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Nicotine-mediated activation of α2 nAChR-expressing OLM cells in developing mouse brains disrupts OLM cell-mediated control of LTP in adolescence

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Abstract

Early postnatal nicotine exposure, a rodent model of smoking during pregnancy, affects hippocampal synaptic plasticity and memory. Here, we investigated the role of α2 nAChR-expressing OLM (α2-OLM) cells in LTP in unexposed and postnatal nicotine-exposed mice. We found that reduced α2 nAChR-dependent activation of OLM cells in α2 heterozygous knockout mice prevented LTP, whereas enhanced α2 nAChR-dependent activation of OLM cells in heterozygous knockin mice expressing hypersensitive α2 nAChRs facilitated LTP. Both optogenetic and chemogenetic activation of α2-OLM cells facilitated LTP as nicotine did. However, in postnatal nicotine-exposed mice, expressing chemogenetic hM3Dq receptors in α2-OLM cells, LTP was facilitated and both nicotinic and chemogenetic activation of α2-OLM cells prevented rather than facilitated LTP. These results demonstrate a critical role of α2-OLM cell activation in LTP as well as altered α2-OLM cell function in postnatal nicotine-exposed mice. To determine whether nicotine-mediated α2 nAChR activation in developing brains causes facilitated LTP and altered nicotinic modulation of LTP in adolescence, we used homozygous knockin mice expressing hypersensitive α2 nAChRs as a way to selectively activate α2-OLM cells. In the knockin mice, postnatal exposure to a low dose of nicotine, which had no effect on LTP in wild-type mice, is sufficient to cause facilitated LTP and altered nicotinic modulation of LTP as found in wild-type mice exposed to a higher dose of nicotine. Thus, the nicotine-mediated activation of α2 nAChRs on OLM cells in developing brains disrupts the α2-OLM cell-mediated control of LTP in adolescence that might be linked to impaired memory.

Keywords

α2 nicotinic acetylcholine receptor; LTP; Nicotine; OLM cells; Optogenetics; Chemogenetics

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1. Introduction

Children of mothers who smoked during pregnancy are at significantly greater risk for cognitive impairments (Batstra et al., 2003; Bruin et al., 2010; Fried et al., 2003; Thompson et al., 2009). In rodent models of smoking during pregnancy, early perinatal exposure to nicotine alone results in persistent deficits in learning and memory (Ankarberg et al., 2001; Sorenson et al., 1991; Yanai et al., 1992) that appears to involve epigenetic mechanisms (Jung et al., 2016). However, it remains to be understood exactly how transient early life exposure to nicotine causes long-lasting cognitive impairments.

Long-term potentiation (LTP) at Schaffer collateral (SC)-CA1 synapses is generally considered 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). Therefore, when memory is impaired, LTP is normally monitored as a first step to identify the underlying mechanism. Oriens lacunosum moleculare (OLM) cells are GABAergic interneurons located in the hippocampal CA1 region. They can control information flow in the hippocampus, facilitating SC input while weakening the temporoammonic (TA) input (Jia et al., 2009; Leao et al., 2012; Nakauchi et al., 2007). They are unique in the hippocampus in that they express the α2 nicotinic acetylcholine receptor (nAChR) (Ishii et al., 2005; Jia et al., 2010; Jia et al., 2009; Leao et al., 2012; Son and Winzer-Serhan, 2006; Wada et al., 1989), which is the most sparsely expressed nAChR subtype in the brain and continuously activated in the presence of nicotine (Jia et al., 2009). This action of nicotine facilitates LTP at SC-CA1 synapses (Nakauchi et al., 2007) via disinhibition (Leao et al., 2012). Given the complexity of nicotine's action in the brain (Jung et al., 2016) and several other nAChR subtypes are expressed in the hippocampal CA1 region (Kenney and Gould, 2008; Kenney et al., 2012), α2 nAChRs may not be the only nAChR subtype involved in the regulation of LTP. However, because several lines of evidence have implicated critical roles of α2 nAChRs in nicotine's effects on LTP (Leao et al., 2012; Lotfipour et al., 2017; Nakauchi et al., 2007), we here further investigated the roles α2-OLM cells in LTP.

During early postnatal development, GABA is an excitatory neurotransmitter and provides an important excitatory drive in the developing hippocampus (Ben-Ari, 2002; Ben-Ari et al., 2004; Flossmann et al., 2019; Gozlan and Ben-Ari, 2003). During this critical period, α2 mRNA expression in OLM cells is upregulated (Son and Winzer-Serhan, 2006), suggesting that continued excitation of α2 nAChR-expressing OLM (α2-OLM) cells via developmental exposure to nicotine may have critical consequences for circuit development. Indeed, early postnatal nicotine exposure disrupts α2-OLM-mediated nicotinic regulation of LTP at SC-CA1 synapses (Chen et al., 2016; Nakauchi et al., 2015). However, it remains unknown whether the inappropriate excitation of α2-OLM cells via developmental nicotine exposure causes this effect. In this study, we addressed this issue utilizing homozygous knockin mice expressing hypersensitive α2 nAChRs as a way to selectively activate α2-OLM cells during postnatal nicotine exposure.

Postnatal nicotine exposure causes the partial (20–40%) loss of detectable α2 mRNA-expressing cells during adolescence (Chen et al., 2016). Although it is unknown whether this

observation is attributed to purely the downregulation of α2 mRNA expression in OLM cells or involves a loss of α2-OLM cells, the observed finding was associated with the almost complete loss of α2 nAChR function in OLM cells (Chen et al., 2016). Further studies are needed to determine why the partial loss of α2 mRNA causes the almost complete loss of α2 nAChR function in OLM cells. In this study, we utilized α2 heterozygous knockout (KO) mice and α2 heterozygous knockin mice expressing hypersensitive α2 nAChRs to address this issue.

OLM cells receive cholinergic input from the medial septum (Klausberger and Somogyi, 2008; Leao et al., 2012; Lovett-Barron et al., 2014) and glutamatergic input from the local CA1 pyramidal cells (Blasco-Ibanez and Freund, 1995; Booker and Vida, 2018). Thus, they are excited via ionotropic and Gq-coupled metabotropic cholinergic and glutamatergic receptors (Hagger-Vaughan and Storm, 2019). However, because it is difficult to selectively activate these receptors expressed in α2-OLM cells by conventional pharmacological approaches, it is unknown whether the activation of these receptors facilitates LTP at SC-CA1 synapses as α2 nAChR activation, and whether early postnatal nicotine exposure disrupts their control of LTP. In this study, we investigated whether optogenetic or chemogenetic activation of α2-OLM cells affects LTP in unexposed and postnatal nicotine-exposed mice.

OLM cells are thought to be critical mediators of the formation of hippocampus-dependent memories (Lovett-Barron et al., 2014; Schmid et al., 2016; Siwani et al., 2018). Furthermore, both maternal nicotine-treated wild-type mice (Nakauchi et al., 2015) and untreated α2 nAChR KO mice (Kleeman et al., 2016) show impairments in long-term object location memory, a CA1-dependent task (Assini et al., 2009; Barrett et al., 2011; Haettig et al., 2013; Lopez et al., 2016). Thus, further understanding the role of α2-OLM cells in LTP at SC-CA1 synapses and how early postnatal nicotine exposure alters their role in LTP may reveal the mechanism underlying maternal nicotine-induced memory impairment.

