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Long-term effects of early postnatal nicotine exposure on cholinergic function in the mouse hippocampal CA1 region

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Abstract

In rodent models of smoking during pregnancy, early postnatal nicotine exposure results in impaired hippocampus-dependent memory, but the underlying mechanism remains elusive. Given that hippocampal cholinergic systems modulate memory and rapid development of hippocampal cholinergic systems occurs during nicotine exposure, here we investigated its impacts on cholinergic function. Both nicotinic and muscarinic activation produce transient or long-lasting depression of excitatory synaptic transmission in the hippocampal CA1 region. We found that postnatal nicotine exposure impairs both the induction and nicotinic modulation of NMDAR-dependent long-term depression (LTD). Activation of muscarinic receptors decreases excitatory synaptic transmission and CA1 network activity in both wild-type and α2 knockout mice. These muscarinic effects are still observed in nicotine-exposed mice. M1 muscarinic receptor activity is required for mGluR-dependent LTD. Early postnatal nicotine exposure has no effect on mGluR-dependent LTD induction, suggesting that it has no effect on the function of m1 muscarinic receptors involved in this form of LTD. Our results demonstrate that early postnatal nicotine exposure has more pronounced effects on nicotinic function than muscarinic function in the hippocampal CA1 region. Thus, impaired hippocampus-dependent memory may arise from the developmental disruption of nicotinic cholinergic systems in the hippocampal CA1 region.

Keywords

nicotinic acetylcholine receptor; muscarinic acetylcholine receptor; LTD; nicotine; development; hippocampus

1. Introduction

Cigarette smoking represents a significant public health risk, and *in utero* exposure to the components of cigarette smoke has serious negative consequences for human brain development, including elevated risk of cognitive deficits in children (Fried et al., 2003;

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Heath and Picciotto, 2009; Thompson et al., 2009). To model cigarette smoking during pregnancy, many studies have opted to administer nicotine to developing rodents. These studies have shown that early perinatal exposure to nicotine alone results in persistent deficits in learning and memory, including hippocampus-dependent spatial memory (Ankarberg et al., 2001; Eppolito and Smith, 2006; Sorenson et al., 1991; Vaglenova et al., 2004; Yanai et al., 1992). However, the underlying mechanism remains to be investigated.

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 (de Graaf-Peters and Hadders-Algra, 2006; Seress, 2007; Seress et al., 2001). This two-week window appears to encompass a “critical period” during which nicotine exposure causes long-lasting cellular/molecular changes and memory impairments (Eriksson et al., 2000; Miao et al., 1998; Zeid et al., 2018). Importantly, this period is a time of rapid development of the cholinergic system in the hippocampus and coincides with a sharp upregulation of nicotinic acetylcholine receptors (nAChRs) in GABAergic neurons (Adams et al., 2002; Shacka and Robinson, 1998; Son and Winzer-Serhan, 2006; Winzer-Serhan and Leslie, 2005; Zoli et al., 1995), which provide an important excitatory drive in the developing hippocampus (Ben-Ari, 2002; Ben-Ari et al., 2004; Gozlan and Ben-Ari, 2003). It is therefore not surprising that nicotine exposure during this period disrupts normal cholinergic roles in the developing hippocampus and produces subsequent lifelong functional changes in the hippocampus. However, it remains unknown what changes underlie the memory impairment.

Memory impairments in rodents are often associated with altered long-term potentiation (LTP) in the hippocampal CA1 region, which is generally thought to be the cellular substrate of learning and memory (Bliss and Collingridge, 1993; Collingridge et al., 2010; Kemp and Bashir, 2001; Malenka and Bear, 2004; Malenka and Nicoll, 1999). We have recently demonstrated that early postnatal nicotine exposure via maternal milk results in impaired hippocampus dependent memory in adolescence (Nakauchi et al., 2015). However, LTP is not diminished. Instead, this memory impairment is associated with altered nicotinic modulation of LTP and the loss of normal α2* nAChR function (Nakauchi et al., 2015). Interestingly, mice lacking the α2* nAChR, like maternal-nicotine-exposed wild-type mice, have impaired hippocampus-dependent memory (Kleeman et al., 2016). These results suggest that altered nicotinic function, especially α2* nAChR function, likely plays a critical role in this memory impairment. However, these findings do not exclude the possibility that altered functions of other nAChR subtypes contribute to memory impairments. In addition, it remains unknown whether nicotine exposure alters muscarinic cholinergic function.

Both N-methyl-D-aspartate receptor (NMDAR)-dependent and group I metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD) are linked to memory mechanisms (Auerbach et al., 2011; Wong et al., 2007). The induction of NMDAR-dependent LTD is regulated by the activation of nAChRs and occurs in mice lacking the α2* nAChR (Nakauchi and Sumikawa, 2014). Activation of muscarinic acetylcholine receptors (mAChRs) using carbachol (CCh) induces transient or long-term depression of excitatory synaptic transmission (Dickinson et al., 2009; Kremin et al., 2006; Leung and Peloquin, 2010; Mann et al., 2005) and m1 mAChR activity is required for mGluR-dependent LTD

(Kamsler et al., 2010; Volk et al., 2007). In the current study, we investigated the effects of early postnatal nicotine exposure on these cholinergic functions.

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. Experiments were carried out using C57BL/6 wild-type mice and α2 knockout (KO) mice in the C57BL/6 background from established colonies of heterozygous breeders, obtained from Dr. Jim Boulter (UCLA). 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 alzet osmotic minipumps (DURECT, Model 1002; approximate nicotine output: 21 mg/kg/day). Others using the same procedure previously reported plasma nicotine levels to be 207 ± 40 ng/ml in dams (Eugenin et al., 2008). Offspring were weaned at P21 and separated by sex into cages of 2–5 mice. Here, we refer to these pups as maternal nicotine (Mat nic)-exposed mice. As controls, mouse pups from dams implanted with saline-containing minipumps were used. As electrophysiological recordings from male and female mice yielded equivalent results, their data were combined for statistical analysis.

