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Nicotine and a positive allosteric modulator of m1 muscarinic receptor increase NMDA/AMPA ratio in the hippocampus and medial prefrontal cortex

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Keywords: Nicotine, VU0453595, m1 muscarinic receptor, NMDA/AMPA ratio, pyramidal cells, parvalbumin interneuron

Abbreviations: Abbreviations: ACSF, artificial cerebrospinal fluid; ACh, acetylcholine; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Chronic nicotine exposure, ChrNic; Chronic VU0453595 exposure, ChrVU; DMSO, dimethylsulfoxide; EPSCs, excitatory postsynaptic currents; fEPSPs, field excitatory postsynaptic potentials; GABA, γ-aminobutyric acid; IR-DIC, infrared differential interference contrast; LE, Long Evans; LTP, long-term potentiation; m1 receptor, m1 muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; NMDAR, N-methyl-D-aspartate receptor; NRG1, neuregulin 1; PAM, positive allosteric modulator; PB, phosphate buffer; PBS, phosphate-buffered saline; mPFC, medial prefrontal cortex; Pv, parvalbumin; SC pathway, Schaffer collateral pathway; SD, Sprague-Dawley; SR, stratum radiatum; TBS, theta burst stimulation

Abstract

Chronic nicotine exposure has been shown to improve memory in rodents. However, the molecular mechanism for such an enhancement remains poorly understood. Chronic nicotine exposure increases NMDA/AMPA ratio due to enhanced NMDAR-mediated responses in hippocampal CA1 pyramidal cells and facilitates LTP. Here, we found that the same nicotine treatment increases NMDA/AMPA ratios in parvalbumin-expressing interneurons in the hippocampus and in layer 5 pyramidal cells in the medial prefrontal cortex (mPFC) of male and female rats. To gain further insight into the nicotine-initiated signaling pathway, we used a positive allosteric modulator (PAM) of m1 muscarinic acetylcholine receptor (m1 receptor), VU0453595. We found that chronic VU0453595 treatment mimics the effects of chronic nicotine exposure, causing increased NMDA/AMPA ratio in hippocampal CA1 pyramidal cells and LTP facilitation. Furthermore, chronic exposure to VU0453595 also caused increased NMDA/AMPA ratio in layer 5 pyramidal cells of mPFC. As the PAM only activates m1 receptors when the endogenous agonist acetylcholine (ACh) is present, the findings suggest that the release of ACh from cholinergic neurons is involved in the effect. Thus, chronic nicotine exposure, by increasing ACh release, may stimulate a signaling pathway in various neuron types, which receive cholinergic input and express m1 receptors, leading to the enhancement of NMDAR responses. The nicotine-initiated signaling pathway, in which ACh and m1 receptors are downstream of nicotinic ACh receptor activation, may represent an important cholinergic pathway involved in cognitive function.

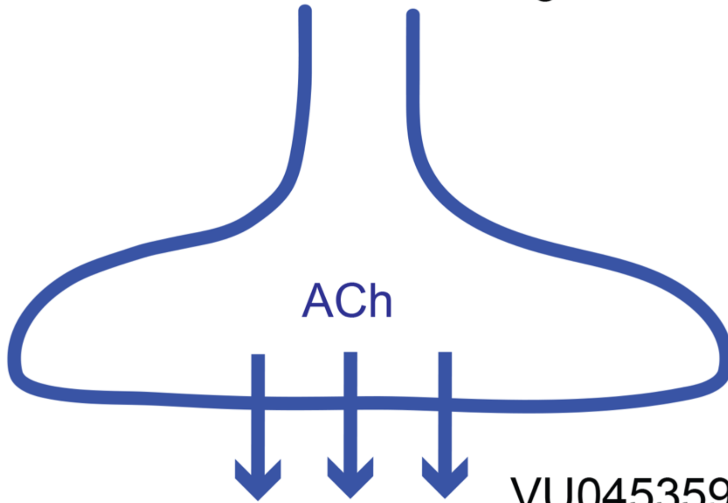
Highlights

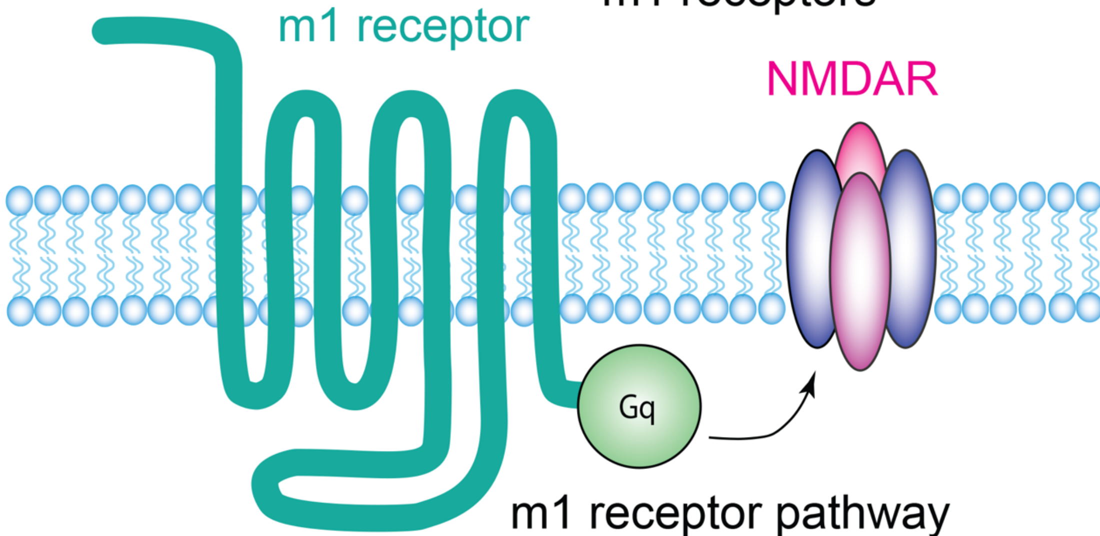
Chronic nicotine exposure increased NMDA/AMPA ratios in CA1 pyramidal cells and Pv-expressing interneurons

Chronic administration of m1 receptor PAM increased NMDA/AMPA ratio in CA1 pyramidal cells

Chronic administration of m1 receptor PAM facilitated LTP as chronic nicotine administration

Both chronic nicotine and m1 receptor PAM exposure increased NMDA/AMPA ratio in layer 5 pyramidal cells in the mPFC


Nicotine excites cholinergic neuron
ACh


VU0453595 activates
m1 receptors
m1 receptor
NMDAR
Gq
m1 receptor pathway
enhances NMDAR response

Introduction

Smoking remains the most important preventable cause of premature death. Although tobacco use has declined in the general population, smoking prevalence among patients with schizophrenia or other mental illnesses that can cause cognitive deficits is still high. Thus, medication development for the treatment of nicotine dependence in the patient population is urgently required. Nicotine is the main active ingredient in tobacco, and although it is an addictive substance, it has also been found to have cognitive benefits in individuals, including schizophrenia patients (Barr et al., 2008; Heishman et al., 1994; Levin, 1992; Rezvani and Levin, 2001). It has therefore been suggested that schizophrenics smoke heavily, despite negative health consequences, to self-medicate cognitive dysfunction. Elucidation of chronic nicotine-induced neuroplasticity mechanisms may provide novel insights into the etiology and treatment of this condition, with the added benefit of facilitating smoking cessation among people with schizophrenia.