2. Materials and methods

2.1. Animals

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. We used Chrna2 KO mice (Lotfipour et al., 2013) and Chrna2 knockin (α2L9'S) mice, expressing hypersensitive α2 nAChRs (Lotfipour et al., 2017), created by Drs. Shahrdad Lotfipour (currently at UCI) and Jim Boulter at UCLA. In addition, we used Chrna2-Cre mice (MMRRC, Stock No: 036502-UCD), Ai32(RCL-ChR2(H134R)/EYFP) mice (JAX Stock No: 024109), and R26-hM3Dq/mCitrine mice (JAX Stock No: 026220). Chrna2-Cre (α2-Cre) mice were crossed with Ai32(RCL-ChR2(H134R)/EYFP) mice (floxed ChR2 mice) or R26-hM3Dq/mCitrine mice (floxed hM3Dq mice) to generate Chrna2-ChR2 (α2-ChR2) mice or Chrna2-hM3Dq (α2-hM3Dq) mice, respectively. α2 heterozygous KO ($\alpha2^{+/-}$) mice, hypersensitive α2L9'S heterozygous and homozygous mice were identified by PCR-based genotyping as described previously (Lotfipour et al., 2013, 2017). Chrna2 KO, Chrna2-Cre, and Ai32(RCL-ChR2(H134R)/EYFP) mice were determined to be congenic

on C57BL/6J. R26-hM3Dq/mCitrine mice were backcrossed to C57BL/6J for at least 3 generations since the strain was obtained from JAX. α2L9'S mice were backcrossed to C57BL/6J for 6 generations after the strain was obtained from Drs. Shahrdad Lotfipour and Jim Boulter.

2.2. Early postnatal nicotine treatment

Female heterozygous α2-Cre mice were crossed with male floxed hM3Dq mice. Litters with different genotypes were adjusted to be five pups total without knowing their sex. Maternal nicotine-exposed α2-hM3Dq mice were identified by PCR-based genotyping. Wild-type mouse litters and α2L9'S homozygous mouse litters were also adjusted to be five pups total without knowing their sex. For experiments involving hypersensitive α2L9'S mouse mothers, after parturition, pups from hypersensitive α2L9'S mouse mothers were fostered to CD1 mouse mothers that had either nicotine or saline minipumps. Five pups (without knowing their sex) from one litter were fostered together to one CD1 dam. Pups were exposed to nicotine through maternal milk during postnatal days 1–15 by subcutaneously implanting nursing CD1 dams with Alzet osmotic minipumps (DURECT, Model 1002; approximate nicotine output: 21 mg/kg/day, 2.1 mg/kg/day, or 0.21 mg/kg/day) as described previously (Kleeman et al., 2016; Nakauchi et al., 2015). Nicotine concentrations are expressed as free-base and we refer to these pups as maternal nicotine-exposed mice. Although the fostering could lead to stress during an early postnatal period, we used this approach to avoid possible differences in maternal care between saline pump- and nicotine pump-implanted hypersensitive α2L9'S mouse mothers as they exhibit hypersensitivity to nicotine. However, the fostering does not eliminate possible differences in maternal care between saline pump- and nicotine pump-implanted CD1 mothers during nicotine exposure. We used mini pumps, because we have previously found that mouse pups exposed to nicotine via the milk of dams implanted nicotine pumps show impairments in long-term object location memory in adolescence (Nakauchi et al., 2015). To investigate the underlying mechanisms, we used the same method of nicotine delivery.

2.3. Maternal behavior

To examine the effect of nicotine on maternal care, 4 dams were implanted with osmotic minipumps continuously releasing nicotine (21 mg/kg/day) or with osmotic minipumps that delivered saline. Observations of dam-pups interaction were conducted from postnatal day 2 to postnatal day 7 as described (Own & Patel, 2013; Orso et al., 2018). Daily observations were carried out six times over a period of 18 min at 3-minute intervals and were performed 3 times a day (9 a.m., 1:00p.m., and 5:00p.m.), totaling 18 daily observations. The behavior of dams was scored as either maternal behaviors (pup handling, licking, nursing, covering, and nesting nursing, licking pups, and contact with pups in the nest) or non-maternal behavior (movement outside/inside nest, grooming, and eating/drinking) by direct visual observations. The percentage of maternal behavior was calculated for each dam and used to compare between nicotine- and saline-exposed dams.

2.4. Extracellular field recordings

LTP experiments were carried out as described previously (Kleeman et al., 2016; Nakauchi et al., 2015). Transverse hippocampal slices were prepared from mice (age 4–6 weeks)

anesthetized with isoflurane inhalation. 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 continually superfused at 2–3 mL/min with oxygenated ACSF at 30°C. A bipolar stimulating electrode was placed at the SC pathway, and the slice was 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 MΩ). 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 μA). 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 theta burst stimulation (weak TBS; two theta bursts of four pulses at 100 Hz) was used to monitor the induction of LTP. To evaluate LTP magnitude, the mean values of the slopes of fEPSPs from 40–50 min after weak TBS were calculated and expressed as a percentage of the mean baseline fEPSPs slopes. Nicotine (1 μM, 0.1 μM, 0.01 μM; Sigma-Aldrich) and clozapine dihydrochloride (5 μM; Hello Bio Inc,) were dissolved in ACSF and bath-applied for approximately 10 min. ChR2 in α2-OLM cells was activated by illumination of hippocampal slices with blue LED light pulses at 10 Hz for 1 min, as OLM cells fire action potentials at approximate theta frequencies (4–12 Hz) in the presence of nicotine (Jia et al., 2009). In experiments, n represents the number of slices. In general, we used two slices from one animal, one for control and the other for acute nicotine/other treatment effect. However, in some experiments, we collected data from one slice per animal due to unexpected technical problems during recording. Both males and females were used in experiments. However, because litters were adjusted to be five pups total without knowing their sex, different numbers of males and females were included in experimental groups. As a rule, at least two males and two females were included in an experimental group. As LTP recordings from male and female mice yielded equivalent results, their data were combined for statistical analysis.

2.5. Immunohistochemistry

α2-ChR2 and α2-hM3Dq mice were perfused with cold saline and 4% paraformaldehyde, and 40 μm thick coronal brain sections were obtained. Fluorescent immunohistochemistry was performed with anti-somatostatin (Sst) rabbit antibody (Peninsula Laboratories, T-4103, 1:1000) by following the standard procedure (Cell Signaling Technology). After washing, sections were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, A11072, 1:1000) secondary antibody. Native EYFP and mCitrine fluorescence were detected to assess the expression of ChR2 and hM3Dq, respectively. Sections were mounted in Vectashield (Vector Laboratories) and fluorescent images were captured using the fluorescence microscope (BIOREVO, Keyence).

2.6. Quantitative Real-Time PCR

We analyzed α2 mRNA levels in wild-type, α2 heterozygous KO, and α2 homozygous KO mice by real-time qRT-PCR. Five hippocampal RNA preparations per each experimental

group were performed using the TRIzol reagent (Invitrogen) and was then used for real-time qRT-PCR with QuantiNova Probe RT-PCR Kit (QIAGEN). Primers used for qPCR reactions were TaqMan Gene Expression Assays (Thermo Fisher Scientific): α2, Mm00460629_m1; glyceraldehyde-3-phosphate dehydrogenase, Mm03302249_g1. Real-time qRT-PCR reactions were carried out according to manufacturer's instructions using the following parameters: 10 min at 45°C, 5 min at 95°C, 40 cycles at 5 sec at 95°C and 30 sec at 60°C. All reactions were performed in triplicate. All data were normalized to glyceraldehyde-3-phosphate dehydrogenase levels as an internal control. Comparison between groups were made using the delta-delta Ct method (Livak and Schmittgen, 2001). The results were presented relative to the control average Ct values.

2.7. Statistical analysis

Electrophysiological data were normalized relative to baseline, expressed as mean ± SEM, and analyzed for significance using a Student's t-test, one-way or two-way ANOVA with post hoc Tukey's test. The percentage of maternal behavior was calculated and data were analyzed for significance using a Student's t-test. A comparison was considered statistically significant if $p < 0.05$. All physiological data were plotted and analyzed using Origin 8.1 (OriginLab).