2.2. Extracellular field recordings in hippocampal slices

Transverse hippocampal slices (300–400 μm) were prepared from mice (age 4–6 weeks) anesthetized with urethane or isoflurane. 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; NaH_2PO_4, 1.25; $MgSO_4$, 2; $CaCl_2$, 2.5; $NaHCO_3$, 22; glucose, 10; and oxygenated with 95% O_2 and 5% CO_2. Slices were submerged in a recording chamber and continuously 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 2 M NaCl (3–8 MΩ). The test stimulus intensity was set to evoke 30–50% of the maximum EPSP slope. NMDAR-dependent LTD was induced by low-frequency stimulation (LFS, 900 pulses at 1 Hz) and mGluR-dependent LTD was induced by bath application of (R.S.)-3,5-dihydroxyphenylglycine (DHPG, 50 μM) for 10 min. To evaluate LTD magnitude, the mean values of the slopes of fEPSPs from 50-55 min after LTD inducing stimulation were calculated and expressed as a percentage of the mean baseline fEPSPs slopes.

2.3. Voltage-sensitive dye imaging

Voltage-sensitive dye (VSD) imaging with Di-4-ANEPPS (Molecular Probes, Carlsbad, CA, USA) was performed as previously described (Nakauchi et al., 2007; Tominaga et al., 2000). 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 and VSD imaging was

performed with a CCD camera (MiCAM02; BrainVision). The SC pathway in VSD-loaded hippocampal slices was stimulated as in the field recordings and the propagation of evoked voltage changes were recorded. Field EPSP recordings were preformed simultaneously with the optical recordings to ensure that the optical response was consistent with the electrical response. Data were analyzed using BV-Analyzer (BrainVision) and the magnitude of voltage changes was illustrated using pseudocolor, in which warmer colors indicate depolarization and red indicates the strongest depolarization. To quantitatively compare optical responses across different slices, the optical responses to a single stimulus were measured as fractional changes of fluorescence (ΔF/F).

2.4. Drug application

Nicotine (Sigma), mecamylamine (Abcam), DHPG (Abcam), and CCh (Sigma) were dissolved in ACSF and bath-applied. To examine the effect of CCh on fEPSPs and optical responses, control responses were first recorded and then CCh was bath-applied for a period of 10 min before recording responses again in the presence of the drug.

2.5. Statistical analysis

Electrophysiological and VSD imaging data were normalized relative to baseline, expressed as mean ± SEM, and analyzed for significance using one-way ANOVA. Two-way ANOVA for two factors of animal groups and CCh treatment was also used. The overall ANOVA was followed by *post hoc* Tukey test. p values of less than 0.05 were considered statistically significant. Sample size n refers to the number of hippocampal slices analyzed in electrophysiological or optical recordings from different mice. In all graphs, p values are depicted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data were plotted and analyzed using Origin 8.1 (OriginLab).

3. Results

3.1 Early postnatal nicotine exposure impairs both the induction and nicotinic modulation of NMDAR-dependent LTD

Given that postnatal nicotine exposure does not impair LTP at SC-CA1 synapses (Chen et al., 2016; Nakauchi et al., 2015), we first investigated its effects on another prevalent form of synaptic plasticity, NMDAR-dependent LTD, and nicotinic modulation of this form of LTD. In hippocampal slices from saline-treated control mice, LFS induced LTD (Fig. 1A, C; 84 ± 4% of basal levels, n = 8) and the magnitude of LTD was significantly enhanced by bath application of nicotine (1 μM) (Fig. 1A, C; Saline/control, 84 ± 4%, n = 8, vs. Saline/acute nicotine, 67 ± 5%, n = 6, $F_{(1,13)} = 6.03$, $p < 0.05$), as previously found in the hippocampus of naïve rats and mice (Fujii and Sumikawa, 2001; Nakauchi and Sumikawa, 2014). Mecamylamine (3 μM), which blocks virtually all subtypes of nAChRs, prevented both the induction of LTD and the acute nicotine's effect (Fig. 1A, C; Saline/acute nicotine, 67 ± 5%, n = 6, vs. Saline/acute nicotine + mecamylamine, 98 ± 3%, n = 6, $F_{(1,11)} = 22.87$, $p < 0.001$). These findings suggest that the activation of nAChRs by endogenous ACh released during LTD-inducing stimulation regulates LTD and the activation of other nAChR subtypes by acute nicotine application enhances LTD. Mecamylamine blocks these effects. In hippocampal slices from Mat nic-exposed mice, however, the same low frequency

stimulus did not induce LTD (Fig. 1B, C; 97 ± 1% of basal levels, n = 5). Thus, early postnatal nicotine exposure blocks the induction of LTD (Fig. 1A-C; Saline/control, 84 ± 4%, n = 8, vs. Mat nic/control, 97 ± 1%, n = 5, $F_{(1,12)} = 5.0$, $p < 0.05$). We then examined whether bath application of nicotine (1 μM) enhances LTD and found that nicotine application has no significant effect on LTD (Fig. 1B, C; Mat nic/control, 97 ± 1%, n = 5, vs. Mat nic/acute nicotine, 92 ± 8%, n = 6, $F_{(1,10)} = 0.32$, $p = 0.59$). Thus, acute nicotine-induced LTD enhancement observed in saline-exposed hippocampi is absent in Mat nic-exposed hippocampi (Fig. 1A-C; Saline/acute nicotine, 67 ± 5%, n = 6, vs. Mat nic/acute nicotine, 92 ± 8%, n = 6, $F_{(1,11)} = 6.08$, $p < 0.05$). These results suggest a strong role for nAChRs in the normal induction and modulation of LTD, as well as a significant impairment of nAChR function involved in LTD induction and modulation following early postnatal nicotine exposure.

3.2 Muscarinic AChR-mediated depression of excitatory responses is still observed in Mat nic-exposed mice

Our previous studies demonstrate that the basal synaptic transmission and the probability of transmitter release at SC-CA1 synapses appear to be normal in both Mat nic mice (Nakauchi et al., 2015) and α2 KO mice (Nakauchi et al., 2007), suggesting that fEPSPs are not significantly altered in these mice. Thus, in the following experiments, we adjusted the strength of stimulus to elicit similar sizes of fEPSPs and examined the effect of Mat nic on muscarinic function and optical responses.