The N-methyl-D-aspartate receptor (NMDAR) plays an important role in learning and memory and NMDAR hypofunction has been implicated in pathophysiology of schizophrenia (Rompala et al., 2013; Snyder and Gao, 2013; Zierhut et al., 2010). Therefore, a potential motivator of tobacco smoking is the enhancement of NMDAR responses. Indeed, we have previously found that chronic nicotine exposure enhances NMDAR responses in CA1 pyramidal cells (Ishibashi et al., 2014; Yamazaki et al., 2006a; Yamazaki et al., 2006b; Yamazaki et al., 2006c). Given that schizophrenia is genetically driven, NMDAR hypofunction most likely occurs in multiple neuron types. While hippocampal dysfunction is observed in individuals with schizophrenia, there is extensive dysfunction throughout the brain, including prefrontal cortical regions (Manoach, 2003; Zierhut et al., 2010). Thus, it is important to determine whether the chronic nicotine-

initiated signaling occurs widely in different neuron types. In the current study, we examined whether the nicotine-initiated signaling occurs in layer 5 pyramidal neurons in the medial prefrontal cortex (mPFC). In addition, as NMDAR hypofunction in inhibitory interneurons is implicated in pathophysiology of schizophrenia (Nakazawa et al., 2012), we also examined whether the nicotine-initiated signaling occurs in parvalbumin (Pv)-expressing interneurons in the hippocampal CA1 region.

Interestingly, chronic exposure to two acetylcholinesterase inhibitors, donepezil and galantamine, and the m1 muscarinic acetylcholine receptor (m1 receptor) agonist RS86 enhance NMDAR responses (Ishibashi et al., 2014). Furthermore, co-administration of the m1 receptor antagonist pirenzepine prevents the effect of cholinergic drugs, including nicotine (Ishibashi et al., 2014). These findings suggest a common cholinergic pathway driving the enhancement. However, the link between nicotine and m1 receptors remains to be established. One possible link is through the excitation of cholinergic neurons by nicotinic acetylcholine receptor (nAChR) activation, which causes the release of acetylcholine (ACh). This in turn stimulates m1 receptor-mediated signaling pathway, leading to the enhancement of NMDAR responses. To gain further insight into the link, as a positive allosteric modulator (PAM) of m1 receptor only activates m1 receptors when the endogenous agonist ACh is present, we examined the effects of chronic exposure to an m1 receptor PAM (VU0453595) on NMDAR-mediated responses in CA1 pyramidal cells and layer 5 pyramidal cells of mPFC, and on hippocampal long-term potentiation (LTP).

Methods

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 wild-type male and female Sprague-Dawley (SD) rats (P28-35). In addition, to identify Pv-expressing interneurons in hippocampal slices for whole-cell recordings, we used Pv-tdTomato male and female rats (P28-42) generated by crossing Pv-Cre mouse line, LE-Tg (Pvalb-iCre)2Ottc (RRRC#773), and floxed tdTomato reporter mouse line, LE-Rosa26 (CAG-LSL-TdTomato)em1Rrrc (RRRC# 938). These transgenic rats are the Long Evans (LE) rat strain. Pv-tdTomato rats were identified by PCR-based genotyping, following the protocols provided by RRRC.

Immunohistochemistry

To verify that tdTomato-positive neurons in Pv-tdTomato rats are Pv-expressing neurons, we carried out immunofluorescent staining. Pv-tdTomato rats were perfused with cold saline and 4% paraformaldehyde, and 40 μm thick coronal brain sections were obtained. Fluorescent immunohistochemistry was performed with parvalbumin (E8N2U) XP® rabbit mAb (Cell Signaling Technology, #80561, 1:1000) by following the standard procedure (Cell Signaling Technology). After washing, sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11008, 1:1000) secondary antibody. Sections were mounted in Vectashield Variance (Vector Laboratories, H-1500) and fluorescent images were captured using a fluorescence microscope (BIOREVO, Keyence). The green channel was used to visualize antibody immunoreactivity and the red channel was used to visualize native tdTomato fluorescence.

Drug treatments

The current study was designed based on our previous results (Ishibashi et al., 2014; Yamazaki et al., 2006a; Yamazaki et al., 2006b; Yamazaki et al., 2006c). Although our model of chronic nicotine exposure, repeated injection of nicotine, may not correspond to the pharmacokinetic characteristics of smoking, we used the same treatment regimen to achieve the same effect of chronic nicotine exposure. Male and female rats were injected subcutaneously with either vehicle or nicotine free-base (~0.5 mg/kg, s.c.) twice daily for at least 7 days and up to 15 days, a treatment known to enhance NMDAR-mediated responses (Yamazaki et al., 2006a), facilitate LTP induction (Fujii et al., 1999; Yamazaki et al., 2006a) and improve spatial learning in the Morris water maze (Abdulla et al., 1996). Smoking several cigarettes delivers an acute dose of 60-300 nM nicotine in the venous blood (Benowitz NL, 1990) and approximately 600 nM nicotine in the arterial blood (Henningfield et al., 1993), which better represents the level of nicotine in the brain. In rats, plasma concentrations of nicotine reach peak values (2.2 μM) within 5-10 min after nicotine (1 mg/kg) administration into the femoral vein, decrease to 0.9 μM 20 min after administration and are maintained at this level for the next 40 min (Sastry et al., 1995). Because the plasma half-life of nicotine in rats is about 45 min and it is about 2 hours in humans (Matta et al., 2007; Sastry et al., 1995), the lower dose (0.5 mg/kg) that was used for subcutaneous injection (intended to be absorbed slowly) in the current study should produce blood levels of nicotine like those found in heavy smokers. In addition, chronic administration of the m1 receptor PAM VU0453595 (MedChemExpress, HY-120023; 3 mg/kg, s.c., twice daily for 7- 10 days) was also carried out. We selected this dose because VU0453595 enhances object recognition in rats at this dose (Moran et al., 2018). The PAM was dissolved in dimethylsulfoxide (DMSO; TCI America, D5293) and diluted in a vehicle of 20% hydroxypropyl-beta-cyclodextrin, (TCI America, H0979) in 100 mM sodium acetate (pH5.4). The final DMSO concentration was no greater than 10%. As a control, either phosphate-buffered saline (PBS) or DMSO-based vehicle was injected. As electrophysiological recordings from PBS- and DMSO-based vehicle-

treated control rats yielded equivalent results, in some experiments, their data were combined for statistical analysis.

Extracellular field recording

Ninety minutes after the last injection of drug, transverse hippocampal slices (~400 μm) were prepared from male and female rats (P28-42) anesthetized with isoflurane inhalation. Slices were maintained at 30˚C for at least 1 h before recordings 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. LTP experiments were carried out as described previously (Chen et al., 2016). Slices were submerged in a recording chamber and continually superfused at 2-3 mL/min with oxygenated ACSF at 30˚C. A bipolar stimulating electrode was placed at the Schaffer Collateral Pathway (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 (SR) 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 (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. In experiments, n represents the number of slices. In general, we used two slices from one animal. 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, as LTP recordings from male and female rats yielded equivalent results, their data were combined for statistical analysis.

