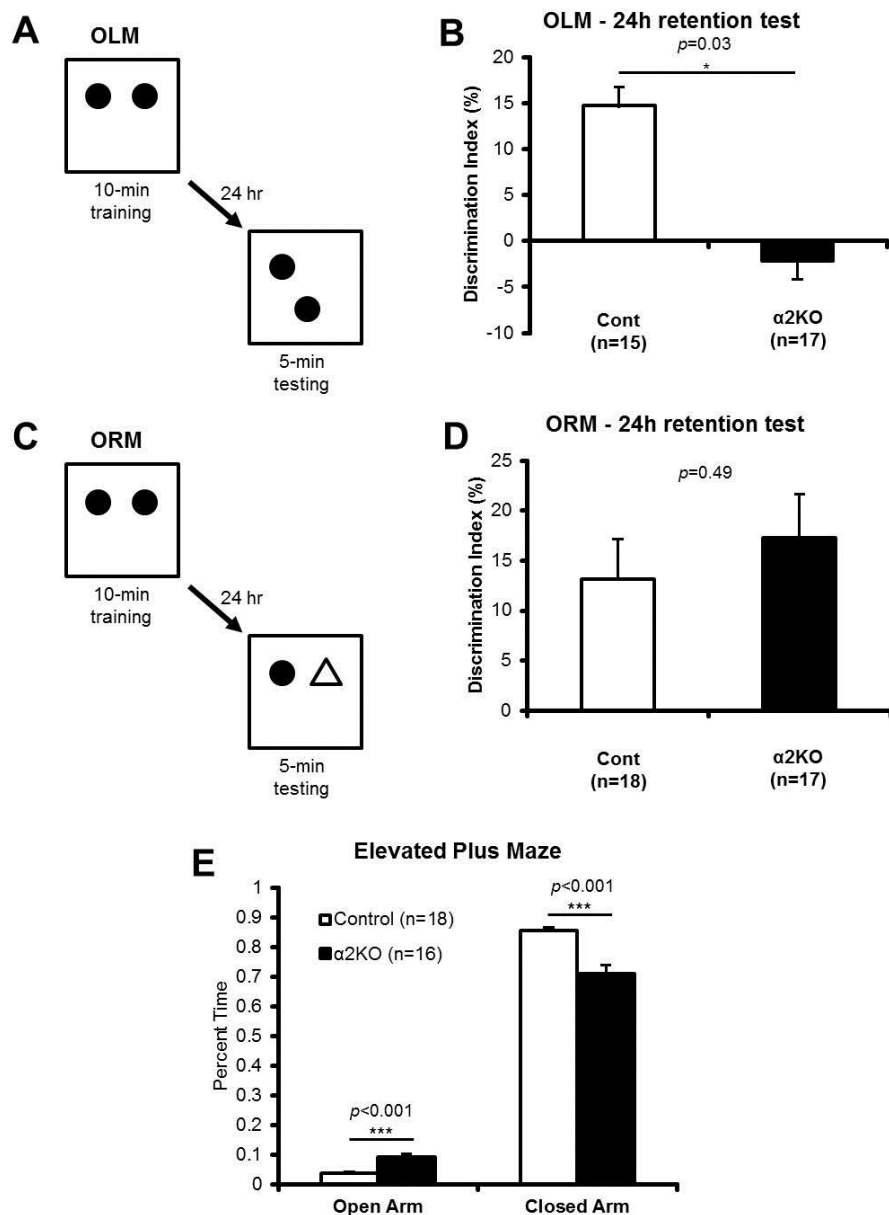
### 3.1 $\alpha 2$ knockout mice have impaired hippocampus-dependent memory

The overall aim of our study was to understand which changes caused by early postnatal nicotine exposure may underlie nicotine-induced memory impairments. Therefore, because nicotine decreases the expression of  $\alpha 2$  nAChR subunit mRNA and impairs the function of  $\alpha 2$ -containing OLM cells (Chen et al., submitted), we first tested whether  $\alpha 2$  knockout mice, like mice that are exposed to early postnatal nicotine, show impairments in long-term object location memory (Fig. 2.1A, B), a CA1-dependent task (Assini et al., 2009; Barrett et al., 2011; McQuown et al., 2011; Haettig et al., 2013).  $\alpha 2$ KO mice exhibited significant deficits in long-term object location memory as compared to wild-type (WT) mice (Fig. 2.1B; WT, *n* = 15, mean DI  $\pm$  SEM: 14.74  $\pm$  3.82;  $\alpha 2$ KO, *n* = 17, mean DI  $\pm$  SEM: -2.17  $\pm$  5.91; Student's *t*-test:  $t_{31} = 2.33$ , *p* = 0.03).

Importantly, there were no differences between the two groups with regards to total exploration time during testing (WT,  $n = 15$ , mean  $\pm$  SEM: 29.48 s  $\pm$  2.39;  $\alpha$ 2KO,  $n = 17$ , mean  $\pm$  SEM: 29.05 s  $\pm$  3.02; Student's  $t$ -test:  $t_{31} = 0.11$ ,  $p = 0.91$ ). These data suggest that the  $\alpha$ 2-containing nAChRs are crucial for the formation of long-term, hippocampus-dependent spatial memory.

To determine whether the memory impairment in  $\alpha$ 2KO mice was global or, as in mice exposed to nicotine during development, was not, we tested them with the object recognition memory task, which is hippocampus-independent (Fig. 2.1C; Norman and Eacott, 2004; Moses et al., 2005).  $\alpha$ 2KO mice showed similar long-term memory for object recognition as did WT mice (Fig. 2.1D; WT,  $n = 18$ , mean DI  $\pm$  SEM: 13.19  $\pm$  3.94;  $\alpha$ 2KO,  $n = 17$ , mean DI  $\pm$  SEM: 17.28  $\pm$  4.40; Student's  $t$ -test:  $t_{34} = -0.69$ ,  $p = 0.49$ ). Both groups showed similar total exploration times during testing (WT,  $n = 18$ , mean  $\pm$  SEM: 38.67 s  $\pm$  1.57;  $\alpha$ 2KO,  $n = 17$ , mean  $\pm$  SEM: 34.37 s  $\pm$  2.67; Student's  $t$ -test:  $t_{34} = 1.41$ ,  $p = 0.17$ ). Thus,  $\alpha$ 2KO mice show normal memory for object recognition, and share the same pattern of hippocampus-specific memory impairment as do early-postnatal-nicotine-exposed mice.

Additionally, because we had previously observed that mice exposed to nicotine during development were more anxious than controls, we also used the elevated plus-maze task to test for anxiety.  $\alpha$ 2KO mice spent significantly more time in the open arms of the platform than did WT mice (Fig. 2.1E; WT,  $n = 18$ , mean  $\pm$  SEM: 3.91%  $\pm$  0.5;  $\alpha$ 2KO,  $n = 16$ , mean  $\pm$  SEM: 9.2%  $\pm$  1.03; Student's  $t$ -test:  $t_{33} = -4.77$ ,  $p < 0.001$ ) and less time in the closed arms (WT,  $n = 18$ , mean  $\pm$  SEM: 85.71%  $\pm$  0.92;  $\alpha$ 2KO,  $n = 16$ , mean  $\pm$  SEM: 71.16%  $\pm$  2.65; Student's  $t$ -test:  $t_{33} = 5.44$ ,  $p < 0.001$ ), suggesting that they



**Figure 2.1.**  $\alpha 2$  knockout mice have impaired long-term spatial memory and decreased anxiety. (A) For the hippocampus-dependent object location memory (OLM) task, mice were trained for 10 min with two identical objects, and tested 24 h later with one object moved to a new location. (B)  $\alpha 2$ KO mice ( $n = 17$ ) showed significantly impaired 24-h long-term OLM compared to controls ( $n = 15$ ), and had a discrimination index not significantly different from zero. There were no significant differences between groups in total exploration time during testing. (C) For the hippocampus-independent object recognition memory (ORM) task, mice were trained for 10 min with two identical object, and tested 24 h later after one object was replaced by a novel item. (D) In the ORM task, there was no difference in the 24-h long-term memory demonstrated by  $\alpha 2$ KO mice ( $n = 17$ ) and controls ( $n = 18$ ), and no significant differences between the groups in total exploration time during testing. (E)  $\alpha 2$ KO mice ( $n = 16$ ) spent significantly more time in the open arms of the elevated plus maze than did control mice ( $n = 18$ ), demonstrating decreased anxiety.

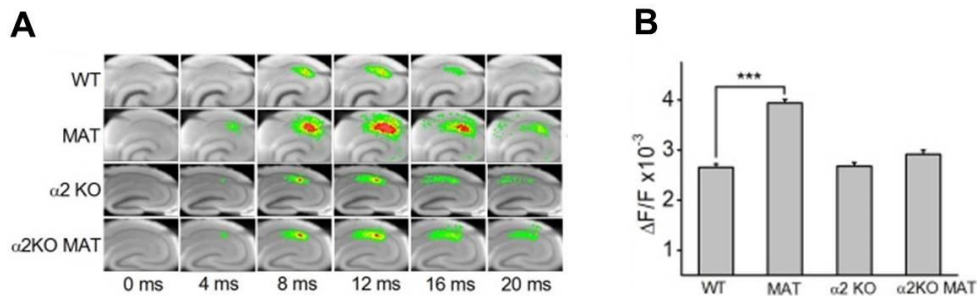


are actually less anxious than controls. This, in combination with the normal exploration times and normal object recognition memory performance of the  $\alpha 2$ KO mice, suggests that their poor performance in the object location memory task is indeed a reflection of impaired long-term, hippocampus-dependent memory.