3. Results

3.1. α2 nAChRs in OLM cells play an essential role in nicotinic facilitation of LTP

Firing of α2-OLM cells causes GABA release onto stratum radiatum interneurons, disinhibiting CA1 pyramidal cells at the SC inputs to facilitate LTP (Leao et al., 2012). In wild-type mice, acute nicotine (1 μM) application facilitates LTP in the SC input to CA1 (Nakauchi et al., 2007) via this mechanism. This nicotinic facilitation of LTP is absent in α2 homozygous KO ($\alpha2^{-/-}$) mice (Nakauchi et al., 2007) and occurs in mice expressing hypersensitive α2L9'S nAChRs with 0.01 μM nicotine application, which is ineffective in wild-type mice (Lotfipour et al., 2017). In the current study, we utilized α2 heterozygous KO mice to determine whether reduced α2 nAChR expression affects nicotinic facilitation of LTP. We found that nicotine-induced facilitation of LTP was absent (Fig. 1A: control, 102.0 ± 3.8%, n = 7, vs. 1 μM nicotine, 101.1 ± 2.8%, n = 7; T(12) = 0.18, p = 0.86) as in α2 homozygous KO ($\alpha2^{-/-}$) mice (Nakauchi et al., 2007), suggesting that one functional α2 gene cannot compensate for a defective one. Although we cannot assume that a level of α2 nAChR expression is reduced linearly with one defective gene, our findings suggest that the expression of α2 nAChRs in the heterozygous KO mice is reduced to the level sufficient to prevent nicotine-induced LTP facilitation.

Subsequently, we examined hypersensitive α2L9'S heterozygous ($\alpha2^{+/L9'S}$) mice to see whether a partial gain of α2 nAChR function is sufficient for nicotinic facilitation of LTP. We first confirmed our previous results that 1 μM, but not 0.1 μM, nicotine facilitates LTP in wild-type mice (Fig. 1B: $F_{(2, 20)} = 16.8$, p = 5.28E-05; control, 106.2 ± 2.7%, n = 6, vs. 1 μM nicotine, 158.0 ± 9.9%, n = 9, p <0.001; control, 106.2 ± 2.7%, n = 6 vs. 0.1 μM nicotine, 108.1 ± 4.6%, n = 8, p = 1.0; one-way ANOVA with post hoc Tukey's test). We then examined whether lower concentrations of nicotine facilitate LTP in hypersensitive

α2L9'S heterozygous ($\alpha2^{+/L9'S}$) mice. We found that 1 and 0.1 μM nicotine, which is ineffective in wild-type mice, facilitated LTP (Fig. 1C: $F_{(3, 19)} = 36.8$, p = 4.13E-08; control, 98.8 ± 0.9%, n = 5, vs. 1 μM nicotine, 136.3 ± 3.7%, n = 6, p <0.001; control, 98.8 ± 0.9%, n = 5, vs. 0.1 μM nicotine, 125.6 ± 2.8%, n = 7, p <0.001; one-way ANOVA with post hoc Tukey's test). However, 0.01 μM nicotine, which facilitates LTP in hypersensitive α2L9'S homozygous mice ($\alpha2^{L9'S/L9'S}$) (Lotfipour et al., 2017), had no effect on LTP (Fig. 1C: control, 98.8 ± 0.9%, n = 5, vs. 0.01 μM nicotine, 103.2 ± 2.8%, n = 5, p = 0.74). These results add to the accumulating evidence that α2 nAChRs in OLM cells play an essential role in nicotinic facilitation of LTP (Leao et al., 2012; Lotfipour et al., 2017; Nakauchi et al., 2007), and suggest that altered expression of α2 nAChRs in OLM cells critically affects LTP at SC-CA1 synapses.

3.2. Both optogenetic and chemogenetic activation of α2-OLM cells facilitated LTP

α2-OLM cell firing can disinhibit CA1 pyramidal cells to facilitate LTP at SC-CA1 synapses (Leao et al., 2012). OLM cells are excited via ionotropic and Gq-coupled metabotropic cholinergic and glutamatergic receptors (Hagger-Vaughan and Storm, 2019). However, because the selective activation of these receptors on OLM cells is difficult to achieve, it is unknown whether the activation of these receptors on OLM cells facilitates LTP at SC-CA1 synapses as α2 nAChR activation. To gain insights into the role of other receptors in LTP at SC-CA1 synapses, we tested whether optical activation of channelrhodopsin-2 (ChR2) and chemogenetic activation of hM3Dq DREADD (designer receptors exclusively activated by designer drug) in α2-OLM cells facilitate LTP. It was previously reported that optical activation of ChR2, expressed in α2 nAChR-containing cells in α2-Cre mice using an adeno-associated viral vector, facilitates LTP (Leao et al., 2012). To gain further insight into the role of α2-OLM cell activation in LTP, we crossed α2-Cre mice with floxed ChR2 (RCL-ChR2(H134R)/EYFP) mice to produce α2-ChR2-EYFP mice. In the hippocampal CA1 region of these mice, the majority of EYFP-positive cells and fibers were almost exclusively located in the stratum oriens/arveus (Fig. 2A, left), where α2 mRNA-expressing cells are detected by in situ hybridization (Ishii et al., 2005; Jia et al., 2010; Son and Winzer-Serhan, 2006; Wada et al., 1989) and the dendrites of OLM cells extend horizontally. In addition, the stratum lacunosum-moleculare, where OLM cell axons densely ramify, was EYFP-positive (Fig. 2A, left). There appears to be a small number of EYFP-positive cells in other areas of the hippocampus, suggesting ectopic expression. We have previously demonstrated that α2-OLM cells express somatostatin (Sst) mRNA (Jia et al., 2010). Immunohistochemical analysis of the hippocampal CA1 region of α2-ChR2 mice revealed that many Sst-positive cells in the stratum oriens/arveus (Fig. 2A, middle) were co-localized with EYFP-positive cells and fibers (Fig. 2A, right). These observations indicate that α2-OLM cells express ChR2-EYFP. We have previously demonstrated that OLM cells fire action potentials at approximate theta frequencies (4–12 Hz) in the presence of nicotine (Jia et al., 2009). We are interested in determining whether OLM cell firing at theta frequencies by different excitatory drive facilitates LTP. We found that the activation of ChR2 in α2-OLM cells by illumination of hippocampal slices with blue LED light pulses at 10 Hz for 1 min facilitated LTP at SC-CA1 synapses in α2-ChR2 mice (Fig. 2B: $F_{(2, 17)} = 16.9$, p <0.001; without blue light, 97.3 ± 1.5%, n = 5, vs. with blue light, 124.5 ± 7.4%, n = 5, p <0.001, one-way ANOVA with post hoc Tukey's test), mimicking the facilitative

effect of nicotine on LTP. While blue light was no effect on LTP in floxed ChR2 mice (Fig. 2B: floxed ChR2 mice, 100.2 ± 1.0%, n=10, vs. α2-ChR2 mice, 124.5 ± 7.4%, n = 5, p <0.001%), confirming that the effect of blue light was mediated by the activation of ChR2 in Cre-expressing α2-OLM cells. These results suggest that different excitatory drive causing OLM cell firing at theta frequencies facilitates LTP. However, it does not necessarily translate into a physiological mechanism involving excitatory ligand-gated ion channels.

Activation of M1/M3, but not M2/M4, muscarinic receptors and the group I mGluRs expressed in α2-OLM cells have been shown to cause depolarizing spike afterpotentials and spontaneous and persistent firing (Lawrence et al., 2006; Hagger-Vaughan and Storm, 2019). They are all coupled to Gq. We are interested in determining whether the activation of M1/M3 muscarinic receptors and the group I mGluRs expressed in α2-OLM cells facilitates LTP as α2 nAChR activation. However, because these receptors are also expressed in other neurons, including pyramidal cells, it is difficult to selectively activate M1/M3 muscarinic receptors and the group I mGluRs expressed in α2-OLM cells by conventional pharmacological approaches during LTP experiments. hM3Dq is a modified form of the human M3 muscarinic receptor with two mutations that can be activated by clozapine-N-oxide (CNO), stimulating the Gq signaling pathway. Therefore, to gain insight into the role of Gq-coupled receptors in α2-OLM cells in LTP at SC-CA1 synapses, we crossed α2-Cre mice with floxed hM3Dq (R26-hM3Dq/mCitrine) mice to express hM3Dq DREADDs in α2-OLM cells. Immunohistochemical analysis of the hippocampal CA1 region of α2-hM3Dq mice revealed that the hM3Dq-mCitrine-positive cells (Fig. 3A, left) in the stratum oriens/arveus were Sst-positive (Fig. 3A, middle, right), indicating that α2-OLM cells express hM3Dq. There appears to be mCitrine-positive cells in other areas of the hippocampus, suggesting ectopic expression as in α2-ChR2 mice (Fig. 2A). In α2-hM3Dq mice, acute nicotine application facilitated LTP at SC-CA1 synapses (Fig. 3B, D: control, 99.1 ± 0.5%, n = 5, vs. 1 μM nicotine, 123.2 ± 5.1%, n = 13, p<0.05, two-way ANOVA with post hoc Tukey's test), suggesting that α2 nAChR-mediated control of LTP is normal. Furthermore, we found that hM3Dq activation by the synthetic ligand CNO (5 μM) mimicked the facilitative effect of nicotine on LTP (Fig. 3B, D: control, 99.1 ± 0.5%, n = 5, vs. 5 μM CNO, 129.2 ± 5.2%, n = 11, p <0.01). Thus, these results suggest that the activation of Gq-coupled receptors on α2-OLM cells can facilitate LTP at SC-CA1 synapses. However, it remains to be determined whether the finding represents a physiological mechanism involving native M1/M3 muscarinic receptors and the group I mGluRs that exists in α2-OLM cells.