Whole-cell recording

Transverse hippocampal slices (~400 um) and coronal brain slices (~400 um) including the prefrontal region were prepared from male and female rats (P28-42) anesthetized with isoflurane inhalation as described for extracellular field recordings. Excitatory postsynaptic currents (EPSCs) were recorded using the whole-cell patch clamp technique as described previously (Ishibashi et al., 2014; Yamazaki et al., 2006a). Slices were placed in a recording chamber on Olympus BX51 microscope stage, submerged, and continuously superfused at 1-2 ml/min with oxygenated ACSF at 30°C. Neurons were visualized for whole-cell recording using a 40× water-immersion objective and an infrared differential interference contrast (IR-DIC) system. tdTomato-expressing neurons in slices were visualized using epifluorescence optics and IR-DIC. Voltage-clamp recordings were then made from the somatic region of CA1 pyramidal cells, Pv interneurons and layer 5 pyramidal cells of mPFC in the presence of the γ-aminobutyric acid $(GABA)_A$ receptor antagonist bicuculline (10 μM), which blocks GABAergic synaptic transmission. The patch electrodes were pulled from borosilicate glass (World Precision Instrument, Sarasota, FL, USA) using a micropipette puller (P-97, Sutter Instrument, Novato, CA, USA). The patch pipettes (5-7 MΩ) were filled with solution containing (in mM) 117 Cs-methanesulfonate, 10 HEPES, 0.5 EGTA, 2.8 NaCl, 5 TEA-Cl, 5 QX-314, 2.5 Mg-ATP, and 0.3 Na-GTP, adjusted to pH 7.3 with CsOH. EPSCs were evoked in CA1 pyramidal cells and Pv interneurons by electrical stimulation of axons in the SR. EPSCs were collected at holding potentials of -70 and +40 mV. While EPSCs were recorded in layer 5 pyramidal cells of mPFC at -70 and +60 mV by stimulating layer 5 approximately 100 μm away from the recording electrode. EPSCs were measured once every 20 seconds and approximately 10

measurements were taken at each holding potentials to obtain averaged EPSC traces for each neuron. Series resistances were monitored throughout experiments by application of hyperpolarizing pulses through the patch pipette; if the series resistances changed more than 20%, the experiments were stopped, and the data were excluded. EPSCs were amplified and filtered (1 kHz) using BVC-700A (Dagan), digitized at 10 kHz using Digidata 1440A (Axon Instruments), stored on a computer, and analyzed using pCLAMP 10 (Axon Instruments). The NMDAR/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) ratio was estimated by calculating NMDAR-mediated EPSC amplitude (EPSC amplitude at a holding potential of +40 mV or +60 mV measured 50-70 msec after the AMPAR-mediated EPSC peak) divided by AMPAR-mediated EPSC amplitude (estimated as the peak EPSC amplitude at a holding potential of -70 mV). The mean values were obtained from these measurements and n represents the number of independent recordings obtained from nearly equal numbers of male and female rats.

Biocytin labeling

During whole-cell recordings, neurons were passively filled with biocytin (0.5%) through the recording pipette for subsequent morphological identification. Following recordings, the slices were incubated for 30 minutes and then fixed overnight in 100 mM phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde. For biocytin staining, the fixed slices were first washed three times for 5 minutes each with 0.1% PB at room temperature. Subsequently, the slices were washed for 5 minutes with 0.1% PB containing 0.2% Triton X-100. The slices were then incubated with Streptavidin Alexa Fluor 488 (Invitrogen, S11223) or Streptavidin Alexa Fluor 546 (Invitrogen, S11225), diluted 1:500, at 4°C for 24 hours or at room temperature for 5 hours. After the final wash with 0.1% PB, the slices were mounted on glass slides with Vectashield Variance (Vector Laboratories, H-1500). Fluorescent images were captured using a fluorescence microscope (BIOREVO, Keyence).

Statistical analysis

The t-test was used to compare the means between two groups, whereas ANOVA was used to compare the means among three groups with Tukey's post hoc test. LTP data were normalized relative to baseline and analyzed using the t-test. NMDA/AMPA ratio data were expressed as mean ± SEM, plotted, and analyzed using the t-test or ANOVA. Data analysis was performed using Origin 2019b (OriginLab) and a comparison was considered statistically significant if $p < 0.05$.

Results

Chronic nicotine exposure increased NMDA/AMPA ratio in CA1 Pv interneurons

Hypofunction of NMDA receptors in GABAergic interneurons is implicated in pathophysiology of schizophrenia (Cohen et al., 2015; Nakazawa and Sapkota, 2020). Furthermore, the previous study found reduced NMDA/AMPA ratio due to decreased NMDAR-mediated responses in hippocampal Pv-expressing interneurons in a mouse model of schizophrenia (Kotzadimitriou et al., 2018). Thus, we examined whether chronic nicotine exposure increases NMDA/AMPA ratio in Pv interneurons. This measure offers the important advantage that it is independent of the number of synapses activated and, therefore, we can compare the synaptic currents recorded in different brain slices. To identify Pv interneurons in hippocampal slices for whole-cell recordings, we generated Pv-tdTomato rats by crossing Pv-Cre rat line with floxed tdTomato reporter rat line. To verify that tdTomato-positive neurons in Pv-tdTomato rats are Pv-expressing cells, we carried out immunofluorescent staining. Native tdTomato fluorescence was detected in hippocampal CA1 region and Pv-expressing cells were fluorescently stained with an anti-Pv antibody (Cell Signaling Technology) and Alexa 488 secondary antibody in the same section (Fig. 1A). The merged image shows that many tdTomato-positive cells express Pv. However,

there appear to be a small number of tdTomato-positive cells that are Pv-negative, suggesting possible ectopic expression of tdTomato in non-Pv interneurons.

We injected nicotine (0.5 mg/kg, s.c., twice daily for 7-10 days) or PBS into Pv-tdTomato rats, which are LE rat strain. Because our previous experiments were conducted with SD rat strain, we first examined whether chronic nicotine exposure enhances NMDA/AMPA ratio in CA1 pyramidal cells of Pv-tdTomato rats as in SD rat strain. Whole-cell patch clamp recordings were obtained from CA1 pyramidal cells, visualized using IR-DIC. Electrical stimulation to SC pathway evoked EPSCs in pyramidal cells from rats exposed either to nicotine or PBS. Inward EPSCs recorded at -70 mV are principally AMPAR mediated, whereas outward EPSCs at +40 mV contain both AMPAR- and NMDAR-mediated EPSCs (Fig. 1B) (Yamazaki et al., 2006b). The NMDA/AMPA ratio was determined by sequentially evaluating EPSC amplitudes at -70 mV (AMPAR) and +40 mV in the same cell. The NMDAR-mediated component of the EPSC at +40 mV was measured 50-70 msec after the AMPAR-mediated EPSC peak. The NMDA/AMPA ratio for PBS-treated pyramidal cells was 0.57 ± 0.10 (n = 13), which was significantly lower than that of 1.10 ± 0.11 (n = 16) for nicotine-exposed (ChrNic) pyramidal cells (Fig. 1C; $t(27) = -3.55$, $p = 0.001$). The result suggests that the nicotine-initiated signaling previously found in pyramidal cells of SD rats also occurs in pyramidal cells of LE rats. Our previous study (Yamazaki et al., 2006a) showed that an increase in the ratio in nicotine-exposed CA1 pyramidal cells of SD rats is due to the enhancement of NMDAR responses, but not the suppression of AMPAR responses. Thus, the enhanced NMDA/AMPA ratio in pyramidal cells of LE rats is most likely due to enhanced NMDAR mediated responses.