### *3.2 Maternal-nicotine-treated $\alpha 2$ KO mice lack increased CA1 network activation after Schaffer collateral stimulation*

One of the significant changes that has been observed in rodent hippocampal function following early postnatal nicotine exposure is an increase in CA1 depolarization and hyperpolarization following Schaffer collateral stimulation (Damborsky et al., 2012; Nakauchi et al., 2015; Chen et al., submitted). This could represent a significant restructuring of neuronal networks, caused by the persistent presence of nicotine at a time when nicotinic receptors are important modulators of the strength of newly forming excitatory synapses (Maggi et al., 2003; Maggi et al., 2004). In order to determine whether nicotine-induced activation of  $\alpha 2$ -containing nAChRs during hippocampal development causes this altered CA1 activity, and whether this change may underlie the memory impairments that result from developmental nicotine exposure, we used voltage-sensitive dye imaging to visualize network activation in response to Schaffer collateral stimulation in hippocampal slices from  $\alpha 2$ KO and maternal-nicotine-treated  $\alpha 2$ KO mice.

Stimulation of the Schaffer collateral pathway, the intensity of which was adjusted to evoke similar sizes of fEPSPs across slices, produced optical signal in all anatomical layers, which could be presented as pseudo-colored images of the dF/F signals (Fig.



**Figure 2.2.** Maternal nicotine exposure increases CA1 depolarization from Schaffer collateral stimulation in wild-type, but not  $\alpha 2$  knockout mice. (A) Voltage-sensitive dye imaging detecting changes in neuronal activity following Schaffer collateral stimulation. Representative pseudocolor images of  $dF/F$  signals in saline-treated wild-type (WT), maternal-nicotine-treated wild-type (MAT), saline-treated  $\alpha 2$  KO ( $\alpha 2$  KO) and maternal-nicotine-treated  $\alpha 2$  KO ( $\alpha 2$  KO MAT) mice.  $\alpha 2$  KO MAT mice do not show the nicotine-induced increase in depolarization detected in MAT mice. (B) Peak optical signals were significantly stronger in MAT mice ( $n = 13$ ) than in WT mice ( $n = 9$ ;  $p < 0.001$ ). There was no significant difference between the optical responses recorded from WT and  $\alpha 2$  KO mice ( $n = 6$ ).  $\alpha 2$  KO MAT mice ( $n = 9$ ) showed a trend for a small increase in CA1 depolarization as compared to  $\alpha 2$  KO-SAL mice, but had significantly weaker responses than WT-MATN mice.

2.2A). As we have previously shown (Nakauchi et al., 2015), in hippocampal slices from both wild-type maternal-saline-treated (WT-SAL) and wild-type maternal-nicotine-treated (WT-MATN) mice, optical responses to a single stimulation peaked at 12 ms, but peak signals were significantly stronger in WT-MATN mice than in WT-SAL mice (WT-SAL mice;  $2.65 \pm 0.07$ ,  $n=9$  vs. WT-MATN mice;  $3.93 \pm 0.07$ ,  $n=13$ ;  $p < 0.001$ ; Fig. 2.2 A, B). These observations confirm that maternal nicotine treatment increases depolarizing neuronal activity in response to stimulation of the Schaffer collateral pathway.

We next observed the responses to Schaffer collateral stimulation in hippocampal slices from  $\alpha 2$  KO mice exposed either to maternal saline ( $\alpha 2$  KO-SAL) or nicotine ( $\alpha 2$  KO-MATN). There was no significant difference between the optical responses recorded from WT-SAL and  $\alpha 2$  KO-SAL mice ( $2.68 \pm 0.06$ ,  $n=6$ ; Fig. 2.2 A, B). However,  $\alpha 2$  KO-MATN mice ( $2.92 \pm 0.07$ ,  $n=9$ ), which showed only a trend for a

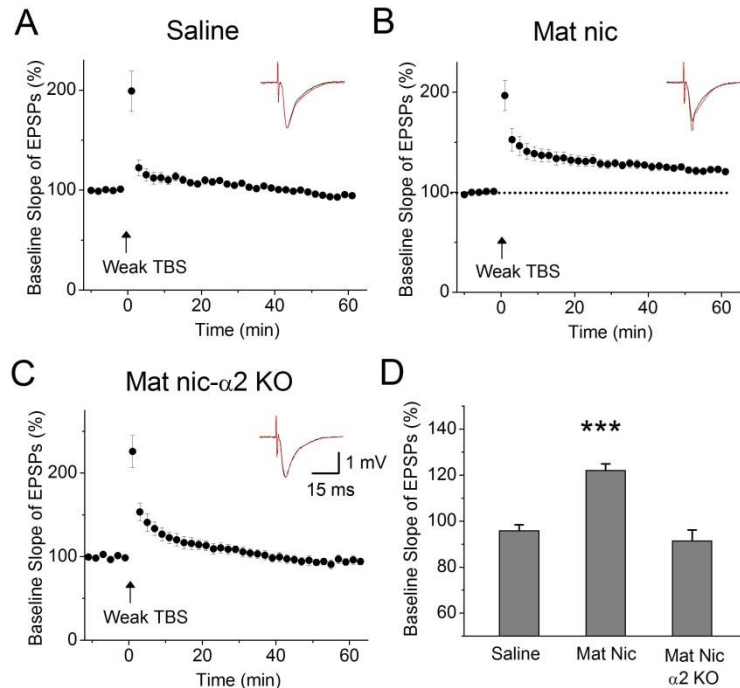
small increase in CA1 depolarization as compared to  $\alpha 2$ KO-SAL mice, had significantly weaker responses than WT-MATN mice.

These findings indicate that it is not the downregulation of  $\alpha 2$ -containing nAChRs that causes increased CA1 network activity in mice exposed to nicotine during development. However, as seen by the near absence of effect in slices from  $\alpha 2$ KO-MATN mice as compared to those from WT-MATN mice, the nicotine-induced activation of  $\alpha 2$ -containing receptors does appear to be necessary to trigger the changes that ultimately do affect CA1 depolarization intensity. Additionally, because both maternal-nicotine-treated wild-type mice and  $\alpha 2$ KO mice have hippocampus-dependent memory impairments, yet maternal-saline- and -nicotine-treated  $\alpha 2$ KO mice have normal responses to Schaffer collateral stimulation, the abnormal CA1 activity observed following early postnatal nicotine exposure is not likely to be the cause of the nicotine-induced memory deficits.

### *3.3 Maternal-nicotine-treated $\alpha 2$ KO mice do not have facilitated induction of LTP*

Because LTP is thought to be one of the cellular mechanisms underlying hippocampal memory, and because we previously found that LTP was not diminished in hippocampal slices from early postnatal nicotine-exposed mice, but was unexpectedly facilitated (Nakauchi et al., 2015), we next investigated whether hippocampal slices from maternal-nicotine-treated  $\alpha 2$ KO mice showed a similar change.

As we have previously shown (Nakauchi et al., 2015), delivery of weak TBS at the Schaffer collateral, which is subthreshold for LTP induction in saline-treated control hippocampi (Fig. 2.3A;  $95.8 \pm 2.6\%$ ,  $n=8$ ), induced LTP in hippocampi from mice that had



**Figure 2.3.** Maternal-nicotine-induced facilitation of LTP occurs in wild-type, but not  $\alpha 2$  knockout mice. (A, D) Weak TBS does not induce LTP in hippocampal slices from maternal-saline-treated control mice ( $n = 8$ ;  $95.8 \pm 2.6\%$ ), but (B, D) does induce LTP maternal-nicotine-treated hippocampi ( $n = 6$ ,  $122.0 \pm 2.9\%$ ,  $p < 0.05$ ). However, (C, D) weak TBS does not induce LTP in maternal-nicotine-treated  $\alpha 2$ KO mice ( $n = 8$ ,  $91.4 \pm 4.7\%$ ).  $***p < 0.001$ .

been exposed to early postnatal nicotine (Fig. 2.3B;  $122.0 \pm 2.9\%$ ,  $n=6$ ,  $p < 0.05$ ). This confirms that maternal nicotine exposure in mice, which impairs CA1-dependent memory, facilitates LTP induction.

We next considered the impact of  $\alpha 2$ -containing nAChRs on this effect of maternal nicotine. Because LTP-inducing Schaffer collateral stimulation does not result in the release of endogenous acetylcholine on OLM cells,  $\alpha 2$  subunit deletion has no effect on LTP induction (Nakauchi et al., 2007). However,  $\alpha 2$ -containing OLM cells do play a role in modulating LTP when activated by bath application of nicotine, and hippocampal slices from  $\alpha 2$ KO mice lack normal nicotine-induced LTP facilitation (Nakauchi et al., 2007). Here we found that, as in hippocampal slices from maternal-saline-treated wild-type mice, weak TBS in slices from maternal-saline-treated  $\alpha 2$ KO

mice did not induce LTP. However, unlike in slices from maternal-nicotine-exposed wild-type mice, weak Schaffer collateral TBS did not induce LTP in maternal-nicotine-exposed  $\alpha 2$ KO hippocampi (Fig. 2.3C;  $91.4 \pm 4.7\%$ ,  $n=8$ ). These findings indicate that maternal-nicotine-induced facilitation of LTP induction is a consequence of inappropriate  $\alpha 2^*$  nAChR activation by early postnatal nicotine exposure, but is not likely the cause of the maternal-nicotine-induced memory impairments. Furthermore, because enhanced CA1 network activation correlates with the facilitation of LTP induction in mice (Nakauchi et al., 2007; Nakauchi and Sumikawa, 2012) and this association of facilitated LTP with enhanced CA1 network activity also occurs maternal-nicotine-exposed wild-type mice (Nakauchi et al., 2015) but not maternal-nicotine-exposed  $\alpha 2$ KO mice, these two changes in hippocampal function may arise from the same cellular mechanism requiring  $\alpha 2^*$  nAChR activation.