Muscarinic AChR activation by bath application of CCh decreases both fEPSPs and excitatory circuit activity in the hippocampus of rats (Mann et al., 2005). We used these measures to assess the impacts of nicotine exposure on muscarinic function. We first examined whether nicotine exposure alters the effect of CCh on fEPSPs in the hippocampal CA1 region. For these experiments, the intensity of stimulation was adjusted to evoke similar sizes of fEPSPs across different slices (Fig. 2A; Saline/control, 101 ± 2%, n = 6, vs. Mat nic/control, 101 ± 1%, n = 10, $p = 0.70$). In both saline- and Mat nic-exposed slices, bath application of 1 and 10 μM CCh significantly decreased fEPSPs (Fig. 2A: Saline/control vs. Saline/1 μM CCh, $p < 0.05$; Saline/control vs. Saline/10 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/1 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/10 μM CCh, $p < 0.001$). However, between the two groups, there were no significant differences in the effects of 1 μM CCh (Fig. 2A; Saline, 54 ± 5%, n = 6, vs. Mat nic, 65 ± 6%, n = 10, $p = 0.87$) and 10 μM CCh (Fig. 2A; Saline, 12 ± 2%, n = 6, vs. Mat nic, 28 ± 5%, n = 10, $p = 0.56$).

We next recorded the muscarinic modulation of CA1 network responses to SC input using VSD imaging. Electrical stimulation in the SC pathway, intensity of which was adjusted to evoke similar amplitudes of fEPSPs in both saline- and Mat nic-exposed slices, caused the spread of optical signal in all anatomical layers, which could be presented as pseudocolored images of the ΔF/F signals (Fig. 2B, D) and traces (Fig. 2C). Depolarizing responses originating from the site of stimulation peaked around 8 ms in slices from both control and Mat nic-exposed mice, followed by delayed hyperpolarization (blue) (Fig. 2B). The peak depolarizing signals were significantly stronger in Mat nic-exposed hippocampi (Fig. 2D;

Saline, 2.52 ± 0.08, n = 6, vs. Mat nic, 3.41 ± 0.11, n = 10, $p < 0.001$). In both saline- and Mat nic-exposed slices, bath application of 1 and 10 μM CCh decreased depolarizing optical responses to similar extents (Fig. 2D: Saline/control vs. Saline/1 μM CCh, $p < 0.001$; Saline/control vs. Saline/10 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/1 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/10 μM CCh, $p < 0.001$). However, perhaps due to higher basal levels of depolarizing responses in Mat nic-exposed mice (Fig. 3D, control), the remaining responses observed in the presence of CCh were significantly stronger in Mat nic-exposed slices (1 μM CCh: Saline, 1.51 ± 0.07, n = 6, vs. Mat nic, 2.51 ± 0.05, n = 10, $p < 0.001$; 10 μM CCh: Saline, 0.65 ± 0.06, n = 6, vs. Mat nic, 1.30 ± 0.04, n = 10, $p < 0.001$). Nevertheless, these observations suggest that the muscarinic modulation of excitatory activity in the hippocampal CA1 region is still maintained in Mat nic-exposed mice.

3.3 Maternal nicotine exposure has no effect on the mAChR-mediated depression of excitatory responses in α2 KO mice

We have previously shown that increased depolarizing responses observed in Mat nic-exposed wild-type mice (Nakauchi et al., 2015) are absent in Mat nic-exposed α2 KO mice (Kleeman et al., 2016). Therefore, to further investigate the effect of maternal nicotine exposure on the muscarinic modulation, we used α2 KO mice. We adjusted the strength of stimulus to elicit similar sizes of fEPSPs in both saline- and Mat nic-exposed slices (Fig. 3A; Saline/control, 100 ± 2%, n = 6, vs. Mat nic/control, 99 ± 3%, n = 10, $p = 0.91$). We observed that CCh depressed fEPSPs in both saline- and Mat nic-exposed α2 KO mice (Fig. 3A: Saline/control vs. Saline/1 μM CCh, $p < 0.001$; Saline/control vs. Saline/10 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/1 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/10 μM CCh, $p < 0.001$. However, there were no significant differences in the effects of 1 and 10 μM CCh between the two groups (Fig. 3A) (1 μM CCh: Saline, 57 ± 5%, n = 6, vs. Mat nic, 55 ± 2%, n = 10, $p = 0.99$; 10 μM CCh: Saline, 14 ± 2%, n = 6, vs. Mat nic, 15 ± 2, n =1 0, $p = 0.98$).

We also recorded the muscarinic modulation of excitatory network responses to SC input using VSD imaging and confirmed that the peak depolarizing signals were not significantly different between saline- and Mat nic-exposed α2 KO mice (Fig. 3B and C, control; Saline, 2.48 ± 0.05, n = 6, vs. Mat nic, 2.42 ± 0.09, n = 10, $p = 0.99$). Bath application of 1 and 10 μM CCh decreased depolarizing optical responses in both saline- and Mat nic-exposed hippocampi (Fig. 3B and C: Saline/control vs. Saline/1 μM CCh, $p < 0.001$; Saline/control vs. Saline/10 μM CCh, $p < 0.001$; Mat nic/control vs. Mat nic/1 μM CCh, $p < 0.01$; Mat nic/control vs. Mat nic/10 μM CCh, $p < 0.001$). There were no significant differences in depression levels between the two groups (1 μM CCh: Saline, 1.95 ± 0.05, n = 6, vs. Mat nic, 1.83 ± 0.08, n = 10, $p = 0.93$; 10 μM CCh: Saline, 1.21 ± 0.05, n = 6, vs. Mat nic, 1.19 ± 0.08, n = 10, $p = 0.99$). These results suggest that, although nicotine exposure causes the increase in CA1 excitatory network responses to SC input, it has no significant effect on the muscarinic modulation of CA1 network responses.

3.4 Maternal nicotine exposure increases hyperpolarizing circuit responses, but does not significantly alter the muscarinic modulation of the responses

In addition to enhanced depolarizing optical responses, we found that Mat nic exposure caused stronger hyperpolarizing responses after SC stimulation in wild-type mice (Fig. 4A; Saline/control, 117.19 ± 10.29, n = 6, vs. Mat nic/control, 331.33 ± 3.94, n = 10, $F_{(1,47)}$ = 516.68, $p < 0.001$). Furthermore, unlike depolarizing responses, Mat nic exposure increased hyperpolarizing responses in α2 KO mice (Fig. 4B; Saline/control, 200.32 ± 12.84, n = 11, vs. Mat nic/control, 268.99 ± 36.45, n = 5, $F_{(1,47)}$ = 4.94, $p < 0.05$), suggesting that enhanced depolarizing responses are not the cause for the stronger hyperpolarizing responses. In both wild-type and α2 KO mice, there were stronger hyperpolarizing responses in Mat nic-exposed slices than in saline-treated slices even in the presence of 1 μM CCh (Fig. 4A; Saline, 275,89 ± 21.09, n = 6, vs. Mat nic, 422.52 ±13.24, n = 10, $F_{(1,47)}$ = 38.56, $p < 0.001$ and Fig. 4B; Saline, 318.88 ± 11.22, n = 11, vs. Mat nic, 374.31 ± 30.10, n = 5, $F_{(1,47)}$ = 4.51, $p < 0.05$) and 10 μM CCh (Fig. 4A; Saline, 150.64 ± 14.87, n = 6, vs. Mat nic, 345.02 ± 7.39, n = 10, $F_{(1,47)}$ = 169.71, $p < 0.001$ and Fig. 4B; Saline, 273.54 ± 8.95, n = 11, vs. Mat nic, 354.31 ± 16.45, n = 5, $F_{(1,47)}$ = 21.89, $p < 0.001$).