3.3. LTP was facilitated in maternal nicotine-exposed α2-hM3Dq mice, and both nicotine and CNO application prevented LTP

In maternal nicotine-exposed wild-type mice (21 mg/kg/day), LTP is facilitated and acute nicotine inhibits rather than facilitates LTP, suggesting that maternal nicotine exposure disrupts normal α2 nAChR-mediated control of LTP (Nakauchi et al., 2015). To gain further insight into the altered control of LTP, we examined whether maternal nicotine exposure also alters the control of LTP by Gq-coupled receptors on α2-OLM cells. For the study, we crossed α2-Cre mice with floxed hM3Dq mice and pups were exposed to nicotine through maternal milk by subcutaneously implanting nursing dams with Alzet osmotic minipumps

(21 mg/kg/day). Maternal nicotine exposed-α2-hM3Dq mice were identified by genotyping. We then examined whether maternal nicotine exposure alters hM3Dq-mediated control of LTP at SC-CA1 synapses in α2-hM3Dq mice. We found that exposure of α2-hM3Dq mouse pups to nicotine caused LTP facilitation (Fig. 3C, D: unexposed control, 99.1 ± 0.5%, n = 5, vs. maternal nicotine-exposed control, 134.6 ± 4.8%, n = 10, p <0.01, two-way ANOVA with post hoc Tukey's test) and acute nicotine (1 μM) prevented LTP (Fig. 3C, D: maternal nicotine-exposed control, 134.6 ± 4.8%, n = 10, vs. acute nicotine, 100.4 ± 2.6%, n = 5, p <0.01) as found in maternal nicotine-exposed wild-type mice (Nakauchi et al., 2015). Furthermore, we found that CNO application also prevented LTP (Fig. 3C, D: maternal nicotine-exposed control, 134.6 ± 4.8%, n = 10, vs. CNO, 111.7 ± 5.6%, n = 10, p <0.05), suggesting that maternal nicotine exposure disrupts the control of LTP by not only α2 nAChRs, but also Gq-coupled receptors on α2-OLM cells. However, it remains to be determined whether maternal nicotine exposure disrupts the function of native M1/M3 muscarinic receptors and the group I mGluRs in α2-OLM cells.

3.4. A lower dose of nicotine still caused LTP facilitation and altered nicotinic control of LTP in hypersensitive α2 nAChR homozygous knockin mice

We have previously demonstrated that weak theta burst stimulation, which is sub-threshold for LTP induction in hippocampal slices from wild-type mice, is able to induce LTP in the presence of 1 μM nicotine. However, in maternal nicotine-exposed wild-type mice (21 mg/kg/day), weak theta burst stimulation alone is capable of inducing LTP and this LTP is prevented in the presence of 1 μM nicotine (Nakauchi et al., 2015). The effects of maternal nicotine exposure were prevented by co-administration of the nonselective nAChR antagonist mecamylamine, suggesting that the nicotine-mediated activation, but not desensitization, of nAChRs in developing brains causes the effects (Chen et al., 2016). To determine whether the activation of α2 nAChRs on OLM cells during maternal nicotine exposure caused LTP facilitation and altered nicotinic control of LTP in adolescence, we used mutant mice expressing hypersensitive α2 nAChRs, which can be selectively activated with low-dose of nicotine too low to activate other nAChR subtypes in neonatal brains. In this way, we are able to determine the role of α2 nAChR activation in the maternal nicotine-induced changes. As controls, we first examined maternal saline-exposed hypersensitive α2L9'S homozygous mice. In the hippocampus of these mice, weak theta burst stimulation alone failed to induce LTP, but induced LTP in the presence of 1 μM nicotine (Fig. 4A, D: control, 94.1 ± 3.7%, n = 5, vs. 1 μM nicotine, 138.5 ± 8.2%, n = 7, p <0.001, two-way ANOVA with post hoc Tukey's test) as in wild-type mice. In maternal nicotine-exposed α2L9'S mice (21 mg/kg/day), LTP was facilitated (Fig. 4B, D: maternal saline-exposed control, 94.1 ± 3.7%, n = 5, vs. maternal nicotine-exposed control, 131.8 ± 3.9%, n = 6, p <0.001) and acute nicotine application (1 μM) prevented LTP (Fig.4B, D: control, 131.8 ± 3.9%, n = 6, vs. 1 μM nicotine, 99.3 ± 2.3%, n = 6, p <0.001; 1 μM nicotine in maternal saline-exposed α2L9'S mice, 138.5 ± 8.2%, n = 7, vs. 1 μM nicotine in maternal nicotine-exposed α2L9'S mice, 99.3 ± 2.3%, n = 6, p <0.001) as in maternal nicotine-exposed (21 mg/kg/day) wild-type mice (Nakauchi et al., 2015). These results demonstrate that the effects of maternal nicotine exposure occur in both wild-type and α2L9'S mice at 21 mg/kg/day dose. We also examined whether 1/100 dose of nicotine (0.21 mg/kg/day) has the effect, and found that LTP was not facilitated and acute nicotine application induced

LTP (Fig. 4C, D: control, 100.3 ± 3.4%, n = 5, vs. 1 μM nicotine, 143.1 ± 3.3%, n = 4, p <0.001; maternal nicotine-exposed (21 mg/kg/day) control, 131.8 ± 3.9%, n = 6, vs. maternal nicotine-exposed (0.21 mg/kg/day) control, 100.3 ± 3.4%, n = 5, p <0.001; 1 μM nicotine in maternal nicotine-exposed (21 mg/kg/day) mice, 99.3 ± 2.3%, n = 6, vs. 1 μM nicotine in maternal nicotine-exposed (0.21 mg/kg/day) mice,143.1 ± 3.3%, n = 4, p <0.001). The results suggest that α2L9'S nAChR activation does not occur in developing brains at the dose. We then used 1/10 dose of nicotine (2.1 mg/kg/day) to treat nursing wild-type mothers. In maternal nicotine-exposed wild-type mice at this dose, we found that LTP was not facilitated and acute nicotine application induced LTP (Fig. 4E, G: control, 102.7 ± 3.0%, n = 9, vs. 1 μM nicotine, 130.6 ± 5.0%, n = 9, p <0.01, two-way ANOVA with post hoc Tukey's test) as in saline-exposed wild-type mice (Nakauchi et al., 2015). These results suggest that the activation of the nAChR subtype responsible for the maternal nicotine-induced alterations in LTP does not occur in wild-type mouse pups at the dose. In contrast, when the same dose was used to expose α2L9'S mouse pups, we found that LTP was facilitated (Fig. 4F, G: saline-exposed α2L9'S mice, 94.1 ± 3.7%, n = 5, vs. maternal nicotine-exposed α2L9'S mice, 128.0 ± 6.3%, n = 11, p <0.01; maternal nicotine-exposed wild-type mice, 102.7 ± 3.0%, n = 9, vs. maternal nicotine-exposed α2L9'S mice, 128.0 ± 6.3%, n = 11, p <0.01) and acute nicotine application (1 μM) prevented LTP (Fig.4F, G: control, 128.0 ± 6.3%, n = 11, vs. 1 μM nicotine, 98.3 ± 0.8%, n = 8, p <0.01; 1 μM nicotine in maternal nicotine-exposed wild-type mice, 130.6 ± 5.0%, n = 9, vs. 1 μM nicotine in maternal nicotine-exposed α2L9'S mice, 98.3 ± 0.8%, n = 8, p <0.01; 1 μM nicotine in saline-exposed α2L9'S mice, 138.5 ± 8.2%, n = 7, vs. 1 μM nicotine in maternal nicotine-exposed α2L9'S mice, 98.3 ± 0.8%, n = 8, p <0.01). These results demonstrate that the activation of α2 nAChRs on OLM cells in developing brains is the cause of altered LTP. In our experiments, we fostered the hypersensitive α2L9'S pups to wild-type dams to avoid possible differences in maternal care between saline pump- and nicotine pump-implanted hypersensitive α2L9'S mothers. However, the fostering does not eliminate possible differences in maternal care between saline pump- and nicotine pump-implanted wild-type mothers during nicotine exposure. Therefore, we observed maternal behavior and compared the index of maternal care between dams implanted with nicotine and saline minipumps. The results revealed that there was no significant group difference (Fig. 4H; Saline, 86.0 ± 2.0%, n =4, vs. Nicotine, 86.0 ± 2.6%, n = 4, t(6) = 0.00, p = 1.00), suggesting that maternal care was not significantly affected during nicotine exposure.