#### **4. Discussion**

Recently, work has begun to emerge suggesting that OLM interneurons in the CA1 region of the hippocampus are important modulators of inputs from the entorhinal cortex and CA3, and play a critical role in the formation of contextual memories (Nakauchi et al., 2007; Leao et al., 2012; Lovett-Barron et al., 2014; Chen et al., submitted). OLM cells are unique in the hippocampus in that they express the  $\alpha 2$ -containing nAChR, which, unlike most, if not all nAChRs, does not appear to desensitize and is thus continually activated in the presence of nicotine. We have previously shown that early life nicotine exposure impairs CA1-dependent memory, disrupts the normal role of OLM cells in hippocampal CA1 function and LTP, and decreases the number of

$\alpha$ 2-containing OLM cells (Nakauchi et al., 2015; Chen et al., submitted). Therefore, here we investigated the possibility that downregulation of  $\alpha$ 2-containing nAChRs underlies the memory impairment induced by early life nicotine exposure. We showed that  $\alpha$ 2 knockout mice, like maternal-nicotine-exposed mice, have impaired hippocampus-dependent long-term memory. However, unlike maternal-nicotine-treated wild-type mice, neither maternal-saline-treated nor maternal-nicotine-treated  $\alpha$ 2 knockout mice showed unusually high CA1 depolarization or facilitated LTP following Schaffer collateral stimulation. Our findings suggest that  $\alpha$ 2-containing nAChRs play a critical role in normal hippocampus-dependent memory, and that their downregulation could be the cause of memory impairments following developmental nicotine exposure. Furthermore, our findings indicate that the activation of  $\alpha$ 2-containing nAChRs by early postnatal nicotine exposure also results in other changes in hippocampal function that are not likely related to nicotine-induced memory impairments.

In part because of their scattered and sparse expression,  $\alpha$ 2-containing nAChRs have not been as well-studied as many other nicotinic receptor types, and much remains to be learned about them in order to fully understand their function in the hippocampus or elsewhere in the brain. For example, it is not known which other subunits combine with  $\alpha$ 2 to form functional nAChRs in hippocampal OLM cells, or where they are located within the interneurons, or their precise properties.  $\alpha$ 2 subunits are able to form functional receptors when co-expressed with  $\beta$ 2,  $\beta$ 3 or  $\beta$ 4 subunits (Sargent, 1993; McGehee and Role, 1995), but, whereas they co-precipitate mostly with  $\beta$ 2 in the interpeduncular nuclei, they are found mostly with  $\beta$ 4 in the olfactory bulbs (Whiteaker et al., 2009), and such differences in receptor composition could have

significant impacts on their function. As well as being strongly expressed in the interpeduncular nuclei and olfactory bulbs,  $\alpha 2$  nAChR mRNA is more sparsely expressed in scattered other regions including the septum, cortical layers V and VI, and the medial nucleus of the amygdala in rodents (Wada et al., 1989; Ishii et al., 2005). However, its distribution is greater in the rhesus monkey brain (Han et al., 2000), suggesting that  $\alpha 2$ -containing nAChRs may play an even more important role in primates.

One interesting possibility is that the scattered expression of  $\alpha 2$  underlies the uneven influence of developmental nicotine exposure on the brain. We have previously shown that early postnatal nicotine treatment in mice impairs long-term hippocampus-dependent object location memory, but does not affect hippocampus-independent object recognition memory (Nakauchi et al., 2015). This was somewhat surprising given the global expression of nAChRs in the brain, including in the perirhinal cortex, though others have also found evidence that the hippocampus is particularly sensitive to the effects of chronic nicotine (Kenney and Gould, 2008). However, it remains to be understood why the hippocampus is so vulnerable. Thus, it is significant that this study found that mice lacking the  $\alpha 2$  nAChR subunit show the same pattern of memory impairment and preservation as do early-nicotine-exposed mice. Notably, whereas  $\alpha 2$  mRNA is found in the CA1 region of the hippocampus, the area critical for object location memory, it does not occur in the perirhinal cortex (Wada et al., 1989; Ishii et al., 2005), which mediates object recognition memory (Norman & Eacott, 2004; Moses et al., 2005). The relative scarcity of  $\alpha 2$  in the brain may also explain why others have observed only limited differences in the behavior of  $\alpha 2$  knockout mice compared to wild-

type mice, although  $\alpha 2$  knockout mice did show increased nicotine withdrawal symptoms (Loftipour et al., 2013). Interestingly, nicotine withdrawal symptoms are normally mediated by the interpeduncular nucleus (Zhao-Shea et al., 2013), the region of greatest  $\alpha 2$  mRNA expression in the rodent brain (Wada et al., 1989; Ishii et al., 2005). It is possible, therefore, that  $\alpha 2$ -containing nAChRs play an outsized role in mediating the effects of developmental and long-term exposure to nicotine.

However, given the complexity of nicotine's action on the brain,  $\alpha 2$ -containing nAChRs are likely not the only nicotinic receptor subtype involved in mediating the memory impairment induced by early postnatal nicotine exposure. Several other nAChR subtypes are abundantly located on both interneurons and projection neurons in the hippocampus, and there is evidence that some of those subtypes are also affected by developmental nicotine exposure. Early postnatal nicotine treatment in rodents increases hippocampal binding of epibatidine (Huang and Winzer-Serhan, 2006; Huang et al., 2007a), an nAChR ligand that binds strongly to  $\alpha 4\beta 2$  (Sullivan and Bannon, 1996), and nicotine also may decrease binding of low-affinity nAChRs (Nordberg et al., 1991), which include  $\alpha 7$  nAChRs, the most common nicotinic receptor subtype in the hippocampus. Furthermore,  $\alpha 2$ -containing nAChRs are not the only nAChR subtype mediating OLM cell activity or TA pathway inhibition – it has also been shown that the activation of OLM cells by fimbria fornix pathway stimulation is partially mediated by  $\alpha 7$  nAChRs (Leao et al., 2012). However, both  $\alpha 7$  knockout mice and  $\beta 2$  knockout mice (which would lack the second-most abundant hippocampal nAChR subtype,  $\alpha 4\beta 2$ ) show normal memory in the hippocampus-dependent Morris water maze task (Zoli et al., 1999; Paylor et al., 1998). It must be noted, though, that as with all studies using



knockout animals, including this one, the impact of confounding compensatory effects cannot be ruled out. Therefore, though this work strongly suggests that  $\alpha 2$ -containing nAChRs play a critical role in the hippocampal memory impairments induced by early life nicotine exposure, it remains possible that other nAChR subtypes are also involved.

One behavioral difference that we did observe between  $\alpha 2$  knockout mice and our previous findings with maternal-nicotine-treated mice was that the knockout mice, unlike the nicotine-treated mice, did not show increased anxiety. In fact,  $\alpha 2$  knockout mice seemed less anxious than wild-type mice, though it should be noted that others using this mouse line did not observe any difference in anxiety compared to controls (Loftipour et al., 2013). Regardless, this data suggests that  $\alpha 2$ -containing nAChRs do not mediate the increased anxiety observed following perinatal nicotine exposure in rodents (Vaglenova et al., 2004; Nakauchi et al., 2015). Increased anxiety could be the result of many factors, including altered maternal behavior during rearing. Though for this behavior study we fostered both the wild-type and  $\alpha 2$  knockout pups with CD1 dams to ensure consistent maternal care, the increased anxiety observed in maternal-nicotine-treated mice may have been due to changes in the dams' conduct while they were exposed to, and eventually withdrawn from, nicotine (Vagelnova et al., 2004).

There are still a great many questions that remain about the effects of developmental nicotine exposure and the role of  $\alpha 2$ -containing nAChRs. Although downregulation of  $\alpha 2$  is a likely cause of the hippocampal memory impairments induced by developmental nicotine exposure, it still needs to be determined whether it is the absence of  $\alpha 2$  during hippocampal development, when neuronal networks are being established (Zancanaro et al., 2001; Danglot et al., 2006; de Graaf-Peters and Hadders-

Algra, 2006; Dwyer et al., 2009), that affects the later ability to form spatial memories, or if it is the lack of  $\alpha 2$  at the time of memory formation itself that is important. Furthermore, though we showed that the activation of  $\alpha 2$ -containing nAChRs is required for early-postnatal-nicotine-induced facilitation of LTP and for heightened CA1 network activity, these changes in hippocampal function are not ultimately due to  $\alpha 2$  downregulation, and the mechanism underlying them remains to be understood. This study is thus an important first step in understanding the significant role that  $\alpha 2$ -containing nAChRs play in hippocampal memory, and that their absence plays in the long-term impacts of early life nicotine exposure.