CCh application significantly increased hyperpolarizing responses in both saline-treated wild-type mice (Fig. 4A; Saline/control, 117.19 ± 10.29, n = 6, vs., Saline/1 μM CCh, 275,89 ± 21.09, n = 6, $F_{(1,35)}$ = 45.72, $p < 0.001$) and α2 KO mice (Fig. 4B; Saline/control, 200.32 ± 12.84, n = 11, vs. Saline/1 μM CCh, 318.88 ± 11.22, n = 11, $F_{(1,65)}$ = 48.34, $p < 0.001$). Enhancing effects of CCh on hyperpolarizing responses were still observed after Mat nic exposure in both wild-type (Fig. 4A; Mat nic/control, 331.33 ± 3.94, n = 10, vs., Mat nic/1 μM CCh, 422.52 ±13.24, n = 10, $F_{(1,59)}$ = 43.60, $p < 0.001$) and α2 KO mice (Fig. 4B; Mat nic/control, 268.99 ± 36.45, n = 5, vs., Mat nic/1 μM CCh, 374.31 ± 30.10, n = 5, $F_{(1,29)}$ =4.96, $p < 0.05$). The implication of these results is that Mat nic exposure does not significantly alter the muscarinic function.

3.5 Maternal nicotine exposure has no effect on mGluR-LTD, but alters nicotinic influence on mGluR-LTD

A brief application of a group I mGluR agonist, DHPG, induces LTD at SC synapses (Bolshakov and Siegelbaum, 1994; Zakharenko et al., 2002) that requires the normal function of m1 mAChRs (Kamsler et al., 2010; Volk et al., 2007). To investigate whether maternal nicotine exposure affects the functional role of m1 mAChRs in mGluR-LTD, we activated mGluRs with DHPG. Application of DHPG caused the maximum transient depression (MTD) of fEPSPs, an electrophysiological measure of mGluR1/5 activation, and induced LTD in saline- and Mat nic-exposed hippocampi (Fig. 5A, B). We observed similar MTD (Saline/control, 21 ± 3%, n = 5, vs. Mat nic/control, 24 ± 6%, n = 9, $F_{(1,13)}$ = 0.10, p = 0.76) and LTD (Saline/control, 83 ± 7%, n = 5, vs. Mat nic/control, 93 ± 8%, n = 9, $F_{(1,13)}$ = 0.43, p = 0.52) in saline- and Mat nic-exposed mice, suggesting that Mat nic exposure does not affect mGluR-LTD. These results also indicate the normal functioning of m1 mAChRs involved in mGluR-LTD.

We also investigated whether altered nicotinic function in Mat nic-exposed mice influences DHPG-induced mGluR-LTD. When experiments were performed in the presence of acute

nicotine, we found that MTD was significantly reduced in Mat nic-exposed mice (Fig. 5A, B; control, 24 ± 6%, n = 9, vs. acute nicotine, 49 ± 5%, n = 7, $F_{(1,15)} = 8.38$, $p < 0.05$), but not in saline-treated mice (Fig. 5A, B; control, 21 ± 3%, n = 5, vs. acute nicotine, 24 ± 6%, n = 7, $F_{(1,11)} = 0.01$, $p = 0.94$). These results suggest that Mat nic exposure affects nicotinic signaling, but the mechanism underlying the nicotine's effect remain to be investigated. In both saline- and Mat nic-exposed mice, the magnitude of LTD induced was not significantly altered by acute nicotine (Fig. 5A, B; Saline/control, 83 ± 7%, n = 5, vs. Saline/acute nicotine, 74 ± 7%, n = 7, $F_{(1,11)} = 1.15$, $p = 0.30$; Mat nic/control, 93 ± 8%, n = 9, vs. Mat nic/acute nicotine, 94 ± 4%, n = 7, $F_{(1,15)} = 8.51$, $p = 0.98$). However, when the magnitudes of LTD induced in saline- and Mat nic-exposed mice in the presence of acute nicotine were compared, we found significantly smaller LTD in Mat nic-exposed mice (Fig. 5A, B; Saline/acute nicotine, 74 ± 7%, n = 7, vs. Mat nic/acute nicotine, 94 ± 4%, n = 7, $F_{(1,13)} = 5.54$, $p < 0.05$). Although mGluR-LTD magnitude is not significantly different between the absence and presence of acute nicotine in Mat saline group (Fig. 5A, B; Mat saline/control vs. Mat saline/acute nicotine), there is a trend towards the enhancement of LTD magnitude in the presence of acute nicotine. This trend, which is absent in Mat nic-exposed group, may be a major factor contributing to the observed significance. These findings suggest that acute nicotine stimulates signaling pathways involved in mGluR-LTD in Mat saline-treated group, but Mat nicotine exposure disrupts this nicotinic influence on mGluR-LTD. However, this needs to be clarified.

4. Discussion

Given the effects of nicotine on maternal behavior in rodents (Chirico et al., 2017; Faure et al., 2019), changes in the dams' conduct while they were exposed to, and eventually withdrawn from, nicotine may have contributed to the observed physiological changes in pups. However, selective effects of Mat nic on nicotinic function, but not muscarinic function, in LTD strongly suggest a specific action of nicotine in the developing brain. In addition, we did not observe obvious abnormal maternal behaviors (such as nest building, nursing, retrieval, and grooming) under conditions used.