Subsequently, tdTomato-expressing neurons in slices from PBS- and nicotine-exposed Pv-tdTomato rats were visualized using epifluorescence optics and IR-DIC for voltage-clamp recordings. These neurons were simultaneously filled with biocytin during recording through

recording pipettes for post-hoc morphological identification of the cells. We selected tdTomato-expressing neurons, which were in and around stratum pyramidale. We electrically stimulated the CA1 SR area and recorded AMPAR- and NMDAR-mediated EPSCs as described above for pyramidal cells. We then calculated NMDA/AMPA ratios to determine whether chronic nicotine exposure enhances the ratio as in pyramidal cells. We found that in tdTomato-expressing neurons exposed to nicotine, there was a small, but significant, increase in the NMDA/AMPA ratio compared to that of PBS control (Fig. 1D; ChrNic, 0.66 ± 0.10, n = 10; Control, 0.43 ± 0.06, n = 19, $t(27) = -2.13$, $p = 0.043$). The results suggest that chronic nicotine exposure stimulates a signaling pathway in Pv interneurons as in pyramidal cells that leads to enhanced NMDAR responses. When biocytin-filled neurons were subsequently visualized with a streptavidin-conjugated fluorophore. Although axons were not clearly visualized, recorded cells had large somata with multipolar dendrites (Fig. 1E). Thus, tdTomato-expressing neurons recorded are likely Pv-expressing basket cells or bistratified cells (Cobb et al., 1997).

Chronic administration of m1 receptor PAM increased NMDA/AMPA ratio in CA1 pyramidal cells and facilitated LTP as chronic nicotine treatment

In the hippocampus, activation of m1 receptors enhances NMDAR-mediated responses (Buchanan et al., 2010; Huang et al., 2001) and induces LTP (Buchanan et al., 2010; Dennis et al., 2016; Shinoe et al., 2005). As co-administration of an m1 receptor antagonist prevents the effect of chronic nicotine treatment on NMDAR function (Ishibashi et al., 2014), both nicotinic and m1 receptor activation may drive the enhancement of NMDAR function via a shared molecular pathway. However, the link between nicotine and m1 receptor activation remains unclear. One possible link between nicotine and m1 receptor activation is through ACh release. As the m1 receptor PAM only activates m1 receptors when the endogenous agonist ACh is present, we tested whether chronic administration of the m1 receptor PAM VU0453595 (3 mg/kg, s.c, twice daily for 7- 10 days) mimics the chronic nicotine’s effect on NMDAR function.

For voltage-clamp recordings, pyramidal cells from rats exposed to nicotine, VU0453595, and vehicle (control) were visualized in hippocampal brain slices by IR-DIC. The SC pathway was stimulated and evoked EPSCs in pyramidal cells, clamped at both -70 mV and +40 mV, were recorded. AMPAR- and NMDAR-mediated EPSCs were then measured to calculate NMDA/AMPA ratios (Fig. 2A, B). A one-way ANOVA revealed that there was a statistically significant difference in mean NMDA/AMPA ratio between at least two groups (Fig. 2B; $F_{2, 47}$ = 24.147, p < 0.001). We first confirmed that chronic nicotine exposure (ChrNic) significantly increased NMDA/AMPA ratio (0.83 ± 0.05, n = 14) compared with control (0.42 ± 0.03, n = 16, p = < 0.001). We also found that NMDA/AMPA ratio was significantly increased in chronic PAM VU0453595 (ChrVU)-exposed pyramidal cells (0.88 ± 0.06, n = 20) compared with vehicle-exposed control pyramidal cells (p = < 0.001), suggesting that PAM VU0453595 mimics the nicotine's effect on NMDAR function. There was no significant difference between chronic nicotine- and PAM VU0453595-exposed groups (p = 0.833). Thus, the link between nicotine and m1 receptor activation is likely through the excitation of cholinergic neurons by nicotine, which causes the release of ACh to activate m1 receptors in CA1 pyramidal cells.

As we have previously found using weak tetanic stimulation that chronic nicotine treatment lowered the threshold for LTP induction (Fujii et al., 1999), we next considered the effect of chronic administration of m1 receptor PAM VU0453595 on LTP. When we delivered 3 theta burst stimulation (TBS), but not 2 TBS, at the SC pathway in hippocampal slices from control rats, LTP was induced (3 TBS, 127.68 ± 3.71, n = 8; 2 TBS, 101.50 ± 0.77, n = 8; t(14) = -6.91, p = < 0.001). This suggests that 2 theta-burst is subthreshold stimulation for LTP induction in control rats. Subsequently, we found that this subthreshold stimulation induces LTP in chronic nicotine-exposed rats (Fig. 3A, B; Control, 101.50 ± 0.77, n = 8; ChrNic, 115.40 ± 2.51, n = 19; t(25) = -3.52, p = 0.001) confirming that chronic nicotine treatment lowered the threshold for LTP induction. To determine whether chronic VU0453595 treatment lowered the threshold for LTP

induction as found with chronic nicotine exposure, we delivered 2 TBS at the SC pathway in hippocampal slices from VU0453595P-exposed rats. We found that unlike in slices from control rats, 2 TBS of the SC pathway induced LTP in VU0453595P-exposed rats (Fig. 3C, D; Control, 100.51 ± 1.41, n = 16; ChrVU, 117.60 ± 3.39, n = 16; $t(30) = -4.65$, $p = < 0.001$). These results suggest that both chronic nicotine and m1 receptor PAM treatments facilitate LTP via stimulating a shared molecular cascade, leading to enhanced NMDAR response.

Both chronic nicotine and PAM VU0453595 exposure stimulate a signaling pathway to cause increased NMDA/AMPA ratios in layer 5 pyramidal cells of mPFC

We next considered whether the chronic nicotine-initiated signaling found in CA1 pyramidal cells occurs widely in neurons, which express m1 receptors. As m1 receptors are expressed in the PFC pyramidal cells (Oda et al., 2018) and the PFC has been implicated in the cognitive effects of nicotine and m1 receptors (Hahn et al., 2003; Ragozzino and Kesner, 1998; Shirey et al., 2009), we examined whether the chronic nicotine-initiated signaling pathway operates in layer 5 pyramidal cells of mPFC. Furthermore, to gain insight into the nicotine-initiated signaling pathway in layer 5 pyramidal cells, we subsequently examined whether chronic VU0453595 treatment mimics the nicotine's effect as in CA1 pyramidal cells. Thus, we prepared coronal brain slices containing mPFC from vehicle-, nicotine-, and PAM VU0453595-exposed rats. We then performed whole-cell voltage-clamp recordings in layer 5 pyramidal cells, identified by the shape of their somata using IR-DIC microscopy and confirmed by post hoc biocytin staining of the recorded cells (Fig. 4A). We electrically stimulated layer 5 to record evoked EPSCs in layer 5 pyramidal cells held at both -70 mV and +60 mV. AMPAR and NMDA receptor components of evoked EPSCs were measured (Fig. 4B, C) to calculate NMDA/AMPA ratios (Fig. 4B). A one-way ANOVA showed that there was a statistically significant difference in mean NMDA/AMPA ratios between at least two groups ($F_{2,65} = 5.539$, $P = 0.006$). We found that the NMDA/AMPA ratio was significantly increased in nicotine-exposed pyramidal cells compared with control