## CHAPTER 3. MOLECULAR AND BEHAVIORAL IMPACTS OF A MODEL OF DEVELOPMENTAL NICOTINE EXPOSURE IN RATS

### 1. Rationale

Although we and several others have used various models of early life nicotine exposure to show that it can cause severe learning and memory deficits, some, using different species, behavior tasks, or methods or timing of drug administration, have not found such clear nicotine-induced impairments (Table 3.1). It remains unclear what the critical factor or factors driving these different outcomes are, thus it is important to consider whether the hippocampal memory impairment we observed after two weeks of postnatal nicotine exposure in mice also occurs in other species.

One of the difficulties of comparing the effects of nicotine across species is that their rates of nicotine metabolism can vary greatly. Whereas in mice, the plasma nicotine half-life is 6-9 minutes, in rats it is 20-45 minutes, and in humans, 2 hours (Matta et al., 2007; Siu and Tyndale, 2006; Sastry et al., 1995; Benowitz et al., 1982). However, we have previously shown that a rat model of early nicotine exposure similar to what we used in mice, with pups receiving nicotine via maternal milk for their first two postnatal weeks, though at a lower concentration than in mice to account for rats' slower nicotine metabolism, causes some of the same changes in hippocampal function in both species. As in mice, this model of early postnatal nicotine in rats yields facilitated LTP induction and increased CA1 depolarization after Schaffer collateral stimulation, and impaired nicotinic modulation of both LTP and CA1 network activity (Nakauchi et al., 2015; Chen et al., submitted). Additionally, in the CA1 region of nicotine-treated rats,

we observed fewer OLM cells expressing  $\alpha 2$  subunit mRNA, and depressed OLM cell activity (Chen et al., submitted), which, as explored in Chapter 2, is associated with significant hippocampal memory impairments. Thus, we expect that that we will observe the same memory impairments in rats following early postnatal nicotine exposure as we have previously found in mice.

Though we have already demonstrated that early developmental nicotine exposure leads to significant changes in the nicotinic modulation of hippocampal CA1 function, in order to fully understand the impact of nicotine on memory it is also important to consider whether it affects hippocampal NMDA receptors, which play a

**Table 3.1.** Summary of published reports of the effects of perinatal nicotine on learning and memory

<b>Study</b>	<b>Model of perinatal nicotine exposure</b>	<b>Impact of nicotine on memory</b>
Peters and Ngan, 1982	M&F Fischer 344 rats treated all gestation via 1.5 or 3 mg/kg/day chronic administration to dam	Impairs shock avoidance in M&F and food maze in F in adults
Sorenson et al., 1991	M&F SD rats treated all gestation via 6 mg/kg/day to dam via drinking water	Impairs 8-RAM at P30
Yanai et al., 1992	M&F HS/lbg mice treated G9-G18 via 1.5 mg/kg twice-daily SC injection to dam	Impairs 8-RAM at P50
Yanai et al., 1992	M&F HS/lbg mice treated P2-P21 via 1.5 mg/kg daily SC injection to pups	Impairs 8-RAM at P50
Levin et al., 1993b	M&F SD rats treated G4-G21 via 2 mg/kg/day to dam via minipump	Minor impairments in 8-RAM at P60
Levin et al., 1996b	M&F SD rats treated G4-G20 via 2 mg/kg/day to dam via minipump	Impairs T-maze in M, subtle impairments in 8-RAM at P50
Cutler et al., 1996	M&F LE rats treated G4-G20 via 4 mg/kg/day to dam via minipump	No effect on WM or 8-RAM at P37-P73
Ankarberg et al., 2001	M NMRI mice treated P10-P15 via 0.0033, 0.033 or 0.066 mg/kg twice-daily SC inj. to pups	No effect on WM or 8-RAM at P120, impairs both at P210 with highest dose
Vaglenova et al., 2004	M&F SD rats treated G3-P0 via 6 mg/kg/day to dam by minipump	Impairs active avoidance at P25-P40
Eppolito and Smith, 2006	M&F LE rats treated G4-P11 via 0.96 mg/kg/day to dam by minipump	Slightly impairs Morris WM in F at P60
Huang et al., 2007b	M&F SD rats treated P1-P7 via 2 mg/kg thrice-daily to pups by gastric intubation	No effect on Morris WM at P60 or T-maze at P90
Nakauchi et al., 2015	M&F C57Bl/6 mice treated P1-P14 via 21 mg/kg/day to dam by minipump	Impaired LTM but not STM for OLM, no effect on ORM at P45-P46

Abbreviations: M = male, F = female, SD = Sprague Dawley, SC = subcutaneous, G = gestational, P = postnatal, 8-RAM = 8-arm radial arm maze, WM = water maze, STM = short-term memory, LTM = long-term memory, OLM = object location memory, ORM = object recognition memory.

critical role in some forms of hippocampal synaptic plasticity, and are strongly associated with hippocampal memory formation (Sakimura et al., 1995; Tang et al., 1999; reviewed in: Nakazawa et al., 2004).

NMDARs are composed of two NR1 subunits, and two modulatory NR2 or NR3 subunits (Kohr, 2006). There are several types of NR2 and NR3 subunits, but hippocampal NMDARs almost exclusively contain either NR2A, or NR2B (Monyer et al., 1994; Wenzel et al., 1997). Because NR2A- and NR2B-containing NMDARs have different  $\text{Ca}^{2+}$  permeability, and trigger different downstream signaling cascades, their relative abundance at the synapse is thought to be an important factor controlling synaptic plasticity (Yashiro and Philpot, 2008).

At nearly the same time as early postnatal nicotine exposure appears to have its most potent effect (Miao et al., 1998; Eriksson et al., 2000), NMDARs in many regions of the brain, including in the hippocampus, are normally experiencing a major shift in subunit composition (Moyner et al., 1994; Sheng et al., 1994; Wenzel et al., 1997; Liu et al., 2004), with significant impacts on the establishment of hippocampal networks. Before this period in brain development, the most abundant NMDARs are those containing NR2B, which are highly  $\text{Ca}^{2+}$  permeable and bind strongly to  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII), the  $\text{Ca}^{2+}$ -triggered autophosphorylating enzyme essential for hippocampal LTP and hippocampus-dependent learning (reviewed in: Hell, 2014). However, during the first three postnatal weeks, as networks and synaptic connections become more established, levels of NR2A begin to climb, surpassing NR2B. This shift in the NR2A/NR2B ratio is associated with the closing of the critical period of sensory development (Quinlan et al., 1999; Chen

et al., 2000; Philpot et al., 2001), and causes a significant decrease in CaMKII binding, and a resulting reduction in synaptic plasticity (Barria and Malinow, 2005). Thus, were nicotine exposure to affect NR2A or NR2B expression in the hippocampus during this period, it could have a significant impact on the normal establishment of hippocampal network connectivity.

Others have shown that prolonged nicotine exposure does affect NMDAR subunit expression in some regions of the brain. In the auditory system, which is impaired in children born to mothers who smoked during pregnancy (Picone et al., 1982; Saxton, 1978), early postnatal nicotine exposure in rats increases NR2A mRNA expression in the auditory cortex and decreases NR2B expression in the thalamus (Aramarkis and Metherate, 1998; Aramarkis et al., 2000; Hseih et al., 2002) – both changes that might precipitate an increase in the NR2A/NR2B ratio and thus a decrease in synaptic plasticity. In the hippocampus, chronic nicotine treatment in adult rats upregulates hippocampal NMDAR binding (Levin et al., 2005) and increases CA1 excitatory postsynaptic current mediated by NR2B-containing NMDARs (Yamazaki et al., 2006a). Additionally, adult chronic nicotine exposure has been shown to alter binding between NR2B subunits and the kinase Src (Yamazaki et al., 2006b), the primary mediator of tyrosine phosphate binding in NMDARs. The interaction between NMDARs and Src family kinases, particularly Src and Fyn, can affect the receptors' localization and function (Kohr and Seeburg, 1996; Lavezzari et al., 2003; Prybylowski et al., 2005; Goebel et al., 2005; Zhang et al., 2008; Sinai et al., 2010), and is crucial for certain forms of synaptic plasticity (Salter and Kalia, 2004). However, it remains to be

determined whether nicotine exposure during development causes any such effects on hippocampal NMDARs.

Thus, in this study we will first test our model of early postnatal nicotine exposure in rats to determine whether it affects hippocampal memory. Additionally, we will take advantage of rats' larger brain size to selectively harvest hippocampal CA1 tissue in order to study whether early life nicotine exposure alters NR2A or NR2B protein levels, or the protein levels of two kinases that are critical to their function, Src and Fyn.

## **2. Methods**

### *2.1. Animals and nicotine treatment*

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with protocols approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. Efforts were made to minimize the number of animals used.

On postnatal day 1 (P1), Sprague Dawley rat litters were adjusted to eight male and female pups, and dams were subcutaneously implanted with osmotic minipumps (Alzet model 2ML2, Durect Corporation) delivering either saline solution for controls, or approximately 6 mg/kg/day of nicotine (Sigma) in saline solution. Thus, nicotine-treated pups were exposed to nicotine through maternal milk from P1-P15, at which time the minipumps were removed. Here, we refer to these pups as maternal-nicotine-exposed (MN) rats. Others using this model have reported that rat pup blood nicotine levels on P8-10 were  $23.9 \pm 3.5$  ng/mL (Chen et al., 2005), similar to blood nicotine levels

achieved by pregnant women who were moderate smokers (15-45 ng/mL; Benowitz and Jacob, 1984).