Nicotine exposure during early development alters the expression of nAChRs, mAChRs, choline transporter, choline acetyltransferase, and vesicular acetylcholine transporter in the hippocampus (Abreu-Villaca et al., 2004; Mao et al., 2008a; Mao et al., 2008b; Nunes-Freitas et al., 2011; Shacka and Robinson, 1998; van de Kamp and Collins, 1994; Zahalka et al., 1992; Zeid et al., 2018). However, physiological and behavioral consequences of these changes have not been investigated. To identify specific cholinergic dysfunction that may be the cause of nicotine-induced cognitive impairments, it is particularly important to use a rodent model with demonstrated learning and memory impairments. We have previously demonstrated that mouse pups exposed to nicotine during the first two postnatal weeks via maternal milk show impaired long-term hippocampus dependent spatial memory during adolescence (Nakauchi et al., 2015). Therefore, in the current study, we used this mouse model and found that early postnatal nicotine exposure impairs both the induction and nicotinic modulation of NMDAR-dependent LTD. Our findings differ from a study that showed increased NMDAR-dependent LTD in rats exposed to nicotine during the gestational period (Parameshwaran et al., 2013). In these rats, NMDA-dependent LTP was

decreased and basal synaptic transmission was also reduced with a concomitant decline in AMPAR mediated synaptic currents. While other studies reported that postnatal nicotine exposure increases excitatory synaptic transmission, but does not alter LTP in the adult rat hippocampus (Damborsky et al., 2012). These findings also contrast with our previous findings that postnatal nicotine exposure resulted in facilitated LTP induction in rats (Chen et al., 2016) and mice (Nakauchi et al., 2015). In our previous study, we recorded fEPSPs in the mouse hippocampus and found that early postnatal nicotine exposure does not significantly alter the basal synaptic transmission and the probability of transmitter release (Nakauchi et al., 2015). Overall, these studies demonstrate that the effects of nicotine exposure during development are complex and sensitive to a combination of factors including species, method of nicotine administration, dose and timing of nicotine exposure. Different experimental protocols used for the induction of LTP and LTD in these studies might be additional factors. Nevertheless, our studies clearly show that impaired hippocampus dependent memory is associated with facilitated LTP, impaired NMDAR-dependent LTD, altered nicotinic modulation of LTP and LTD.

Cholinergic signaling controls synaptic plasticity and consequently hippocampus-dependent learning and memory (Drever et al., 2011; Kenney et al., 2012). Because LTP at the SC pathway is a cellular correlate of hippocampus-dependent memory (Bliss and Collingridge, 1993; Collingridge et al., 2010; Kemp and Bashir, 2001; Malenka and Bear, 2004; Malenka and Nicoll, 1999), we had expected that we would see an association of memory impairments with impaired LTP. However, postnatal nicotine exposure, which impairs hippocampus-dependent memory, resulted in facilitated LTP induction (Chen et al., 2016; Nakauchi et al., 2015). This could be explained if facilitated LTP induces behavioral impairments by strengthening synapses that compete with those required for spatial memory. The current study showed that postnatal nicotine exposure disrupts NMDAR-dependent LTD, but not mGluR-dependent LTD. Increasing evidence suggests that NMDA-dependent LTD plays an important role in hippocampus-dependent learning and memory (reviewed in Collingridge, 2010). Especially interesting are several studies suggesting that hippocampal LTD is essential for spatial memory (Ge et al., 2010; Kemp and Manahan-Vaughan, 2007). NMDAR-dependent LTD may be important for the clearing of old memory traces. Without NMDAR-dependent LTD, synapses may reach a maximum level of strength, preventing memory formation. However, it remains unknown whether disrupted LTD observed in the current study contributes to the Mat nic-induced memory impairments.

Relatively little is known about the cellular and molecular mechanisms underlying LTD. However, several studies have shown a role for acetylcholine and nAChRs in LTD (Fujii and Sumikawa, 2001; Kirkwood et al., 1999; Nakauchi and Sumikawa, 2014). In hippocampal slices from naïve mice, LTD is enhanced in the presence of the α7 nAChR antagonist methyllycaconitine, and LTD is larger in α7KO mice as compared to wild-type mice (Nakauchi and Sumikawa, 2014). These results indicate that the activation of α7 nAChRs occurs during LTD-inducing stimulation that in turn regulates LTD induction. Furthermore, bath application of nicotine enhances LTD, suggesting the involvement of other nAChR subtypes. This nicotine's effect is blocked by the non-selective nAChR antagonist mecamylamine, but not the α4*nAChR antagonist dihydro-β-erythroidine, and is still present in α2 KO and β2 KO mice (Nakauchi and Sumikawa, 2014). These findings

suggest the involvement of α3β4* nAChR activation in the nicotinic modulation of LTD. Therefore, it seems possible that the Mat nic-induced changes in LTD are directly related to long-term changes in α7 and α3β4*nAChR function in the hippocampal CA1 region. However, the involvement of other subtypes cannot be excluded. Interestingly, enhanced LTP in Mat nic-exposed mice and rats was accompanied by the loss of facilitative effect of acute nicotine on LTP. This effect of acute nicotine is mediated by α2 nAChR activation, suggesting that postnatal nicotine exposure also causes the long-lasting change in α2 nAChR function (Chen et al., 2016; Nakauchi et al., 2015). Our current study also shows that Mat nic exposure affects nAChR signaling, which interacts with the pathways involved in mGluR-MTD and LTD. The observed effects of Mat nic may be attributed to changes in the expression levels of nAChR subtypes, which need to be further clarified by carrying out pharmacological experiments and in situ hybridization.

Four subtypes of muscarinic receptors, m1-m4, are expressed in the hippocampus with the limited expression of m5 (Cea-del Rio et al., 2011; Cea-del Rio et al., 2010; Dasari and Gulledge, 2011; Levey et al., 1995). These receptors can influence hippocampal function by modulating neurotransmitter release and a variety of ionic conductances (Dasari and Gulledge, 2011; Dutar and Nicoll, 1988; Giessel and Sabatini, 2010; Leung and Peloquin, 2010; McQuiston and Madison, 1999). Exposure to nicotine prenatally or neonatally increased the number of quinuclidinyl benzilate, a pan-muscarinic receptor antagonist, binding in the mouse hippocampus (Yanai et al., 1992), but no effect on pirenzepine, m1 muscarinic receptor antagonist, binding in rats exposed to nicotine during the gestational period (Zahalka et al., 1993). Muscarinic receptor activation strongly depresses excitatory synaptic transmission and CA1 network activity (Mann et al., 2005). Our current study showed that these muscarinic effects were still observed in Mat nic-exposed mice, suggesting that muscarinic cholinergic systems are less affected by postnatal nicotine exposure than nicotinic cholinergic systems.

mGluR-dependent LTD at SC synapses are linked to cognitive impairments (Auerbach et al., 2011). Mat nic exposure has no effect on mGluR-induced LTD, suggesting that Mat nic exposure-induced memory impairments are not due to altered mGluR-dependent LTD. Presynaptic m1 mAChRs provide basal activation of PKC, which is further activated by DHPG for the induction of mGluR-dependent LTD (Kamsler et al., 2010). Thus, the normal function of presynaptic m1 mAChRs is necessary for mGluR-dependent LTD. The normal induction of mGluR-dependent LTD in Mat nic-exposed mice suggests the normal function of the m1 mAChRs in Mat nic-exposed hippocampi.