pyramidal cells (Fig. 4C, D; ChrNic, 0.82 ± 0.07, n = 26; Control, 0.55 ± 0.06, n = 20; p = 0.0142). The finding suggests that chronic nicotine exposure causes the enhancement of NMDAR function in layer 5 pyramidal cells as in CA1 pyramidal cells. We also found that the NMDA/AMPA ratio for PAM VU0453595-treated pyramidal cells was 0.83 ± 0.06 (n = 22), which was significantly higher than that of control pyramidal cell (Fig. 4C, D; p = 0.0119), suggesting that chronic PAM VU0453595 treatment mimics the nicotine's effect in layer 5 pyramidal cells as in CA1 pyramidal cells. There was no significant difference between chronic nicotine- and PAM VU0453595-exposed groups (p = 0.981). These findings imply that a common cholinergic signaling pathway is stimulated by chronic nicotine and PAM VU0453595 exposure in CA1 and mPFC layer 5 pyramidal cells to enhance NMDAR-mediated responses.

Discussion

A core feature of schizophrenia is cognitive dysfunction. Therefore, a potential motivator of tobacco smoking is pro-cognitive effects of nicotine. Chronic nicotine exposure has been shown to improve hippocampal memory in rodents (Kenney and Gould, 2008; Levin et al., 1990; Levin and Rose, 1990). However, the underlying molecular mechanism remains poorly understood. In the current study, we explored the potential mechanism of pro-cognitive effects of chronic nicotine exposure. The NMDAR plays an important role in cognitive function and NMDAR hypofunction has been postulated to be a mechanism underlying the pathophysiology of schizophrenia. Thus, the pro-cognitive effect of nicotine likely involves the enhancement of NMDAR responses. Indeed, we have previously found that chronic nicotine exposure enhances NMDAR responses in CA1 pyramidal cells (Ishibashi et al., 2014; Yamazaki et al., 2006a; Yamazaki et al., 2006b; Yamazaki et al., 2006c). We also found that chronic administration of several cholinergic cognitive-enhancing drugs (acetylcholinesterase inhibitors and an m1 muscarinic agonist) all induce significant increases in NMDAR-mediated responses (Ishibashi et

al., 2014). Furthermore, we have found that co-administration of the m1 receptor antagonist pirenzepine prevents the effect of these cholinergic drugs (Ishibashi et al., 2014). The observations suggest that in vivo nicotine exposure stimulates the intrinsic cholinergic pathway involved in cognitive function and that nAChRs, ACh, and m1 receptors all converge for the subsequent enhancement of NMDAR function. Thus, nicotine indirectly stimulates m1 receptor-mediated signaling cascade through increased ACh release from cholinergic neurons. In the present study, to gain further understanding of the link between the release of ACh and the activation of m1 receptors, we examined the effect of chronic exposure to VU0453595, a PAM of m1 receptor. We found that chronic VU0453595 treatment mimics the effects of chronic nicotine exposure, causing the enhancement of NMDAR responses and LTP facilitation. Although we have not tested whether co-administration of the m1 receptor antagonist pirenzepine prevents the effect, as the PAM selectively activates m1 receptors when the endogenous agonist ACh is present, the current findings reinforce an idea that ACh and m1 receptors are downstream of nAChR activation in chronic nicotine-initiated signaling pathway.

While hippocampal dysfunction is observed in individuals with schizophrenia, there is extensive dysfunction throughout the brain, including prefrontal cortical regions (Lieberman et al., 2018). Given that schizophrenia is genetically driven, NMDAR hypofunction likely occurs in multiple neuron types. Thus, we expect that a nicotinic mechanism that counteracts NMDAR hypofunction in schizophrenia occurs widely not only in the hippocampus, but also PFC. Indeed, in the current study, we found that chronic nicotine exposure enhances NMDA/AMPA ratio in PFC pyramidal cells as in hippocampal pyramidal cells. In addition, the current study shows that chronic nicotine exposure enhances NMDA/AMPA ratio in CA1 Pv interneurons. As hypofunction of NMDA receptors in Pv interneurons is implicated in pathophysiology of schizophrenia (Cohen et al., 2015; Nakazawa and Sapkota, 2020), the observed nicotine’s effect may be a mechanism that counteracts NMDAR hypofunction in schizophrenia. As

pyramidal cells and Pv interneurons express m1 receptors (Oda et al., 2018; Yi et al., 2014), we speculate that the effect of chronic nicotine occurs widely in various neuron types, which receive cholinergic input and express m1 receptors. The role of nicotine in the signaling is the excitation of cholinergic neurons via nAChRs for the release of ACh.

Previous studies suggest that cholinergic innervation of the PFC plays critical roles in PFC-dependent cognitive function (Ghoshal and Conn, 2015; Luchicchi et al., 2014; Ragozzino and Kesner, 1998; Shirey et al., 2009) and cholinergic signaling is disrupted in schizophrenia patients and animal models of schizophrenia (Berman et al., 2007; Dean et al., 2002; Scarr and Dean, 2009). Furthermore, the m1 PAM VU0453595 fully restores deficits in cognitive function in a mouse model of schizophrenia (Ghoshal et al., 2016). The nicotine-initiated signaling involving m1 receptors, found in the current study, may be cholinergic signaling involved in PFC-dependent cognitive function and the restoration of deficits in cognitive function by the PAM.

Previous studies show that ACh release from cholinergic fibers facilitates LTP via the activation of m1 receptors and nAChRs (Gu and Yakel, 2011; Shinoe et al., 2005). Furthermore, previous work shows that m1 receptor activation leads to an increase in NMDAR function in CA1 pyramidal cells by inhibiting SK potassium channels that negatively regulates NMDAR function and facilitates LTP (Buchanan et al., 2010). In addition, m1 receptor activation can increase NMDAR responses in CA1 pyramidal cells by stimulating the Gq signaling pathway, which signals through protein kinase C and cell adhesion kinase-β/proline-rich tyrosine kinase 2. This in turn activates Src, leading to upregulation of NMDAR responses and facilitation of LTP (Ali and Salter, 2001; Huang et al., 2001; Salter and Kalia, 2004). This second mechanism appears to play an important role in prompting chronic nicotine's effects. We have previously found that chronic nicotine exposure-induced enhancement of NMDAR responses (Ishibashi et al., 2014;

Yamazaki et al., 2006a; Yamazaki et al., 2006b; Yamazaki et al., 2006c) is associated with increased NMDAR tyrosine phosphorylation via Src (Ishibashi et al., 2014; Yamazaki et al., 2006a; Yamazaki et al., 2006c). Thus, current results, together with previous findings, suggest that chronic nicotine exposure stimulates m1 receptor-mediated Gq signaling cascades to enhance NMDAR responses. This pathway begins by the activation of cholinergic neurons via nAChRs, which causes the release of ACh to stimulate the m1 receptor-mediated Gq signaling cascade. However, enhanced NMDAR function caused by chronic nicotine administration is distinct from the effect of normal stimulation of m1 receptor-mediated Gq signaling cascades. The effect emerges over the course of 5 days of nicotine exposure and this enhancement is maintained during chronic nicotine exposure, and it can persist well after the drug has cleared from the hippocampus (Yamazaki et al., 2006a; Yamazaki et al., 2006b). Understanding the underlying mechanism is critical if we are to identify a motivator of tobacco smoking in schizophrenia patients.