## *2.2 Morris Water Maze*

Beginning at P49, maternal-nicotine- and -saline-treated male rats were handled 2 minutes per day for 5 days. Water maze testing was conducted in a quiet, dimly lit room. The rats were moved into the behavior room to habituate for about 1 h each day before training began. All training was conducted in a large circular tank filled with water at 24 °C ( $\pm$  2) until the platform, which was a clear plastic disc approximately 15 cm in diameter, was submerged by 2 cm and thus not visible from the surface.

For the spatial water maze task, which began at P54, the platform was located at a fixed location through all the trials. Each animal was trained daily in two rounds of four trials each, with the rounds separated by about 1 h, for a total of three days. In each trial, the rat was released into the water facing the tank wall at one of five semi-randomly selected start points, such that it never began at the same point twice in a row. The rat was given 60 sec to find the submerged platform, and the time it took to reach the target was recorded. Rats unable to find the platform on their own were manually guided toward the target and allowed to sit atop it for 10 sec before being removed. Trials were separated by a 20 sec inter-trial interval. After each round of training, animals were temporarily moved to heated cages before being returned to their home cage. On the second, third and fourth days of training, each rat was given a probe test before the spatial memory or reversal memory (see below) tasks began. For the probe test, the platform was removed, and the animals were released from one of five



randomly selected locations in the tank and allowed to swim for 60 seconds while their path was being recorded.

For the spatial memory reversal task, after the spatial water maze training described above, the submerged platform was moved to a new location. On this day, at P57, rats were trained for 6 trials of up to 60 seconds each. For each trial, the rat was released into the water facing the tank wall at one of five semi-randomly selected start points, such that it never began at the same point twice in a row. The time to reach the platform was recorded, and rats unable to find the platform after 60 seconds were manually guided to it and allowed to sit atop it for 10 seconds. Trials were separated by a 20 second inter-trial interval.

The cued water maze task, at P58, followed the previous four days of training described above. For this task, the platform was marked by a green tennis ball on the surface of the water above it. Each rat was trained in two rounds of four trials per round, with the second round beginning about 1 hour after the first. For every trial, both the platform location and rat starting point were semi-randomly selected such that neither the rat's starting point nor the platform location was the same twice in a row. Each trial lasted 60 seconds, and the rat's time to reach the platform was recorded. Rats unable to find the platform after 60 seconds were manually guided to it before being removed. Trials were separated by a 20 second inter-trial interval, and after each round, rats were temporarily placed in a heated cage before being returned to their home cage.

### *2.3 Western blotting*

Rats were anesthetized with isoflourane and immediately sacrificed. Their brains were quickly removed and chilled in ice-cold oxygenated sucrose-artificial cerebral spinal fluid containing (in mM): NaCl, 425; KCl, 12.5; sucrose, 375; MgSO<sub>4</sub>, 20; CaCl<sub>2</sub>, 2.5; NaH<sub>2</sub>PO<sub>4</sub>, 6.25; dextrose, 125; NaHCO<sub>3</sub>, 120. Once each brain had chilled for two minutes, their hippocampi were microdissected to remove the CA1 region, which was flash-frozen in liquid nitrogen before being stored at -80°C until later use.

Frozen CA1 samples were briefly processed with a polytron homogenizer in an ice-cold lysis solution (10 mM Tris/HCl, 320 mM sucrose, protease and phosphatase inhibitor cocktail tablets (Roche); pH 7.4). 10 µg of total protein lysate from each sample was run on a 4-12% Bis-Tris gradient gel (Life Technologies) at 200 V for 50 minutes, then transferred onto a non-autofluorescing PVDF membrane (Millipore) at 25 V for 1 hour. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% w/v nonfat milk, before being probed overnight at 4°C with mouse antibody to NR2A (1:2,000; Millipore), or rabbit antibody to NR2B (1:2,000; Millipore) or β-tubulin (1:80,000; Millipore). Membranes were washed and probed for 1 hour at room temperature with either HRP-conjugated donkey antibody to mouse (1:5,000; Jackson) or fluorescent goat antibody to rabbit (1:2000 Alexa Fluor 594 or 488; Invitrogen). Membranes were with HRP secondary antibodies were developed using Pierce SuperSignal West Pico Chemiluminescent Substrate (Pierce), and chemiluminescent and fluorescence detection was conducted with ImageStation 4000MM Pro (Carestream). Band intensities were measured using Carestream Molecular Imaging software (Carestream). There were between 6 and 9 samples tested

for each treatment group at each timepoint. NR2A, NR2B, Src and Fyn values were normalized against  $\beta$ -tubulin intensities.

## 2.4 Statistical analysis

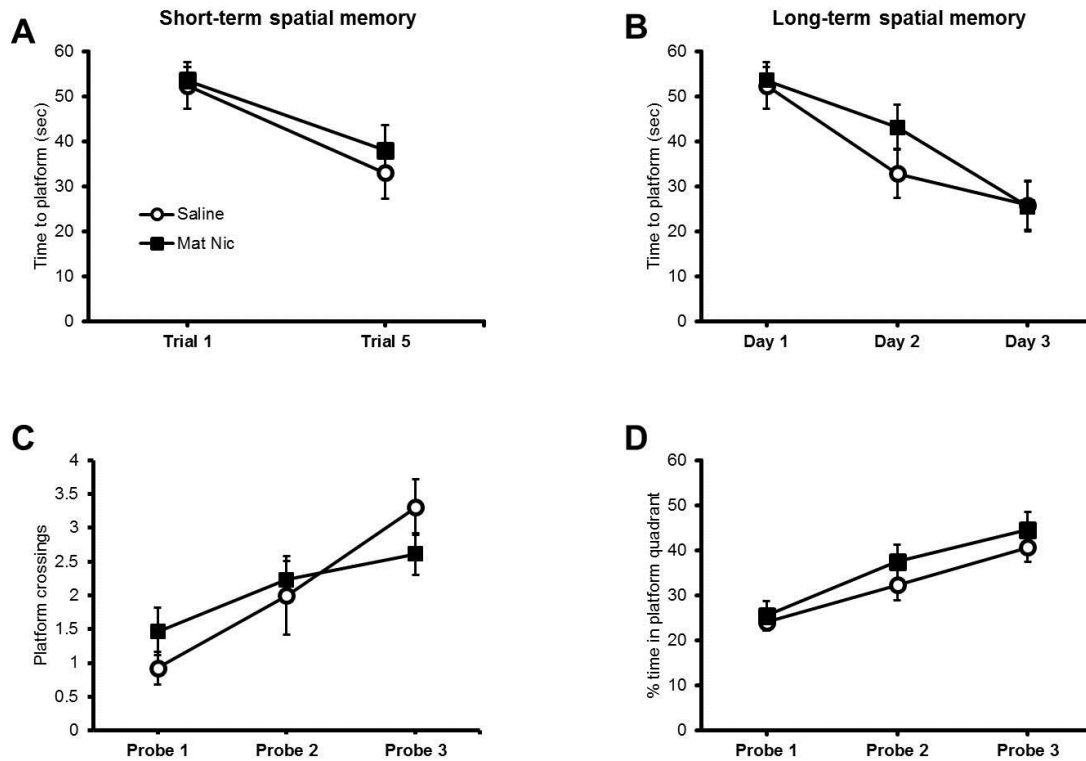
Morris water maze behavior data was analyzed using two-way ANOVAs with repeated measures on one factor. Because male and female rats yielded similar results in the protein quantification experiments, they were combined for statistical analysis. Western blot data was expressed relative to saline controls, and analyzed using two-way ANOVAs. In all graphs,  $p$  values are depicted as follows:  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

## 3. Results

### 3.1 Early postnatal nicotine exposure does not affect spatial, cued or reversal memory in the Morris water maze task

In order to determine the impact of early postnatal nicotine exposure on memory in rats, we first tested maternal-nicotine- and -saline-treated rats using the hippocampus-dependent Morris water maze spatial memory task (Morris et al., 1982; Logue et al., 1997). Rats exposed to maternal nicotine ( $n=13$ ) showed no change in short-term memory compared to saline-treated controls ( $n=13$ ), as shown by their latency to reach the hidden platform at the start of their second round of training during the first day, approximately one hour after their initial training trial (Fig. 3.1A; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,24)} = 0.39$ ,  $p = 0.54$ , significant effect of Trial  $F_{(1, 24)} = 12.33$ ,  $p = 0.002$ , no interaction  $F_{(1, 24)} = 0.15$ ,  $p = 0.70$ ). Likewise,