Using voltage sensitive dye to visualize hippocampal activity, we have previously found that early postnatal nicotine exposure results in enhanced CA1 depolarization and hyperpolarization after SC stimulation (Nakauchi et al., 2015). The increased depolarizing response observed in Mat nic-exposed wild-type mice is absent in Mat nic-exposed α2 KO mice (Kleeman et al., 2016). In the current study, we found that Mat nic exposure still increased hyperpolarizing responses in α2 KO mice, demonstrating that enhanced depolarizing and hyperpolarizing responses observed in Mat nic-exposed mice are caused by distinct mechanisms.

5. Conclusions

The current study shows that postnatal nicotine exposure disrupts nicotinic cholinergic modulation of NMDAR-dependent LTD and alters neuronal activity within the hippocampal CA1 circuits without significantly affecting muscarinic cholinergic function. Furthermore, postnatal nicotine exposure had no significant effect on the induction/baseline of mGluR-dependent LTD, but impaired nicotinic modulation of mGluR-dependent LTD. Our previous studies identified significant α2 nAChR dysfunction and altered nicotinic modulation of LTP and CA1 activity (Chen et al., 2016; Nakauchi et al., 2015). Together, one of the most pervasive long-term impacts of developmental nicotine exposure appears to be the altered nicotinic modulation of CA1 function. However, the outstanding question remains: which nicotinic changes induced by developmental nicotine exposure underlie the resulting memory impairment?

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Abbreviations:

ACSF	artificial cerebrospinal fluid
CCh	carbachol
DHPG	(R.S.)-3,5-dihydroxyphenylglycine
ERK	extracellular signal-regulated protein kinase
fEPSPs	field excitatory postsynaptic potentials
KO	knockout
LFS	low-frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
Mat nic	maternal nicotine
mGluR	metabotropic glutamate receptor
MTD	maximum transient depression
nAChR	nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate receptor
SC	Schaffer collateral

VSD	voltage-sensitive dye

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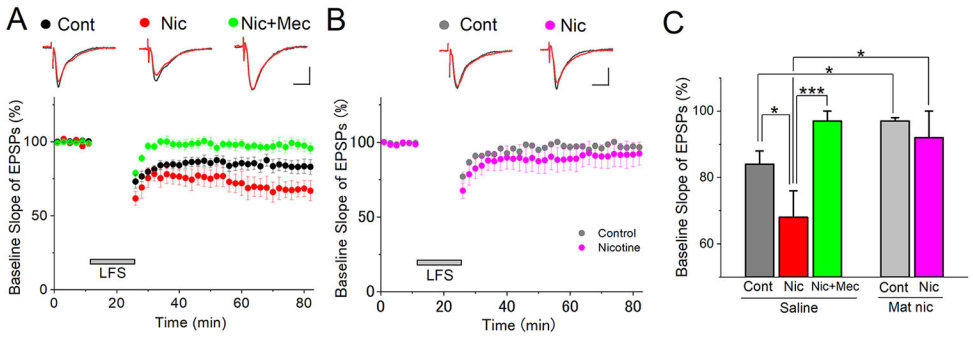

Figure 1. Maternal nicotine exposure disrupts NMDAR-dependent LTD induction and its nicotinic modulation

(A) At SC synapses in control mice, LFS induced NMDAR-dependent LTD and bath application of 1 μM nicotine (Nic) facilitated LTD. Co-application of mecamylamine (Mec, 3 μM) with nicotine prevented LTD. (B) At SC synapses in Mat nic-exposed mice, LFS failed to induce NMDAR-dependent LTD and bath application of 1 μM nicotine had no significant effect on LTD. In (A) and (B), changes in the slope of fEPSPs are plotted as the percentage change from initial baseline responses. Each trace above the graph was recorded before LFS (black) and at 55 min after LFS (red). LTD-inducing stimulation (LFS) and drugs were delivered at the time indicated by the horizontal bars. Scale bars are 10 ms and 1 mV. (C) Histograms show the percent change in the slope of fEPSPs measured 50-55 min after delivery of LFS. *p< 0.05, *** p< 0.001