4. Discussion

Acute nicotine application facilitates LTP in the hippocampal CA1 region. Although several nAChR subtypes, including α2-, α3-, α4-, α5-, and α7-containing nAChR subtypes are expressed in the hippocampal CA1 region (Alkondon and Albuquerque, 2001; Jia et al., 2010; Kenney and Gould, 2008), accumulating evidence suggests that this effect of nicotine requires α2 nAChR activation (Leao et al., 2012; Lotfipour et al., 2017; Nakauchi et al., 2007). In the current study, we found that the effect of nicotine was absent in α2 heterozygous KO mice and was observed in hypersensitive α2L9'S heterozygous mice with a lower concentration of nicotine, which is ineffective for LTP facilitation in wild-type mice.

These results confirm the essential role of α2 nAChR activation in nicotine-induced LTP facilitation.

Maternal nicotine exposure caused the partial (20–40%) loss of detectable α2 mRNA-expressing cells that was associated with the almost complete loss of α2 nAChR function in OLM cells (Chen et al., 2016). It is possible that the reduction of α2 mRNA results in decreased cell surface expression of α2 nAChRs due to a less efficient subunit assembly or cellular trafficking. Our current study demonstrates that the facilitative effect of nicotine on LTP is absent in α2 heterozygous KO mice. Inactivation of α2 gene was accomplished via insertion of a neomycin cassette into exon 5, but not complete elimination of α2 gene (Lotfipour et al., 2013). Because α2 mRNA containing a neomycin cassette is likely expressed, we are unable to determine whether the expression of α2 gene is reduced linearly with one defective α2 gene. Although it remains to be determined whether this mouse line has lower α2 subunit protein level, our findings suggest that small changes in α2 mRNA expression may have a profound impact on functional α2 nAChRs expression. However, we are unable to exclude the possibility that the change observed in α2 heterozygous KO mice is due to developmental adaptations to low α2 subunit protein expression.

OLM cells receive glutamatergic input from the local CA1 pyramidal cells (Blasco-Ibanez and Freund, 1995; Booker and Vida, 2018) and are excited via ionotropic and Gq-coupled metabotropic glutamate receptors (Le Duigou et al., 2015; Le Vasseur et al., 2008; Viaene et al., 2013). OLM cells also receive cholinergic input from the medial septum (Klausberger and Somogyi, 2008; Leao et al., 2012; Lovett-Barron et al., 2014) and are excited by nicotinic (Jia et al., 2009; Leao et al., 2012) and Gq-coupled muscarinic receptors (Lawrence et al., 2006; Le Duigou et al., 2015). These receptors have previously been reported to facilitate LTP at glutamatergic synapses on OLM cells (Jia et al., 2010; Le Duigou et al., 2015; Le Vasseur et al., 2008; Nicholson and Kullmann, 2021), potentially enhancing OLM cell output to postsynaptic cells. Our current study shows that early nicotine exposure disrupts not only α2 nAChR function, but also hM3Dq function in α2-OLM cells. The data implicate functions of Gq-coupled muscarinic and metabotropic glutamate receptor in OLM cells as being altered by maternal nicotine exposure. This implies that maternal nicotine exposure disrupts not only the excitatory control of OLM cells, but also their output to postsynaptic cells, resulting in hippocampal circuit dysfunction. However, in the current study, as we only tested hM3Dq-mediated modulation, it remains to be tested whether the data are relevant to native Gq-coupled muscarinic and metabotropic glutamate receptors in OLM cells.

Our current study demonstrates that OLM cell excitation via ChR2 and hM3Dq activation facilitates LTP at SC-CA1 synapses, suggesting that the properly timed activation of cholinergic and glutamatergic receptors on OLM cells could facilitate LTP at SC-CA1 synapses via the disinhibition mechanism. OLM cells can respond as coincidence detectors of glutamatergic and cholinergic input, resulting in strongly enhanced output to postsynaptic cells (Hagger-Vaughan and Storm, 2019). Furthermore, OLM cells are unique in that they express the α2 nAChR (Jia et al., 2010; Leao et al., 2012), which is continuously activated in the presence of nicotine (Jia et al., 2009). Thus, in the presence of nicotine, the depolarization mediated by glutamatergic input will result in greater depolarization than

that induced by the activation of glutamatergic input alone. Enhanced OLM cell output to postsynaptic cells could more easily facilitates LTP at SC-CA1 synapses. Furthermore, our results demonstrate that maternal nicotine exposure disrupts this critical role of OLM cell function in CA1 circuits.

CA1 pyramidal cells receive two major excitatory inputs, one from area CA3 (the SC pathway) and the other from the the entorhinal cortex (TA pathway). Excitation of OLM cells inhibits CA1 pyramidal cells on distal apical dendrites, which are the terminus of the TA pathway, and disinhibits pyramidal cells on proximal dendrites (Leao et al., 2012), where the SC pathway terminates. Thus, activation of OLM cells can shift the balance of information flow into the CA1 region (Jia et al., 2009; Leao et al., 2012; Nakauchi et al., 2007), decreasing direct entorhinal cortex layer III inputs (from the TA pathway) in favor of indirect entorhinal cortex layer II inputs (from the trisynaptic circuit, ending with SC input from the CA3). Our results suggest that the OLM cell-mediated control of information flow is disrupted in maternal nicotine-exposed mice. Cholinergic activation of OLM cells is required for encoding hippocampus-dependent contextual fear conditioning (Lovett-Barron et al., 2014) and chemogenetic silencing of OLM cells impairs memory (Schmid et al., 2016). Maternal nicotine exposure, which decreases the expression of α2 mRNA, impairs the normal function of α2 nAChRs (Chen et al., 2016) and hippocampus dependent memory (Nakauchi et al., 2015). Furthermore, α2 KO mice, like mice that are exposed to early postnatal nicotine, show impairments in long-term object location memory (Kleeman et al., 2016). Thus, the loss of normal function of α2-OLM cells in CA1 circuits might underlie maternal-nicotine-induced impairment of hippocampus dependent memory.

Early postnatal exposure of mouse pups to nicotine via maternal milk impairs hippocampus-dependent memory during adolescence (Nakauchi et al., 2015). In these mice, LTP at the SC pathway is not diminished, but unexpectedly facilitated (Nakauchi et al., 2015). Furthermore, acute nicotine inhibits rather than facilitates LTP. The suppressive effect of acute nicotine was blocked by the non-specific nAChR antagonist mecamylamine, but not by DHβE, an antagonist of β2-containing nAChRs, or by MLA, an antagonist of α7 nAChRs (Nakauchi et al., 2015). We have previously shown that LTP facilitation by acute nicotine is absent in α2 and β2 KO mice and inhibited by DHβE, in wild-type mice (Nakauchi and Sumikawa, 2012). These findings suggest that maternal nicotine exposure alters nAChR or circuit function to such a degree that a different nAChR subtype now plays the dominant role in LTP control, with the completely opposite effect.