NMDARs exist as a macromolecular complex and their function can be affected by interactions among many proteins linked to various signaling pathways (Salter and Kalia, 2004). Schizophrenia patients were often found to have enriched de novo mutations in genes encoding postsynaptic density proteins at glutamatergic synapses, particularly components of the NMDAR complex (Fromer et al., 2014; Hahn, 2011). These genes could participate in NMDAR hypofunction. One putative schizophrenia risk gene is neuregulin 1 (NRG1), which signals via ErbB4 receptor, itself is a schizophrenia risk gene and a component of NMDAR complexes (Banerjee et al., 2010). The previous study found reduced NMDA/AMPA ratio due to decreased NMDAR-mediated currents in hippocampal Pv basket cells in NRG1-overexpressing transgenic mice (Kotzadimitriou et al., 2018). In the current study, we showed that chronic nicotine exposure stimulates a signaling pathway in Pv interneurons that leads to increased

NMDA/AMPA ratio. The result suggests that nicotine exposure may counteract schizophrenia-linked NMDAR hypofunction in Pv interneurons.

Postmortem human studies suggest increased neuregulin function in prefrontal and hippocampal regions of brain tissue from individuals with schizophrenia (Chong et al., 2008; Hahn et al., 2006; Law et al., 2006). The excessive NRG1-ErbB4 signaling reduces the phosphorylation of Src at tyrosine 416, the active form of Src, in acute human brain slices (Zhu et al., 2017). Furthermore, NRG1-ErbB4 signaling participates in NMDAR hypofunction by suppressing Src-mediated enhancement of NMDAR function in pyramidal cells in the hippocampus and PFC (Pitcher et al., 2008; Pitcher et al., 2011; Salter and Pitcher, 2012; Shamir et al., 2012). These findings suggest that Src is a downstream target of NRG1-ErbB4 signaling. We have previously found that chronic nicotine exposure, which stimulates Src to enhance NMDAR function (Ishibashi et al., 2014; Yamazaki et al., 2006a; Yamazaki et al., 2006c), counteracts the excessive NRG1-ErbB4 signaling in hippocampal CA1 pyramidal cells (Yamazaki and Sumikawa, 2017). The current study suggests that chronic nicotine exposure may stimulate Src to enhance NMDAR function and counteract the excessive NRG1-ErbB4 signaling in PFC pyramidal cells as in hippocampal pyramidal cells.

Our current results provide critical new insights into a potential motivator of tobacco smoking in individuals with schizophrenia and support a role of m1 receptor activation in nicotine-initiated signaling. The finding, a non-addictive m1 receptor PAM mimics the effect of nicotine, is encouraging for the development of smoking cessation treatments among people with schizophrenia.

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Figure legends

Fig. 1. Chronic nicotine exposure increased NMDA/AMPA ratios in CA1 pyramidal cells and Pv-expressing interneurons

(A) Pv- and tdTomato-expressing neurons in the hippocampal CA1 region. Pv-expressing cells were detected with an anti-parvalbumin antibody and Alexa Fluor 488 secondary antibody (left, green). In the same section, native tdTomato fluorescence was detected (middle, red). The merged image is shown on the right (scale bar, 100 μm). Many neurons in and around stratum pyramidale (SP) express both Pv and tdTomato. Magnified images of the boxed area are shown below the low-power images. (B) Example EPSC traces recorded from CA1 pyramidal cell from a control rat. Negative traces represent inward currents from a cell voltage-clamped at −70 mV, positive traces represent outward currents from a cell voltage-clamped at +40 mV. Gray traces indicate repeated trials, while black traces show average EPSC traces. A gray bar indicates windows used to measure NMDAR-mediated component. (C) Mean EPSC traces at −70 and +40 mV recorded in pyramidal cells from PBS- (left) and chronic nicotine-exposed (right) rats. NMDA/AMPA ratios measured in CA1 pyramidal cells from control and chronic nicotine exposed rats. (D) Example of average EPSC traces at −70 and +40 mV recorded in tdTomato-expressing neurons from PBS- (left) and chronic nicotine-exposed (right) Pv-tdTomato rats. NMDA/AMPA

ratios measured in tdTomato-expressing neurons from control and chronic nicotine exposed Pv-tdTomato rats. (E) Biocytin staining of tdTomato-expressing neuron. The data are presented as the means ± SEM. Statistical significance (* $P < 0.05$, ** $P < 0.01$) was evaluated using paired Student's t-test. Scale bar in B, C, D; 50 pA, 100 ms

Fig. 2. Chronic administration of m1 receptor PAM increased NMDA/AMPA ratio in CA1 pyramidal cells as chronic nicotine administration

(A) Mean EPSC traces at −70 and +40 mV recorded in pyramidal cells from vehicle- (left), chronic nicotine- (middle), and chronic VU0453595-exposed (right) rats. Scale bar, 50 pA, 100 ms (B) A bar graph shows NMDA/AMPA ratios measured in CA1 pyramidal cells from control, chronic nicotine-, and VU0453595-exposed rats. Statistical significance was evaluated using ANOVA with Tukey's post hoc test. *** $P < 0.001$

Fig. 3. Chronic administration of m1 receptor PAM facilitated LTP as chronic nicotine administration

(A) In PBS-exposed control hippocampal slices, two theta-burst stimulation failed to induce LTP, whereas in chronic nicotine-exposed slices, the same stimulation induced LTP. (B) A bar graph shows the percent change in the slope of fEPSPs measured 50-55 min after delivery of two theta-burst stimulation. (C) In hippocampal slices exposed to DMSO-based vehicle control, two theta-burst stimulation failed to induce LTP. While in hippocampal slices from chronic VU0453595-exposed rats, two theta-burst stimulation induced LTP. A bar graph in D shows the percent change in the slope of fEPSPs measured 50-55 min after delivery of two theta-burst stimulation. In A and C, 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 2 theta-burst stimulation (red). Two theta-burst stimulation (2 theta) was delivered at the time indicated. Scale bars are 10 ms and 1 mV. ** $p < 0.01$, *** $p < 0.001$

Fig. 4. Both chronic nicotine and PAM VU0453595 exposure stimulate a signaling pathway to cause increased NMDA/AMPA ratio in layer 5 pyramidal cells in the mPFC