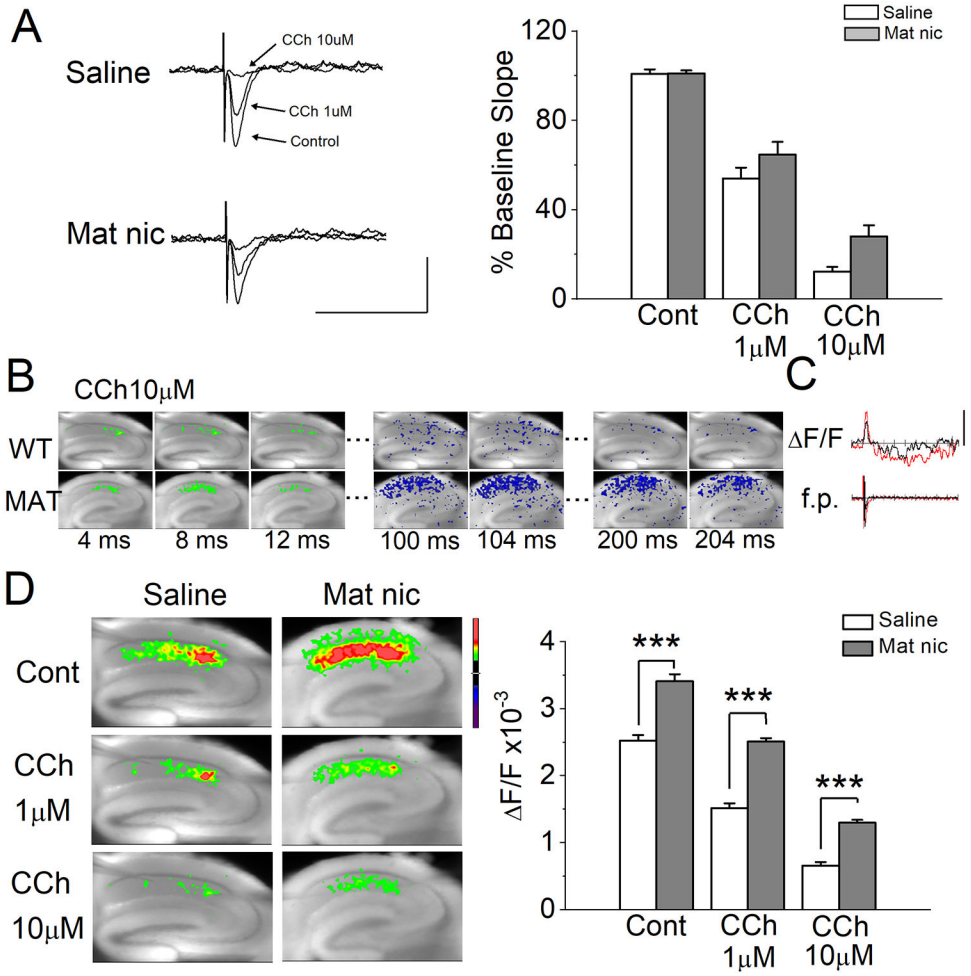

Figure 2. Maternal nicotine exposure does not disrupt mAChR-mediated suppression of excitatory responses

(A) In both saline- and Mat nic-exposed mice, carbachol (CCh) application decreased fEPSPs recorded in the stratum radiatum in response to electrical stimulation of the SC. The stimulus intensity was adjusted to elicit similar sizes of fEPSPs across different slices. (A, left) Representative fEPSP traces recorded in saline (control)- and Mat nic-exposed slices in the absence and presence of 1 μM or 10 μM of CCh. Scale bars are 50 ms and 1 mV. (A, right) A bar graph shows comparisons of the suppressive effects of CCh on the slope of fEPSPs between saline- and Mat nic-exposed slices. Two-way ANOVA: animal group, $F(1, 49) = 5.92$, $p = 0.01$; CCh treatment, $F(2, 49) = 67.02$, $p < 0.001$; interaction, $F(2, 49) = 0.07$, $p = 0.92$ (B) Time courses of voltage sensitive dye imaging detecting changes in neuronal activity following SC stimulation. Representative pseudocolor images (green: depolarization, blue: hyperpolarization) of ΔF/F signals in saline (WT)- and Mat nic (MAT)-exposed slices in the presence of 10 μM CCh are shown. (C) Representative traces of simultaneous optical (ΔF/F) and fEPSP (f.p.) recordings in saline (black)- and Mat nic (red)-exposed slices in the presence of 10 μM CCh. Despite similar sizes of fEPSPs, both depolarizing and hyperpolarizing responses were larger in Mat nic-exposed slices than in saline-treated slices (also see B). Scale bar: 1.0×10^{-3}. (D) In both saline- and Mat

nic-exposed mice, CCh application decreased depolarizing responses. (D, left) Pseudocolor representations of maximum depolarizing responses after SC stimulation, recorded in the absence and presence of 1 μM or 10 μM of CCh, in saline- and Mat nic-exposed slices. Warmer colors indicate depolarization and red indicates the strongest depolarization. (D, right) Comparisons of peak depolarizing optical signals recorded in saline- and Mat nic-exposed slices in the absence and presence of 1 μM or 10 μM of CCh. Maximum depolarizing responses observed in the absence and presence CCh were significantly larger in Mat mic-exposed slices than saline-treated slices. Two-way ANOVA: animal group, $F(1, 47) = 180.55$, $p < 0.001$; CCh treatment, $F(2,47) = 333.91$, $p < 0.001$; interaction, $F(2, 47) = 2.62$, $p = 0.08$

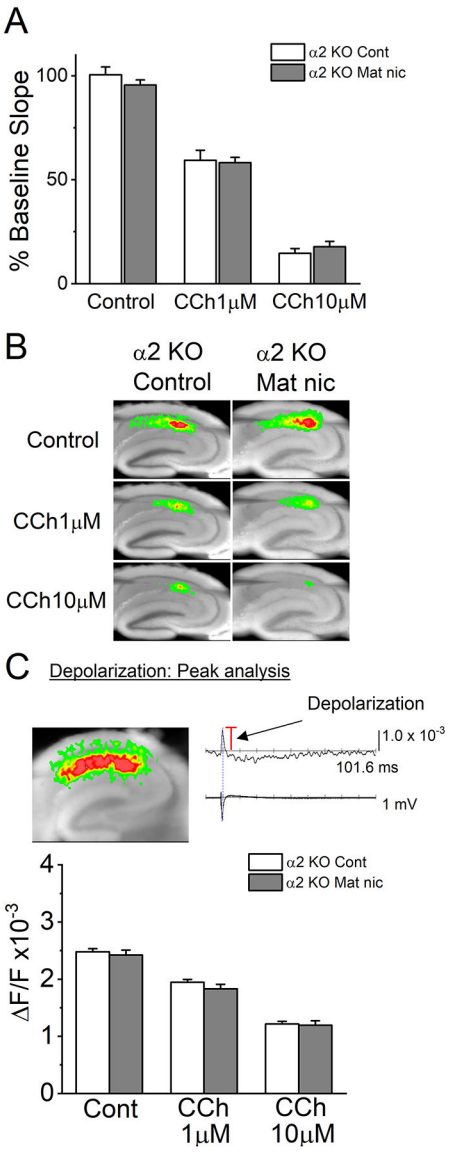

Figure 3. Maternal nicotine exposure does not affect mAChR-mediated suppression of excitatory responses in α2 KO mice

(A) fEPSPs were recorded as in Figure 2A. CCh application decreased fEPSPs in saline- and Mat nic-exposed slices as in wild-type mice. A bar graph shows the percent change in the slope of fEPSPs in saline- and Mat nic-exposed α2 KO mice in the absence and presence of 1 μM or 10 μM CCh, demonstrating that Mat nic exposure does not alter the muscarinic modulation of fEPSPs. Two-way ANOVA: animal group, $F(1,35) = 0.11$, $p = 0.73$; CCh treatment, $F(2,35) = 272.34$, $p < 0.001$; interaction, $F(2,35) = 0.66$, $p = 0.52$ (B) Pseudocolor representations of maximum depolarizing responses after SC stimulation, recorded in the absence and the presence of 1 μM or 10 μM of CCh, in saline- and Mat nic-exposed α2 KO mice. (C) Top, a *schematic illustration of* the measurement of the peak of membrane depolarization. An example of pseudocolor image of maximum depolarizing responses (left) and simultaneous optical (ΔF/F) and fEPSP recordings (right). Bottom, comparisons of peak optical signals recorded in saline- and Mat nic-exposed α2 KO mice in the absence and

presence of 1 μM or 10 μM of CCh. Maximum depolarizing responses observed in the absence and presence CCh and the degrees of suppression by CCh were similar between the two groups. Two-way ANOVA: animal group, $F(1,38) = 0.81$, $p = 0.37$; CCh treatment, $F(2,38) = 105.40$, $p < 0.001$; interaction, $F(2,38) = 0.14$, $p = 0.86$