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, might have contributed to the observed physiological changes in pups. However, selective effects of postnatal nicotine treatment on α2 nAChR function (Chen et al., 2016) without significantly affecting muscarinic function (Nakauchi et al., 2021), suggest a specific action of nicotine in the developing brain. In addition, we did not observe obvious abnormal maternal behaviors under conditions used. We are unable to exclude the possibility that the hypersensitive alpha2 nAChRs also induce developmental changes due to hypersensitivity to acetylcholine. Our previous (Lotfipour et al., 2013) and current studies demonstrate that 1 μM nicotine facilitates LTP in hypersensitive α2

nAChR homozygous knockin mice as in wild-type mice. In maternal nicotine-exposed hypersensitive α2 nAChR homozygous knockin mice (21 mg/kg/day), LTP was facilitated and acute nicotine application (1 μM) prevented LTP, as found with nicotine-treated wild-type mice. Thus, it appears that the control mechanism of LTP is not significantly affected by hypersensitivity to acetylcholine. In the current study, we found that a lower dose of nicotine, which did not cause the LTP-related changes in wild-type mice, in hypersensitive α2L9'S mice causes the same LTP related changes as found in wild-type mice exposed to a higher dose of nicotine. These findings suggest that nicotine-mediated OLM cell excitation via α2 nAChRs in the developing brain is the cause of these maternal nicotine-induced changes.

During early postnatal development, GABA is an excitatory neurotransmitter and can generate spontaneous activity in the developing hippocampal circuits (Ben-Ari et al., 1989; Flossmann et al., 2019) that appears to be essential for proper circuit development (Tolner et al., 2012; Zhang et al., 2011). Activation of α2 nAChRs on OLM cells can increase intracellular Ca^{2+} levels at least in part via Ca^{2+} entry through their channels (Jia et al., 2010). Thus, our current study strongly suggests that disturbances of early circuit activity and Ca^{2+} signaling by the inappropriate activation of α2-OLM cells can lead to persistent alterations in OLM cell excitability and circuit activity, affecting LTP and memory in adolescence.

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Abbreviations:

ACSF	artificial cerebrospinal fluid
ChR2	channelrhodopsin-2
CNO	clozapine-N-oxide
DREADD	designer receptors exclusively activated by designer drug
fEPSPs	field excitatory postsynaptic potentials
KO	knockout
LTP	long-term potentiation
nAChR	nicotinic acetylcholine receptor
OLM	oriens-lacunosum moleculare
SC	Schaffer collatera
Sst	somatostatin
TA	temporoammonic

TBS	theta burst stimulation

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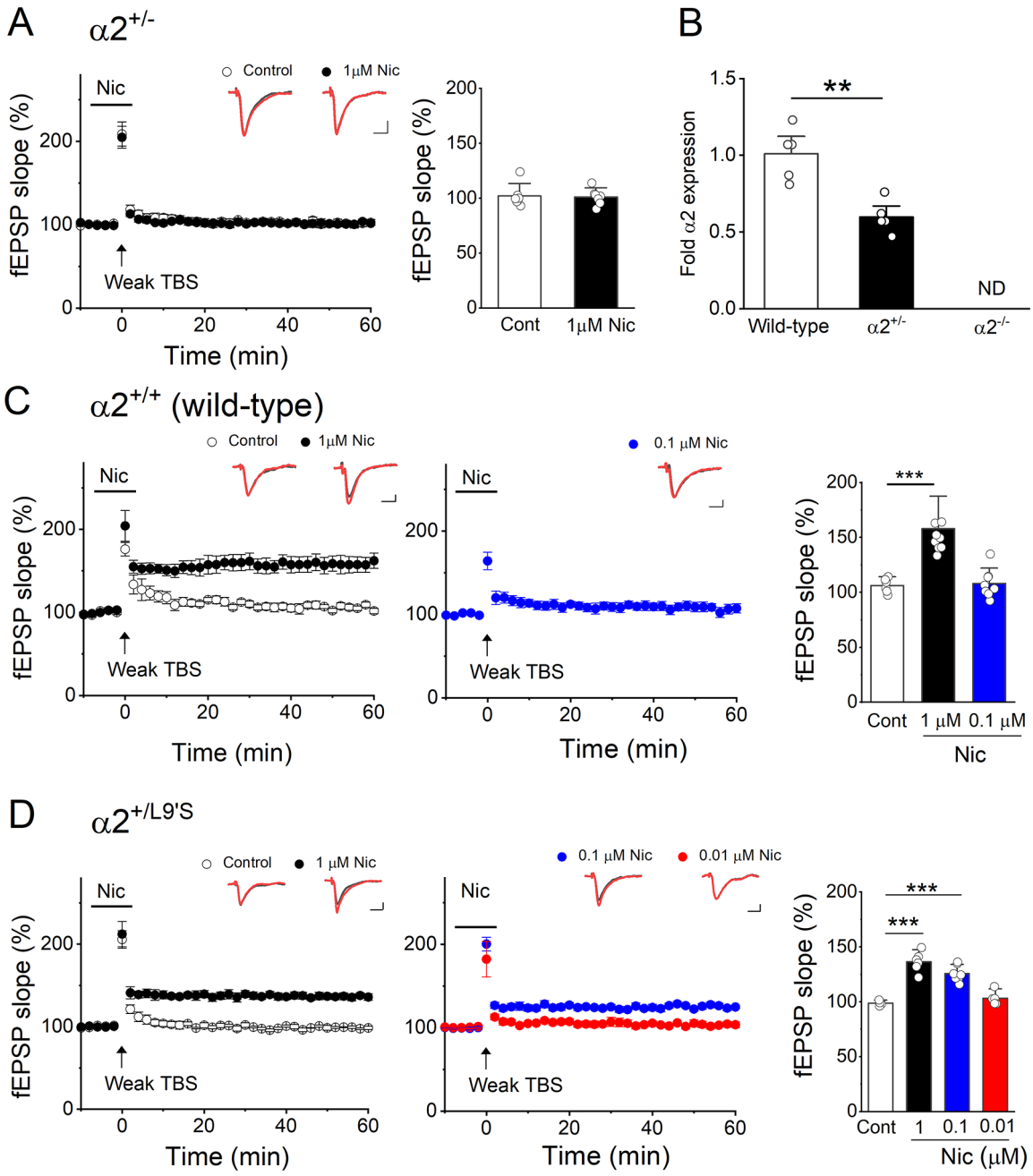

Figure 1. α2 nAChRs in OLM cells play an essential role in nicotine-induced LTP facilitation

(A) Acute nicotine (1 μM) application had no effect on LTP in α2 heterozygous KO ($\alpha2^{+/-}$) mice. (B) The real-time RT-PCR results confirmed that a level of α2 transcript is reduced with one defective α2 gene ($\alpha2^{+/-}$ mice). α2 transcript was not detected (ND) in $\alpha2^{-/-}$ mice. (C) Bath application of 1 μM, but not 0.1 μM, nicotine facilitated LTP in wild-type mice. (C) Acute application of 1 μM and 0.1 μM, but not 0.01 μM, nicotine facilitated LTP in hypersensitive α2L9'S heterozygous ($\alpha2^{+/L9'S}$) mice. In (A),(C), (D) and the following figures, changes in the slope of fEPSPs were plotted as the percentage change from initial baseline responses. Each trace above the graph was recorded before (black) and at 55 min

after weak TBS (red). Weak TBS and drugs were delivered at the time indicated. Scale bars are 10 ms and 1 mV. Bar graphs show the percent change in the slope of fEPSPs measured 50–55 min after delivery of weak TBS. *** $p < 0.001$