(A) Schematic illustration of the prefrontal cortex slice preparation. Whole-cell recordings were made from layer 5 pyramidal cells and the stimulation electrode was placed on layer 5, approximately 100 μm away from the recording electrode (left). PrL, prelimbic. Biocytin staining of layer 5 pyramidal neuron (right, top). Voltage responses to hyperpolarizing and depolarizing current injections to the soma of a layer 5 pyramidal neuron (right). Scale bar, 20 mV, 200 ms (B) Example EPSC traces recorded from layer 5 pyramidal cell from a control rat. Gray traces indicate repeated trials, while black traces show average EPSC traces. A gray bar indicates windows used to measure NMDAR-mediated component. (C) Mean EPSC traces at −70 and +60 mV recorded in pyramidal cells from vehicle- (left), nicotine- (middle), and VU0453595-exposed (right) rats. (D) A bar graph shows NMDA/AMPA ratios measured in pyramidal cells from control, chronic nicotine (ChrNic)-, and VU0453595 (ChrVU)-exposed rats. Statistical significance was evaluated using ANOVA with Tukey's post hoc test. * $P < 0.05$

Scale bar in B, C; 50 pA, 100 ms

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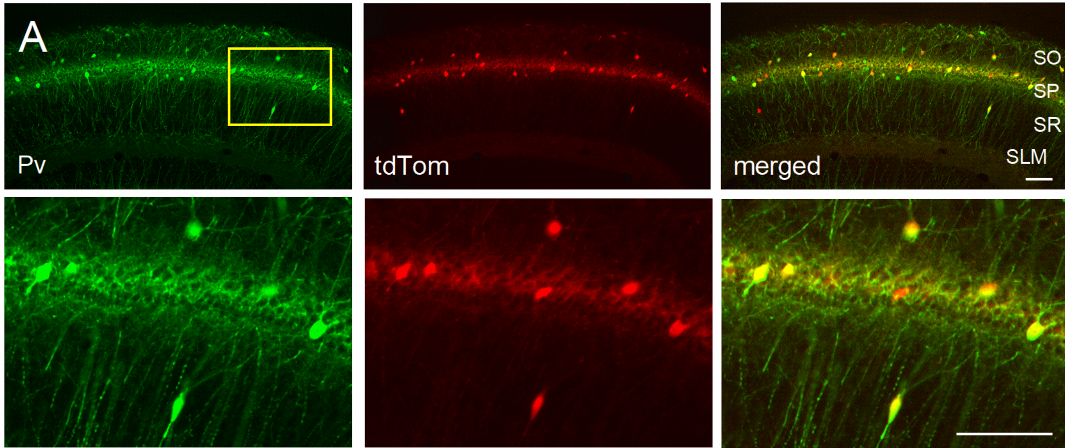
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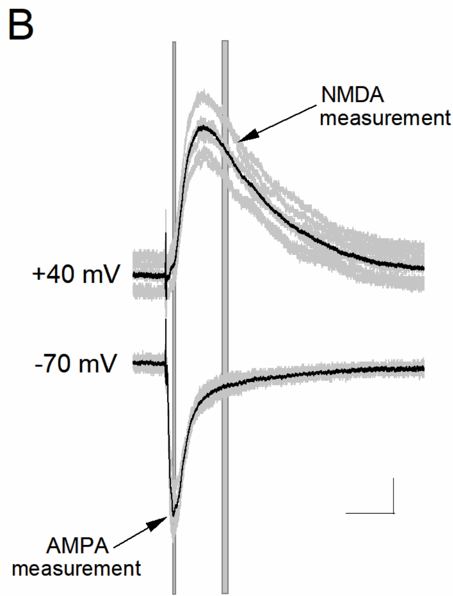
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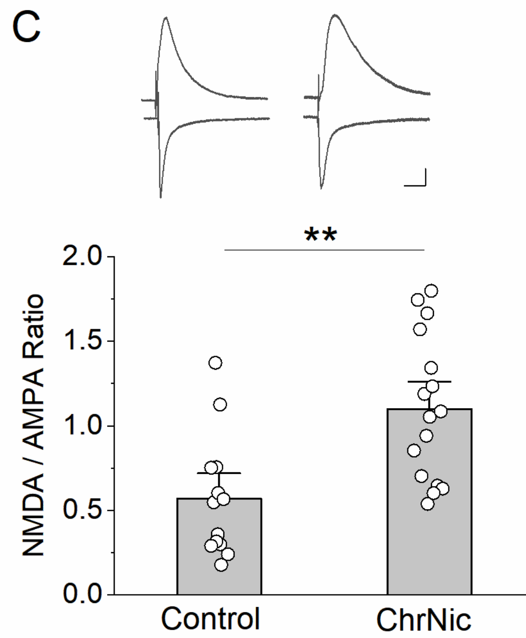
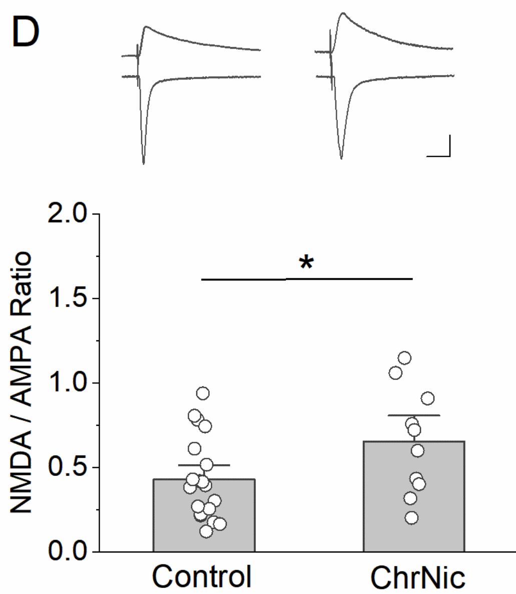
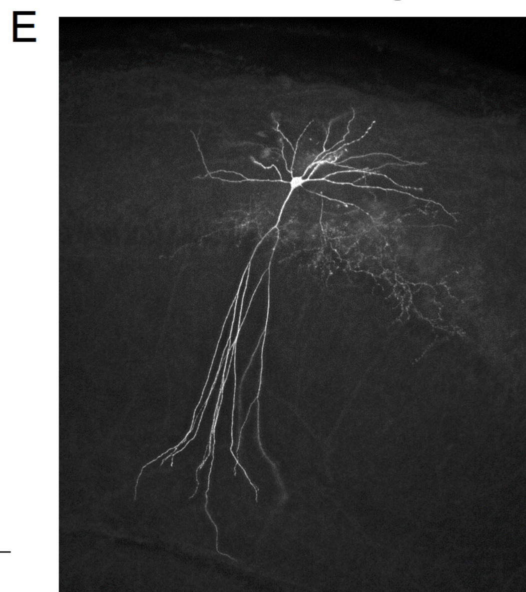
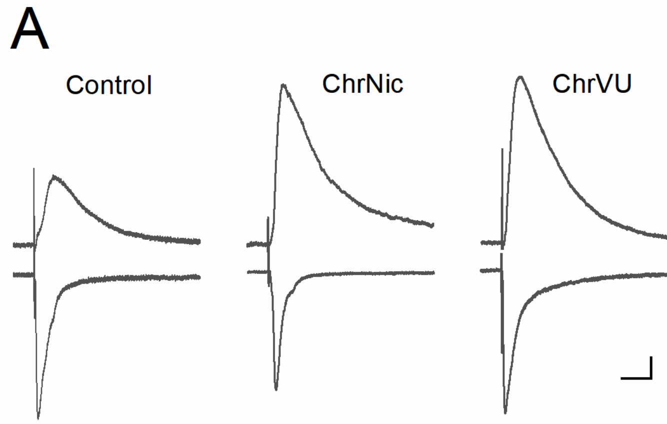
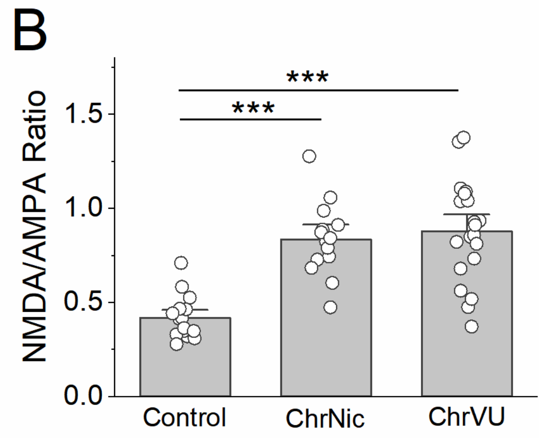
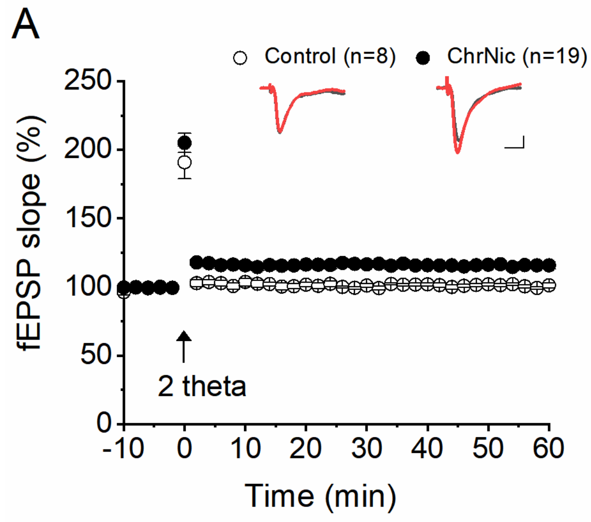