as

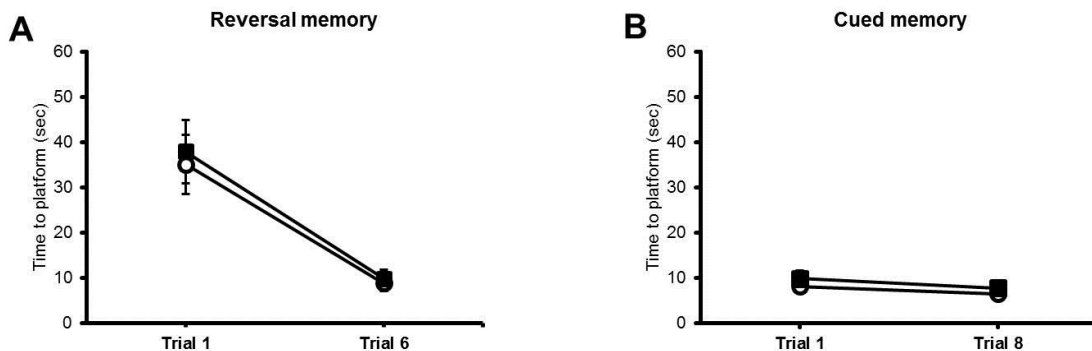


**Figure 3.1.** Maternal-nicotine-treated rats do not show any changes in spatial memory during the Morris water maze task. (A) MN rats ( $n = 13$ ) did not show any difference in spatial short-term memory compared to saline-treated controls ( $n = 13$ ), as demonstrated by their time to platform during their fifth training trial, approximately 1 hour after their initial training round (two-way repeated measures ANOVA: no effect of treatment  $F_{(1,24)} = 0.39$ ,  $p = 0.54$ , significant effect of trial  $F_{(1, 24)} = 12.33$ ,  $p = 0.002$ , no interaction  $F_{(1, 24)} = 0.15$ ,  $p = 0.70$ ). (B) MN rats ( $n = 13$ ) did not show any difference in spatial long-term memory compared to saline-treated controls ( $n = 13$ ), as measured by their time to platform during the first trial of each of the three spatial memory training days (two-way repeated measures ANOVA: no effect of treatment  $F_{(1,48)} = 0.66$ ,  $p = 0.43$ , significant effect of day  $F_{(2,48)} = 17.08$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 0.75$ ,  $p = 0.48$ ). (C-D) In the probe test, another measure of long-term spatial memory in which the platform is removed, MN rats ( $n = 13$ ) showed no difference from controls ( $n = 13$ ) in either (C) their number of platform crossings (two-way repeated measures ANOVA: no effect of treatment  $F_{(1,48)} = 0.00$ ,  $p = 1.00$ , significant effect of day  $F_{(2,48)} = 14.14$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 1.85$ ,  $p = 0.17$ ), or (D) the percentage of time they spent in the platform quadrant (two-way repeated measures ANOVA: no effect of treatment  $F_{(1,48)} = 0.98$ ,  $p = 0.33$ , significant effect of day  $F_{(2,48)} = 14.62$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 0.19$ ,  $p = 0.83$ ).

indicated by the latency to platform for the first training trial of each day, there was no difference in long-term spatial memory between the groups (Fig. 3.1B; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,48)} = 0.66$ ,  $p = 0.43$ , significant effect of Day  $F_{(2,48)} = 17.08$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 0.75$ ,  $p = 0.48$ ). The absence of any change in long-term memory following early postnatal nicotine exposure

was also observed during the probe tests in which the platform was removed. Nicotine-treated rats showed no difference compared to saline-treated rats in either their number of platform crossings (Fig. 3.1C; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,48)} = 0.00$ ,  $p = 1.00$ , significant effect of Day  $F_{(2,48)} = 14.14$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 1.85$ ,  $p = 0.17$ ), or in the percentage of time they spent in the platform quadrant (Fig. 3.1D; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,48)} = 0.98$ ,  $p = 0.33$ , significant effect of Day  $F_{(2,48)} = 14.62$ ,  $p < 0.0001$ , no interaction  $F_{(2,48)} = 0.19$ ,  $p = 0.83$ ). Together, these results show that maternal nicotine treatment does not affect either short-term or long-term hippocampus-dependent spatial learning.

We next considered whether developmental exposure to nicotine affected memory in other ways. After the spatial memory training was complete, we used a spatial memory reversal task in order to assess the rats' flexibility in learning new

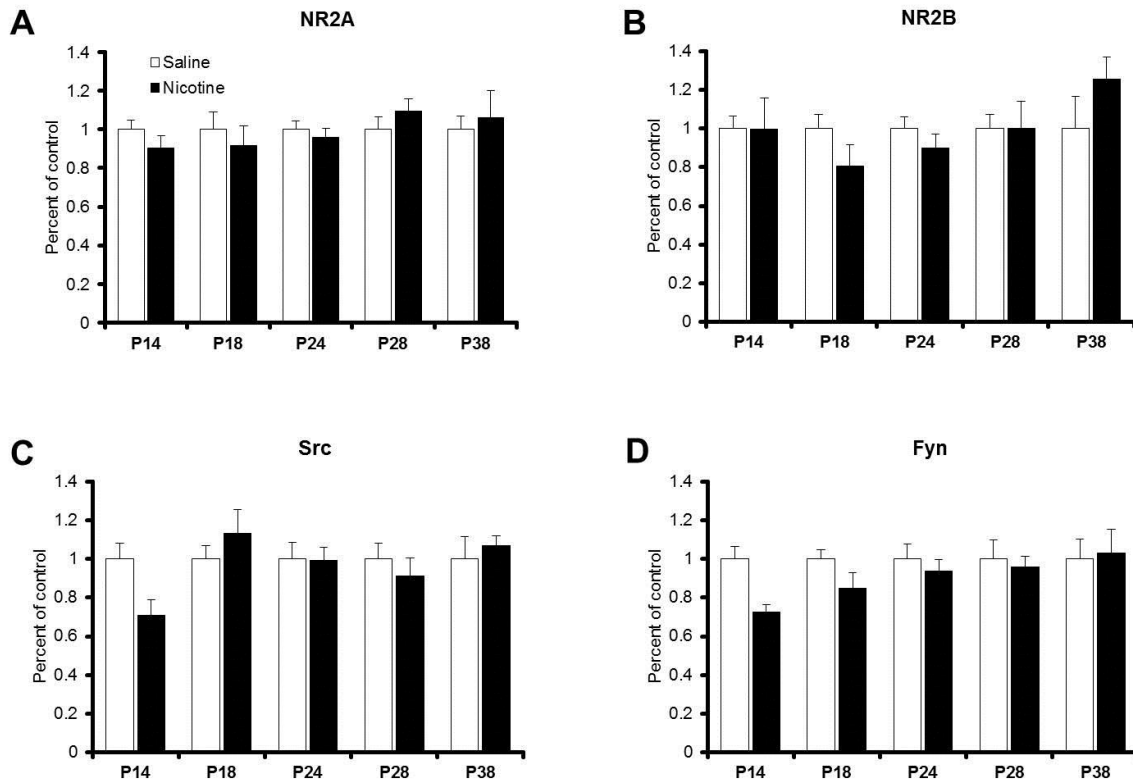


**Figure 3.2.** Maternal-nicotine-treated rats do not show any changes in reversal or cued memory during the Morris water maze task. (A) After three days of spatial memory training, the hidden platform was moved to a new location and rats were tested for their latency to reach the platform over the course of six trials. MN rats ( $n = 13$ ) demonstrated the same reversal memory as did saline-treated rats, as measured by their time to platform at the first and last trials ( $n = 13$ ; two-way repeated measures ANOVA: no effect of treatment  $F_{(1,24)} = 0.14$ ,  $p = 0.71$ , significant effect of trial  $F_{(1,24)} = 30.9$ ,  $p < 0.0001$ , no interaction  $F_{(1,24)} = 0.04$ ,  $p = 0.84$ ). (B) MN rats ( $n = 13$ ) also showed no difference from controls ( $n = 13$ ) in cued memory when the platform was made visible and moved between trials, as measured at by their platform latency at the beginning and end of eight training trials (two-way repeated measures ANOVA: no effect of treatment  $F_{(1,24)} = 0.14$ ,  $p = 0.71$ , significant effect of trial  $F_{(1,24)} = 30.9$ ,  $p < 0.0001$ , no interaction  $F_{(1,24)} = 0.04$ ,  $p = 0.84$ ).

information. We found that maternal-nicotine-treated rats showed no difference in spatial reversal learning relative to controls, as determined by comparing their latency to reach the platform in the first and last training trials (Fig. 3.2A; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,24)} = 0.14$ ,  $p = 0.71$ , significant effect of Trial  $F_{(1, 24)} = 30.9$ ,  $p < 0.0001$ , no interaction  $F_{(1,24)} = 0.04$ ,  $p = 0.84$ ). Additionally, we tested both groups of rats in a cued memory task, and also found no difference between the groups when comparing their time to platform in the first and final training trials (Fig. 3.2B; two-way repeated measures ANOVA: no effect of Treatment  $F_{(1,24)} = 0.14$ ,  $p = 0.71$ , significant effect of Trial  $F_{(1,24)} = 30.9$ ,  $p < 0.0001$ , no interaction  $F_{(1,24)} = 0.04$ ,  $p = 0.84$ ). Thus, maternal nicotine exposure has no apparent effect on either hippocampus-dependent spatial memory or hippocampus-independent cued memory in rats during their late adolescence.

### *3.2 Early postnatal nicotine exposure may alter CA1 protein levels of Fyn, but not Src or the NMDAR subunits NR2A and NR2B*

In order to determine the effect of early postnatal nicotine on NMDARs in the CA1 region of the hippocampus, we investigated whether maternal nicotine exposure altered NR2A or NR2B subunit protein levels at five time points during development. We found no difference between the saline- and nicotine-treated groups for either NR2A (Fig. 3.3A; two-way ANOVA: no effect of Treatment  $F_{(1,62)} = 0.09$ ,  $p = 0.76$ , no effect of Day  $F_{(4,62)} = 0.62$ ,  $p = 0.65$ , no interaction  $F_{(4,62)} = 0.59$ ,  $p = 0.67$ ), or NR2B (Fig. 3.3B; two-way ANOVA: no effect of Treatment  $F_{(1,64)} = 0.02$ ,  $p = 0.87$ , no effect of Day  $F_{(4,64)} = 1.26$ ,  $p = 0.29$ , no interaction  $F_{(4,64)} = 1.15$ ,  $p = 0.34$ ) protein expression.