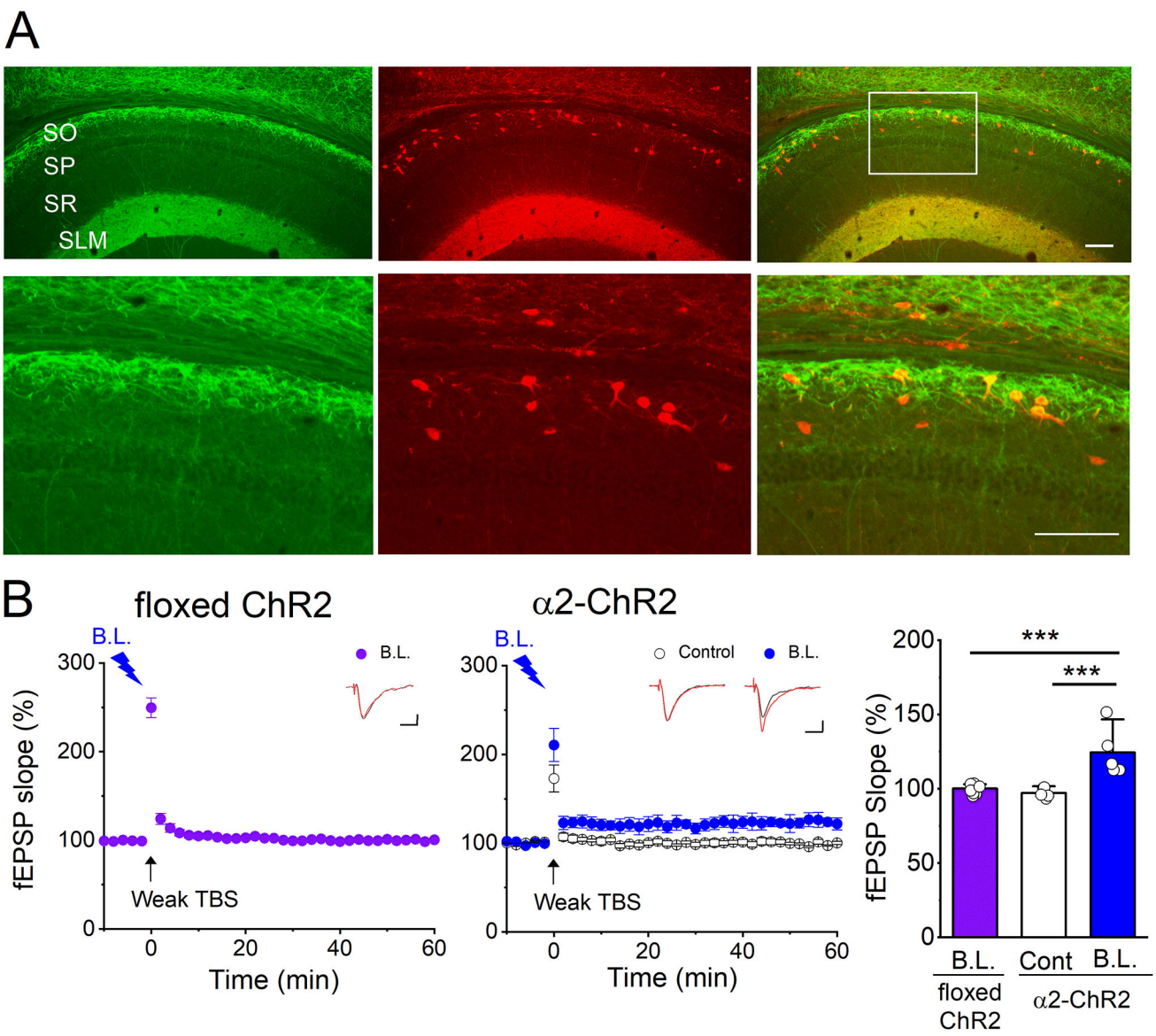

Figure 2. Optogenetic activation of α2-OLM cells facilitated LTP

(A) Native EYFP fluorescence (left, green) were detected to assess the expression of ChR2-EYFP in the hippocampal CA1 region. There are EYFP-positive fibers in the stratum oriens/arveus (SO), where the dendrites of OLM cells extend horizontally, and the stratum lacunosum-moleculare (SLM), where OLM cell axons densely ramify. SP, the stratum pyramidale; SR, the stratum radiatum. In the same section, somatostatin-expressing cells were detected with an anti-somatostatin antibody (middle, red). The merged image is shown on the right (scale bar, 100 μm). Magnified images of the boxed area are shown below the low-power images. (B) In floxed ChR2 mice, blue light had no effect on LTP (left). In α2-ChR2 mice, blue light facilitated LTP (middle). A bar graph shows the percent change in the slope of fEPSPs measured 50–55 min after delivery of weak TBS (right). One-way ANOVA with post hoc Tukey's test, *** $p < 0.001$

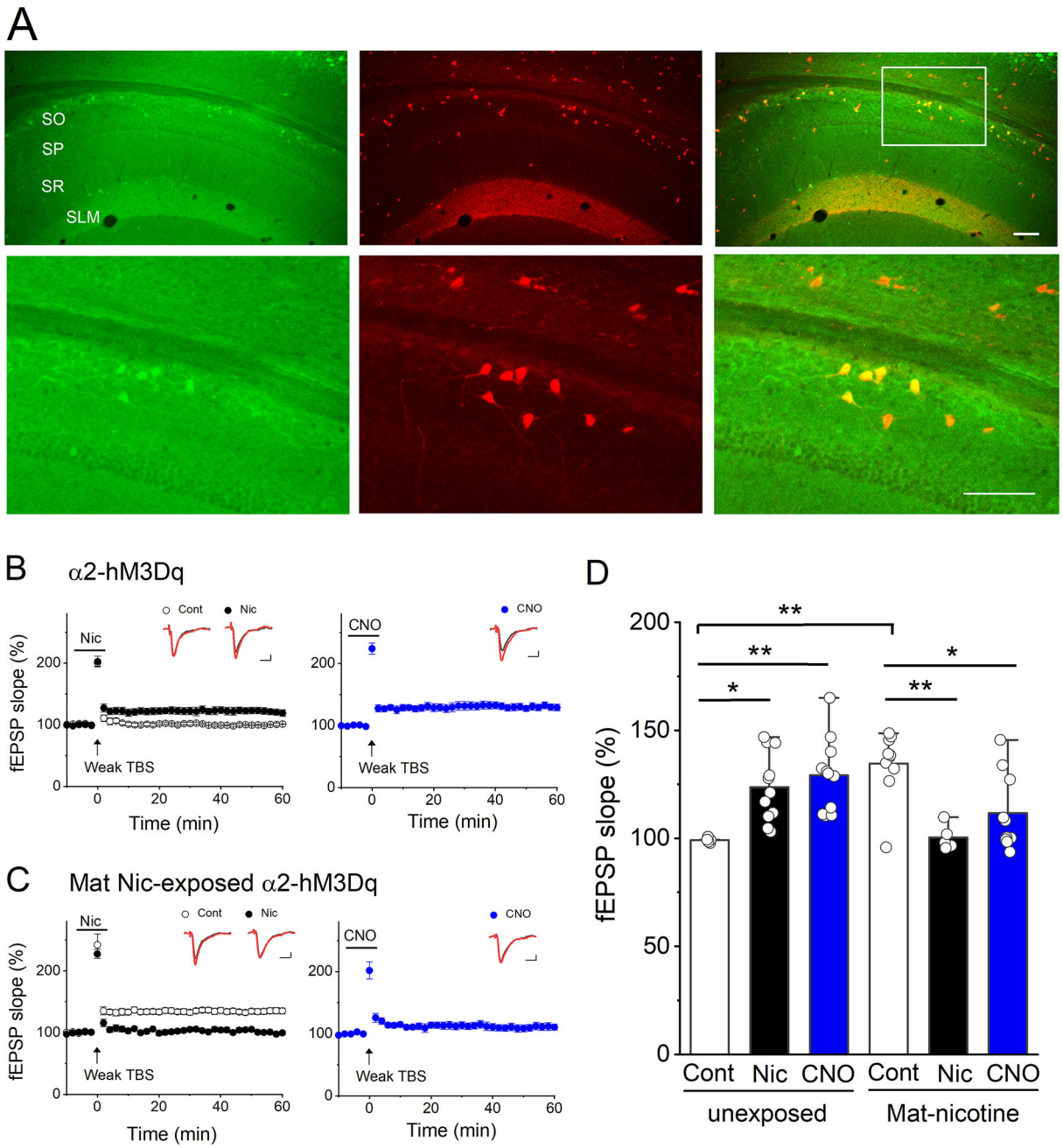

Figure 3. Maternal nicotine exposure alters the chemogenetic control of LTP in α2-hM3Dq mice