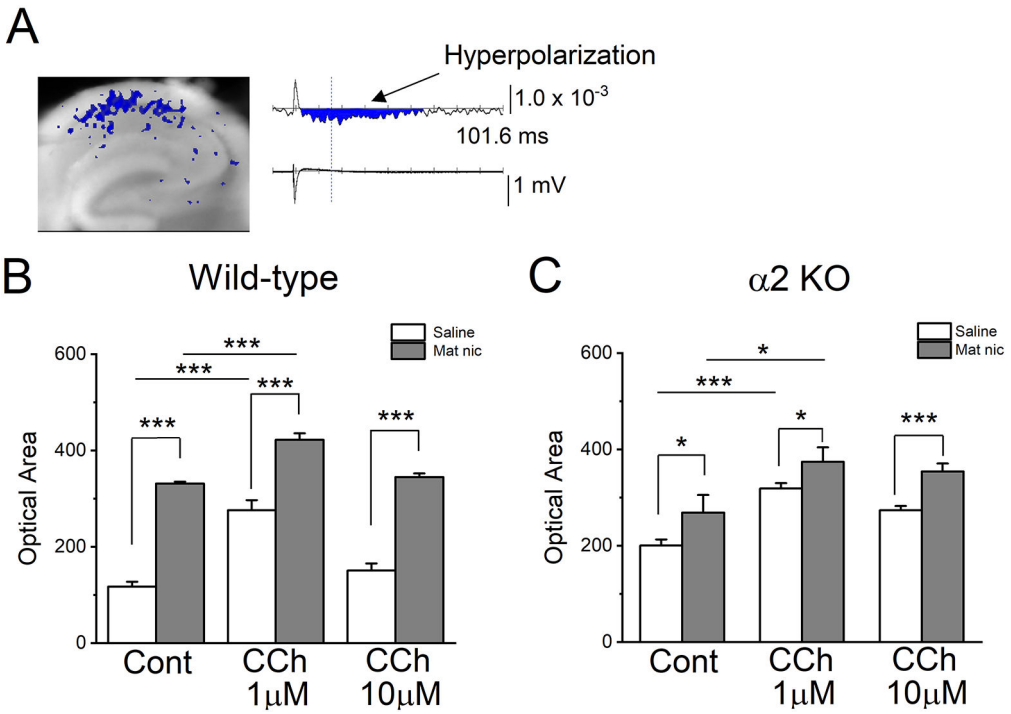

Figure 4. Maternal nicotine exposure and CCh application increase hyperpolarizing responses in wild-type and α2 KO mice

Voltage sensitive dye imaging detecting changes in neuronal activity following SC stimulation was performed as in Figure 2B in slices from saline- and Mat nic-exposed wild-type and α2 KO mice in the absence and presence of 1 μM or 10 μM CCh. (A) A schematic illustration of the measurement of the areas of hyperpolarization (as shown by blue region in optical trace). An example of pseudocolor image of hyperpolarizing optical response (left), alongside simultaneous optical (ΔF/F) and fEPSP recordings (right). (B, C) Histograms show comparisons of the areas of hyperpolarizing responses recorded in slices from saline- and Mat nic-exposed wild-type (B) and α2 KO (C) mice. Mat nic exposure increased hyperpolarizing responses in both wild-type and α2 KO mice. CCh (1 μM) application enhanced hyperpolarizing responses in saline-treated slices from both wild-type and α2 KO mice and the effects of CCh application were still observed in Mat nic-exposed slices from both wild-type and α2 KO mice, suggesting that Mat nic exposure has no significant effect on the muscarinic function. *p< 0.05, *** p< 0.001

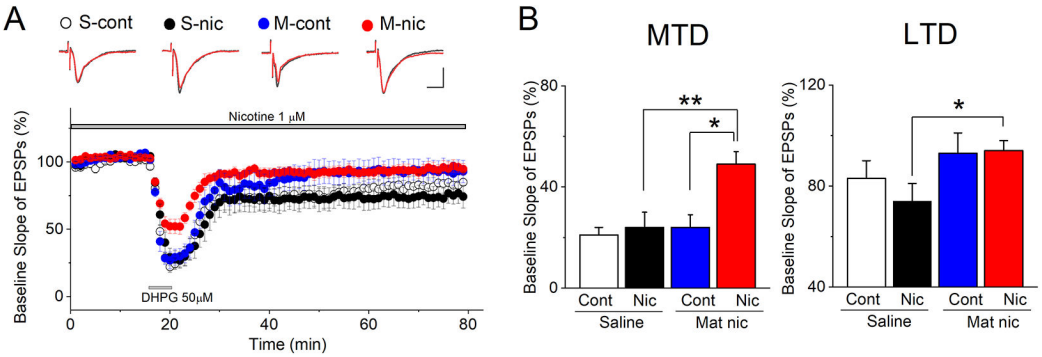

Figure 5. Maternal nicotine exposure has no effect on mGluR-LTD, but alters nicotinic influence on mGluR-LTD

(A) At SC synapses, fEPSPs were recorded as in Figure 2A. DHPG application caused the transient depression of fEPSPs and induced mGluR-dependent LTD that were not significantly different between control and Mat nic-exposed mice. Scale bars are 10 ms and 1 mV, and administration of drugs is indicated by the horizontal bars. Each trace above the graph was recorded before DHPG application (black) and at 50-55 min after DHPG application (red). (B) Histograms show the percent change in the slope of fEPSPs measured 50–55 min after DHPG application. Bath application of nicotine (1 μM) suppressed DHPG-induced maximum transient depression (MTD) of fEPSPs in Mat nic-exposed mice, but not in control mice. In the presence of nicotine, LTD was smaller in Mat nic-exposed slices than in saline-treated slices. *p< 0.05, **p< 0.01