**Figure 3.3.** Maternal nicotine exposure in rats does not affect total protein levels of NR2A, NR2B or Src in the CA1, but may alter levels of Fyn. (A-D) Quantification of Western blot for levels of NMDAR-associated proteins in hippocampal CA1 tissue;  $n$  = between 6 and 9 for each measure. Maternal nicotine treatment did not alter protein levels in the CA1 of (A) NR2A (two-way ANOVA: no effect of treatment  $F(1,62) = 0.09$ ,  $p = 0.76$ , no effect of day  $F(4,62) = 0.62$ ,  $p = 0.65$ , no interaction  $F(4,62) = 0.59$ ,  $p = 0.67$ ), (B) NR2B (two-way ANOVA: no effect of treatment  $F(1,64) = 0.02$ ,  $p = 0.87$ , no effect of day  $F(4,64) = 1.26$ ,  $p = 0.29$ , no interaction  $F(4,64) = 1.15$ ,  $p = 0.34$ ) or (C) Src (two-way ANOVA: no effect of treatment  $F(1,64) = 0.54$ ,  $p = 0.47$ , no effect of day  $F(4,64) = 1.95$ ,  $p = 0.11$ , no interaction  $F(4,64) = 1.91$ ,  $p = 0.12$ ). However, (D) there was a main effect of nicotine, but not an interaction effect, on Fyn expression (two-way ANOVA: main effect of treatment  $F(1,64) = 4.21$ ,  $p = 0.04$ , no effect of day  $F(4,64) = 1.10$ ,  $p = 0.37$ , no interaction  $F(4,64) = 1.09$ ,  $p = 0.37$ ). The greatest mean difference we observed in Fyn expression was at the earliest time point we measured, P14 (Saline,  $n = 8$ ; Mat Nic,  $n=8$ ).

Additionally, we investigated whether maternal nicotine exposure affected protein levels of two Src family kinases that alter NMDAR function by binding to NR2A or NR2B. We observed no difference between nicotine- and saline-treated rats in CA1 Src protein levels (Fig. 3.3C; two-way ANOVA: no effect of Treatment  $F_{(1,64)} = 0.54$ ,  $p = 0.47$ , no effect of Day  $F_{(4,64)} = 1.95$ ,  $p = 0.11$ , no interaction  $F_{(4,64)} = 1.91$ ,  $p = 0.12$ ). However, we found weak evidence for a main effect of nicotine, but not an interaction

effect, on Fyn expression (Fig. 3.3D; two-way ANOVA: main effect of Treatment  $F_{(1,64)} = 4.21$ ,  $p = 0.04$ , no effect of Day  $F_{(4,64)} = 1.10$ ,  $p = 0.37$ , no interaction  $F_{(4,64)} = 1.09$ ,  $p = 0.37$ ). The greatest mean difference we observed in Fyn expression was at the earliest time point we measured, P14 (Saline,  $n = 8$ , mean  $\pm$  SEM:  $1 \pm 0.07$ ; Mat Nic,  $n=8$ , mean  $\pm$  SEM:  $0.73 \pm 0.04$ ). Thus, although maternal nicotine exposure in rats does not appear to affect CA1 protein levels for the NMDAR subunits NR2A or NR2B, or for the kinase Src, it may alter expression of the Src family kinase Fyn, particularly at early time points.

#### **4. Discussion**

Because maternal nicotine treatment in rats causes several of the same changes in hippocampal function as in maternal-nicotine-treated mice, which have impaired hippocampus-dependent spatial memory (Nakauchi et al., 2015; Chen et al., submitted), we strongly expected that nicotine-treated rats would also show impaired spatial memory. However, this study found that rats exposed to our model of early postnatal nicotine exposure have normal performance in Morris water maze tasks testing short-term and long-term spatial memory, as well as in those testing cued and reversal memory.

It is difficult to interpret the significance of these negative results, as they might represent a lack of effect of our model of developmental nicotine exposure on spatial memory in rats, a lack of effect of any form of developmental nicotine exposure on spatial memory in rats, or an inability to detect the effect of nicotine with this task. Because several research groups have previously reported that perinatal nicotine exposure in rats causes long-lasting deficits in spatial memory (Sorenson et al., 1991;



Levin et al., 1993b; Levin et al., 1996b; Vagelnova et al., 2004; Eppolito and Smith, 2006; Huang et al., 2007b), it is highly unlikely that our results reflect a complete inability of nicotine to cause hippocampal memory deficits. However, as reflected in the range of behavioral outcomes that have been observed using various models of perinatal nicotine exposure and various forms of testing (Table 1), there are many other possible factors that may have influenced our results. The rats' age during testing may be important – whereas normal radial and Morris water maze performance was observed in 4-month-old mice exposed to 5 days of postnatal nicotine, the same mice showed impaired memory relative to controls when tested again three months later (Ankarberg et al., 2001). Alternatively, any nicotine-induced memory impairment may have been sex-dependent and limited to females, as has been previously been observed given certain nicotine-delivery and testing conditions (Peters and Ngan, 1982; Eppolito and Smith, 2006).

However, given the clear deficits observed in long-term object location memory in both adolescent male and female mice exposed to a similar model of nicotine administration (Nakauchi et al., 2015), as well the considerable parallels in hippocampal mis-function between the mouse and rat models (Nakauchi et al., 2015; Chen et al., submitted), and the observed decrease in  $\alpha 2$ -containing OLM cell function and  $\alpha 2$ -containing cell numbers in this rat model, we believe that it is most likely that our inability to observe any nicotine-induced effect is a reflection of a lack of sensitivity of the Morris water maze task for this type of deficit. Indeed, though others have successfully used other tests to demonstrate perinatal-nicotine-induced memory impairments in rats, investigations using Morris water maze have rarely demonstrated

nicotine-induced deficits. It should also be noted that we and others have found that Sprague Dawley rats, which are albino and therefore have poor vision, have particular difficulty performing spatial memory tasks (Harker and Whishaw, 2002). This, too, may have masked any developmental-nicotine-induced effects we were hoping to observe.

In this study, we also investigated the effect of maternal nicotine treatment on hippocampal CA1 levels of NR2A, NR2B, Src and Fyn, which all play important roles in hippocampal LTP and memory. We saw no nicotine-induced alteration in total protein levels for NR2A, NR2B or Src, but some evidence for an effect of nicotine on the kinase Fyn. As with our behavior data, it is hard to determine whether this lack of clear change is because our model of early life nicotine exposure did not alter protein levels, or because any change was below the detection limit of our approach, or because no form of early life nicotine exposure alters expression of these proteins.

It is very possible, though, that developmental nicotine exposure does affect NMDAR function without changing total protein levels of NR2A, NR2B, Src or Fyn. We have previously shown in adolescent rats that chronic nicotine exposure altered excitatory postsynaptic current mediated by NR2B-containing NMDARs, without changing the total amount of NR2A or NR2B protein in the CA1 (Yamazaki et al., 2006a). NMDAR function within neurons can be dramatically affected by several changes that do not affect subunit protein expression, including shifts in receptor localization, receptor channel properties, or in downstream protein-protein interactions. Each of these changes can be mediated by tyrosine phosphorylation of NR2A or NR2B, and can therefore be driven by altered expression or phosphorylation of Src family kinases such as Src and Fyn (Wang et al., 1996; Kohr and Seeberg, 1996; Lavezzari et

al., 2003; Prybylowski et al., 2005; Goebel et al., 2005; Nakazawa et al., 2006; Zhang et al., 2008; Sinai et al., 2010). It is therefore interesting that we found some weak evidence for a developmental-nicotine-induced change in Fyn expression, with the largest mean difference between saline- and nicotine-treated animals occurring at P14, shortly after the conclusion of postnatal nicotine exposure. It may therefore be valuable to revisit this early time point in future studies of developmental nicotine's effect on Fyn and NMDAR function.

Thus, although our model of early postnatal nicotine exposure in rats caused no observable spatial memory or cued memory impairment, no changes in NR2A, NR2B or Src protein expression, and only weak evidence for a change in Fyn expression, the full impact of developmental nicotine exposure on the hippocampus remains unresolved.

## CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

In order to understand the mechanisms underlying cognitive impairment in children of mothers who smoked during pregnancy, here we studied the hippocampal impacts of a model of early postnatal nicotine exposure in mice and rats. Models of nicotine exposure can vary greatly, resulting in a large variety of behavioral outcomes. Thus, it was important to first study whether our method of nicotine delivery resulted in the cognitive impairments we were trying to understand. For these experiments, mice and rats were exposed to nicotine during their first two postnatal weeks, a time of brain development roughly corresponding to the third trimester in human pregnancies (Dobbing and Sands, 1973; Seress et al., 2001; de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007). Nicotine was delivered by maternal milk from dams implanted with osmotic minipumps, at different concentrations in mice (21 mg/kg/day) and rats (6 mg/kg/day) to account for their dissimilar rates of nicotine metabolism. In mice, these maternal-nicotine-exposed pups, when tested a month after drug administration had ceased, were found to have impaired long-term memory for the hippocampus-dependent object location task. However, nicotine-treated mice demonstrated normal short-term memory for object location, and normal long-term memory for hippocampus-independent object recognition. Importantly, their ability to perform the short-term object location task and long-term object recognition task to the same level as controls indicates that nicotine-treated mice did not have any performance deficits that may have confounded the interpretation of results for the long-term object location memory task.