(A) Native mCitrine fluorescence (left, green) was detected to assess hM3Dq-mCitrine expression in the hippocampal CA1 region. There are mCitrine-positive cells in the stratum oriens/arveus (SO), where OLM cells are located (left, green). SP, the stratum pyramidale; SR, the stratum radiatum; SLM, the stratum lacunosum-moleculare. In the same section, somatostatin-expressing cells were detected with an anti-somatostatin antibody (middle, red). The merged image shows that mCitrine-positive cells in the stratum oriens/arveus express somatostatin (right, scale bar, 100 μm). Magnified images of the boxed area are shown below the low-power images. (B) In α2-hM3Dq mice, bath application of nicotine

(1 μM) facilitated LTP as found in wild-type mice (left). Activation of hM3Dq by CNO (5 μM) also facilitated LTP (right). (C) In maternal nicotine-exposed α2-hM3Dq mice, LTP was facilitated and acute nicotine inhibited rather than facilitated LTP (left) as found in maternal nicotine-exposed wild-type mice. Bath application of CNO (5 μM) inhibited rather than facilitated LTP (right). (D) A bar graph shows the percent change in the slope of fEPSPs measured 50–55 min after delivery of weak TBS. Two-way ANOVA treatment effect (unexposed, Mat-nicotine): $F_{(1, 46)} = 0.15$, $p = 0.70$; acute drug effect (control, nicotine, CNO): $F_{(2, 46)} = 1.27$, $p = 0.29$; interaction effect: $F_{(2, 46)} = 16.12$, $p < 0.001$. Two-way ANOVA with post hoc Tukey's test, *$p< 0.05$, ** $p< 0.01$

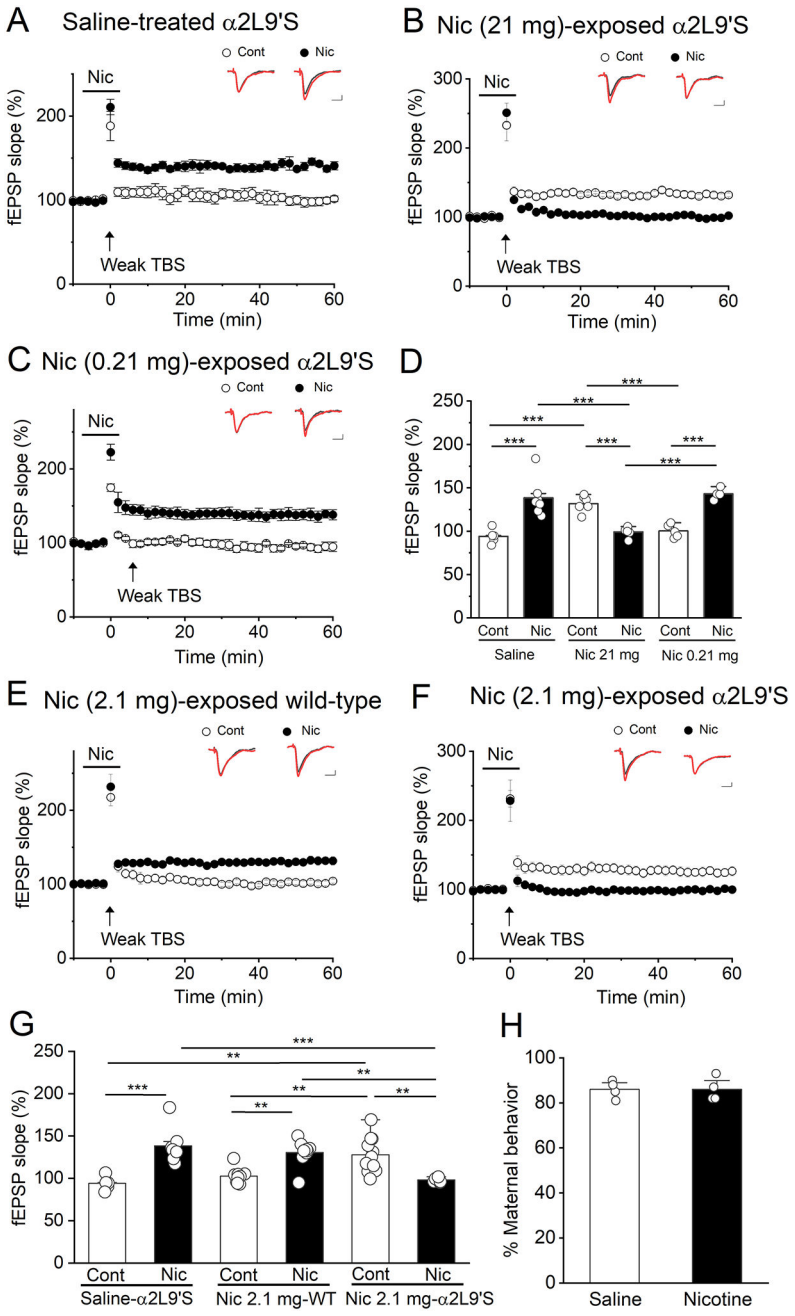

Figure 4. Maternal nicotine-mediated α2 nAChR activation on OLM cells causes LTP facilitation and altered nicotinic control of LTP in adolescence

(A) In maternal saline-treated α2L9'S mice, bath application of nicotine (1 μM) facilitated LTP. (B) In maternal nicotine-exposed (21 mg/kg/day) α2L9'S mice, weak TBS alone induced LTP and acute nicotine (1 μM) application prevented LTP. (C) In maternal nicotine-exposed α2L9'S mice with a very low dose (0.21 mg/kg/day), weak TBS alone had no effect on LTP and bath application of nicotine (1 μM) facilitated LTP as in untreated wild-type mice. (D) A bar graph shows the percent change in the slope of fEPSPs measured 50–55 min after delivery of weak TBS. Two-way ANOVA treatment effect (saline, Nic 21 mg, Nic 0.21 mg): $F_{(2, 27)} = 0.73$, $p = 0.49$; acute drug effect (control, nicotine): $F_{(1, 27)} = 17.71$,

p <0.001; interaction effect: $F_{(2, 27)}$ = 36.78, p < 0.001. Two-way ANOVA with post hoc Tukey's test, ***p< 0.001 (E) In maternal nicotine-treated wild-type mice with a lower dose (2.1 mg/kg/day), weak TBS alone had no effect on LTP and bath application of nicotine (1 μM) facilitated LTP as in untreated wild-type mice. (F) In maternal nicotine-exposed α2L9'S mice with the low dose (2.1 mg/kg/day), weak TBS alone induced LTP and acute nicotine (1 μM) application prevented LTP as in maternal nicotine-exposed α2L9'S mice with the high dose (21 mg/kg/day). (G) A bar graph shows the percent change in the slope of fEPSPs measured 50–55 min after delivery of weak TBS. Saline-treated α2L9'S data used in D were reused in G for comparisons. Two-way ANOVA treatment effect (saline, WT-Nic 2.1 mg, α2L9'S-Nic 2.1 mg): $F_{(2, 43)}$ = 0.28, p = 0.76; acute drug effect (control, nicotine): $F_{(1, 43)}$ = 10.03, p <0.01; interaction effect: $F_{(2, 43)}$ = 26.19, p < 0.001. Two-way ANOVA with post hoc Tukey's test, **p< 0.01, *** p< 0.001. (H) Effects of nicotine exposure on the index of maternal behavior. No significant difference was observed when the index of maternal behavior from the saline and nicotine dams was compared.