C
**
NMDA / AMPA Ratio
2.0
1.5
1.0
0.5
0.0
Control
ChrNic

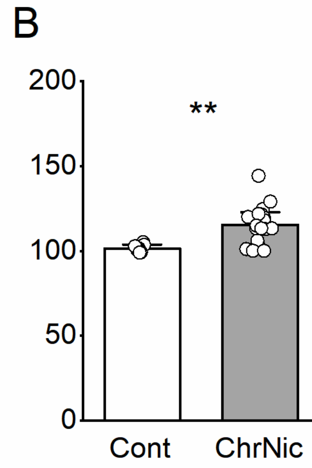

D
*
NMDA / AMPA Ratio
2.0
1.5
1.0
0.5
0.0
Control
ChrNic

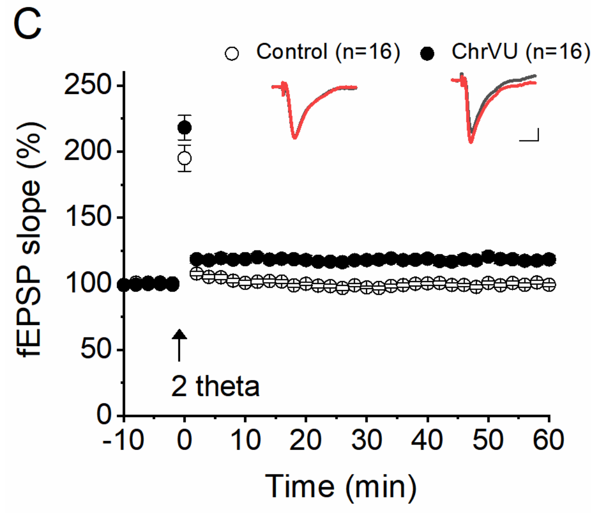

E

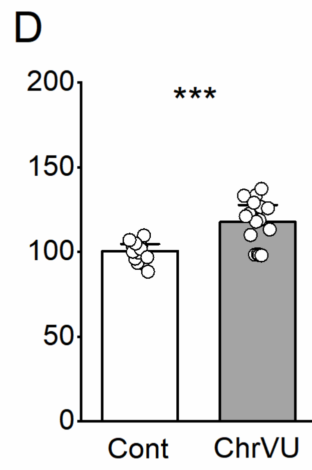

A
Control
ChrNic
ChrVU


B
***
***
NMDA/AMPA Ratio
1.5
1.0
0.5
0.0
Control
ChrNic
ChrVU

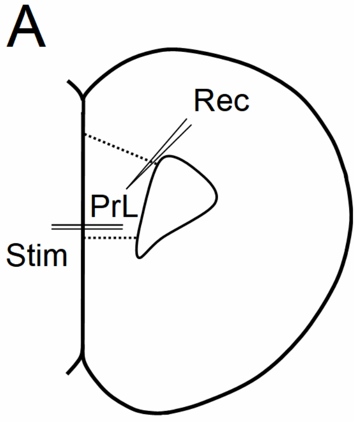

A
Control (n=8)
ChrNic (n=19)
fEPSP slope (%)
250
200
150
100
50
0
2 theta
-10
0
10
20
30
40
50
60
Time (min)

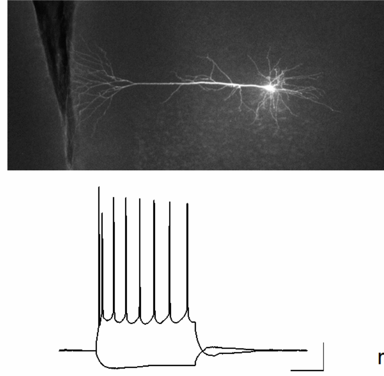

B
200
150
100
50
0
**
Cont
ChrNic


C
Control (n=16)
ChrVU (n=16)
fEPSP slope (%)
250
200
150
100
50
0
2 theta
-10
0
10
20
30
40
50
60
Time (min)

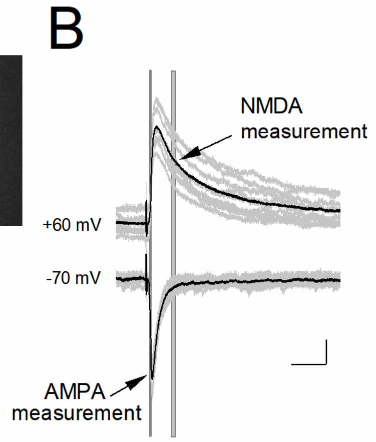

D
200
150
100
50
0
***
Cont
ChrVU

A

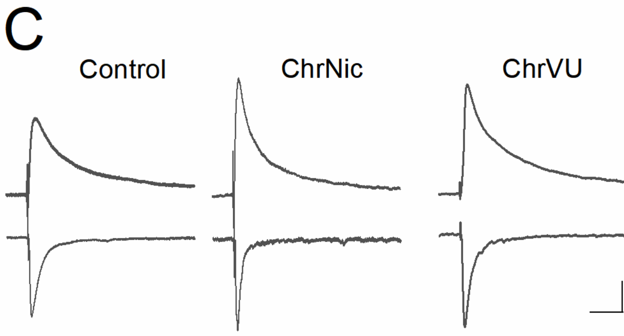




B



C



D