Thus, maternal-nicotine-exposed mice do have impaired long-term hippocampus-dependent spatial memory.

Surprisingly, however, when we tested our rat model of maternal nicotine exposure using the Morris water maze task, we did not observe any memory deficits, either in long- or short-term spatial memory, or in reversal memory or cued memory. It is difficult to interpret these negative results, as they could indicate that our rat model does not accurately mimic the conditions of developmental nicotine exposure that cause cognitive impairment in humans, that the Morris water maze task is not a sensitive-enough measure to detect any changes in cognitive impairment, or that rats do not experience nicotine-induced impairments in hippocampus-dependent memory. However, because many research groups have demonstrated perinatal-nicotine-induced spatial memory deficits in rats (Sorenson et al., 1991; Levin et al., 1993b; Levin et al., 1996b; Vagelnova et al., 2004; Eppolito and Smith, 2006; Huang et al., 2007b), and maternal-nicotine-treated rats show significant dysfunction in  $\alpha$ 2-OLM cell activity (Chen et al., submitted) and have many of the same changes in hippocampal function as we have observed in our similar mouse model of postnatal nicotine exposure, which impairs spatial memory, we believe that our rat model of developmental nicotine exposure likely does result in spatial memory impairments that would be observed with a more sensitive test. Going forward, it will be important to further explore this discrepancy in our results, to confirm the translatability of our mouse work to other species, including humans, and determine the behavioral relevance of those changes in hippocampal function that we have observed using this rat model of developmental nicotine exposure.

Having demonstrated that our mouse model of early life nicotine exposure impaired hippocampus-dependent memory, we next explored the hippocampal function of these mice to identify the changes that might underlie this cognitive deficit. Because LTP at the Schaffer collateral pathway has been strongly implicated as a cellular mechanism of hippocampal learning and memory, we expected that we might observe nicotine-induced impairments in LTP. Surprisingly, however, in hippocampal slices from maternal-nicotine-treated mice, LTP induction at the Schaffer collateral was facilitated, and there was weak evidence for a small increase in LTP magnitude. Maternal-nicotine-treated slices, when imaged with voltage-sensitive-dye, also showed significantly heightened depolarization and hyperpolarization in the CA1 following Schaffer collateral stimulation. However, one of the most pervasive long-term impacts of developmental nicotine exposure that we observed was the altered nicotinic modulation of CA1 activity and LTP. Whereas in slices from control mice, bath application of nicotine potentiates CA1 activation and facilitates LTP, bath nicotine had no effect on CA1 activation in nicotine-treated slices, and reversed the maternal-nicotine-induced facilitation of LTP. Furthermore, whereas bath nicotine normally blocks LTP induced by temporoammonic pathway stimulation, it had no effect on this form of LTP in slices from early-postnatal-nicotine-exposed mice.

We also observed that early postnatal nicotine exposure altered which nAChR subtypes were driving the nicotinic modulation of Schaffer collateral LTP, such that it was no longer caused by the activation of either  $\alpha 7$ - or  $\beta 2$ -containing receptors. In adolescent and adult mice,  $\alpha 2$ -containing nAChRs, which, in the hippocampus, are found only in OLM interneurons, are important mediators of the effect of nicotine

(Nakauchi et al., 2007; Jia et al., 2009; Leao et al., 2012). Furthermore, other research underway in our lab using our rat model of maternal nicotine exposure indicated that these animals had significantly decreased OLM interneuron function, as well as fewer  $\alpha 2$ -containing OLM cells in the CA1 region (Chen et al., submitted). Additionally, there is growing evidence to suggest that OLM cells, which directly inhibit temporoammonic pathway inputs and indirectly facilitate Schaffer collateral inputs to pyramidal cells (Nakauchi et al., 2007; Leao et al., 2012; Chen et al., submitted), are critically important for hippocampal learning and memory (Leao et al., 2012; Lovett-Barron et al., 2014). Together, these findings suggested that the changes in hippocampal function and memory resulting from early postnatal nicotine exposure may be caused by nicotine-induced downregulation of  $\alpha 2$ -containing nAChRs.

Thus, we next investigated the impact  $\alpha 2$  downregulation by studying hippocampal memory and function in an  $\alpha 2$  knockout mouse line. We found that mice lacking  $\alpha 2$ -containing nAChRs showed the same pattern of memory impairment as did maternal-nicotine-treated mice, with normal hippocampus-independent long-term object recognition memory, but defective hippocampus-dependent long-term object location memory. These findings are strong evidence that  $\alpha 2$ -containing nAChRs play a critical role in the consolidation of long-term spatial memory in the hippocampus, and are also crucial mediators of the effects of developmental nicotine.

Additionally, in these  $\alpha 2$  knockout mice, we investigated some of the maternal-nicotine-induced alterations in hippocampal function that we had previously observed, in order to determine whether these changes could be linked to  $\alpha 2$ -containing nAChRs, and to understand whether they might underlie impairments in hippocampal

memory. We found that  $\alpha 2$  knockout mice did not show the increase in CA1 network activation in response to Schaffer collateral stimulation that occurs following maternal nicotine treatment in wild-type mice, suggesting that the nicotine-induced effect is not the direct result of downregulated  $\alpha 2$  expression. Additionally, we found that  $\alpha 2$  knockout mice exposed to early postnatal nicotine also did not show this change in hippocampal network activity, indicating that the effect does require the abnormal activation of  $\alpha 2$  nAChRs by developmental nicotine. However, the absence of heightened CA1 network activity in  $\alpha 2$  knockout mice, which do have hippocampal memory impairments, is an indication that this nicotine-induced alteration in hippocampal function may not be associated with the cognitive impacts of developmental nicotine exposure. Likewise, maternal-nicotine-treated  $\alpha 2$  knockout mice, unlike maternal-nicotine-treated wild-type mice, did not show facilitated LTP after Schaffer collateral stimulation, suggesting that this change too, though initially dependent on the activation of  $\alpha 2$ -containing nAChRs, is neither the result of  $\alpha 2$  downregulation, nor a mechanism underlying nicotine-induced memory impairments.

In total, the work presented here indicates that early postnatal nicotine exposure causes several changes to hippocampal function, and impairs hippocampus-dependent long-term memory. It further suggests that  $\alpha 2$ -containing nAChRs in OLM interneurons are crucial mediators of hippocampal memory and of some of the nicotine-induced changes in hippocampal function. Finally, it is likely that the downregulation of  $\alpha 2$ -containing nAChR subtypes by developmental nicotine exposure is a cause of nicotine-induced memory impairments.



Thus, this work adds to a growing body of evidence that  $\alpha 2$ -containing hippocampal OLM cells play a crucial role in hippocampal function and dysfunction. Normally, OLM cells are important mediators of spatial memory, but alterations in their activity or number are hypothesized to underlie not only certain effects of developmental nicotine exposure, but also hippocampal neurological disorders including epilepsy-induced memory impairments (Dugladze et al., 2007), age-associated learning and memory deficits (Stanley et al., 2012) and schizophrenia (Neymotin et al., 2011). However, many questions remain about the role of OLM cells and  $\alpha 2$ -containing nAChRs in developmental-nicotine-induced cognitive impairments.

One major question that remains to be explored is the timing during which developmental-nicotine-induced downregulation of  $\alpha 2$ -containing nAChRs affects hippocampal memory. Because hippocampal development is still underway during the third trimester of human pregnancy and the first postnatal weeks in rats (Dobbing and Sands, 1973; Seress et al., 2001; de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007), and because nAChRs play an important role in modulating the strength of newly forming synapses (Liu et al., 2006), it is possible that a nicotine-induced change in  $\alpha 2$ -containing nAChRs at this time could cause long-lasting alterations in hippocampal connectivity and, therefore, long-lasting cognitive impairments. However, another likely possibility is that it is the persistent downregulation of  $\alpha 2$ -containing nAChRs that directly results in cognitive deficits by preventing the proper function of OLM interneurons at the time of attempted spatial memory formation. Because we used a constitutive knockout mouse line for our experiments, we were unable to differentiate between these two potential causes of nicotine-induced cognitive impairment. Future

work using hippocampal injection of  $\alpha 2$ -targeting siRNA or oligoneucleotides would allow for these alternate possibilities to be tested. Additionally, once this timing had been established, viral-mediated upregulation of  $\alpha 2$  would be expected to rescue developmental-nicotine-induced memory impairments, further supporting our hypothesis that  $\alpha 2$ -containing nAChRs underlie nicotine-induced deficits in hippocampal memory. Such work would both elucidate the function of  $\alpha 2$ -containing OLM cells in the hippocampus, and provide valuable information to facilitate the development of therapies to counter the effects of developmental nicotine exposure.

Furthermore, we still do not know the details of the pathway by which  $\alpha 2$  downregulation results in impaired memory, or even why chronic nicotine exposure during development impairs spatial memory when chronic exposure during adulthood facilitates it (Poincheval-Fuhrman and Sara, 1993; Arendash et al., 1995; Socci et al., 1995; Levin et al., 1996a; Levin and Torry, 1996). And, ultimately, in order to begin to develop treatments for the 400,000 children born each year in this country to mothers who smoke, it will be crucial to understand whether these changes in hippocampal function that we have observed in mice and rats also underlie developmental-nicotine-induced cognitive impairments in humans.

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