

# Neonatal nicotine exposure increases excitatory synaptic transmission and attenuates nicotine-stimulated GABA release in the adult rat hippocampus



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## ABSTRACT

Developmental exposure to nicotine has been linked to long-lasting changes in synaptic transmission which may contribute to behavioral abnormalities seen in offspring of women who smoke during pregnancy. Here, we examined the long-lasting effects of developmental nicotine exposure on glutamatergic and GABAergic neurotransmission, and on acute nicotine-induced glutamate and GABA release in the adult hippocampus, a structure important in cognitive and emotional behaviors. We utilized a chronic neonatal nicotine treatment model to administer nicotine (6 mg/kg/day) to rat pups from postnatal day (P) 1–7, a period that falls developmentally into the third human trimester. Using whole-cell voltage clamp recordings from CA1 pyramidal neurons in hippocampal slices, we measured excitatory and inhibitory postsynaptic currents in neonatally control- and nicotine-treated young adult males. Neonatal nicotine exposure significantly increased AMPA receptor-mediated spontaneous and evoked excitatory signaling, with no change in glutamate release probability in adults. Conversely, there was no increase in spontaneous GABAergic neurotransmission in nicotine-males. Chronic neonatal nicotine treatment had no effect on acute nicotine-stimulated glutamate release in adults, but acute nicotine-stimulated GABA release was significantly attenuated. Thus, neonatal nicotine exposure results in a persistent net increase in excitation and a concurrent loss of nicotinic acetylcholine receptor (nAChR)-mediated regulation of presynaptic GABA but not glutamate release, which would exacerbate excitation following endogenous or exogenous nAChR activation. Our data underscore an important role for nAChRs in hippocampal excitatory synapse development, and suggest selective long-term changes at specific presynaptic nAChRs which together could explain some of the behavioral abnormalities associated with maternal smoking.

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## 1. Introduction

In the United States, it is estimated that more than 15% of women smoke during pregnancy (SAMHSA, 2010), exposing their offspring to a variety of harmful substances, including nicotine, which may alter neuronal development (Abbott and Winzer-Serhan, 2012; Winzer-Serhan, 2008). Previously, we have shown that chronic neonatal nicotine exposure during a time period equivalent to the third trimester of human brain development

(Winzer-Serhan, 2008) results in an overall increase in field excitatory postsynaptic potential (EPSP) responses within the adult male rat hippocampus (Damborsky et al., 2012). Based on these data, as well as other studies showing endogenous nicotinic cholinergic signaling is a critical component of excitatory but not inhibitory synapse formation in the hippocampus (Lozada et al., 2012a, 2012b), we hypothesized that the increase in excitation in the adult hippocampus following chronic neonatal nicotine exposure is due to an increase in glutamatergic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) signaling rather than a decrease in GABAergic signaling.

There is also strong evidence that developmental exposure to nicotine leads to long-term loss of nicotinic acetylcholine receptor (nAChR) responsiveness to nicotine, essentially eliminating nicotine-stimulated release of transmitters such as norepinephrine and dopamine (Britton et al., 2007; Kane et al., 2004; Liang

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et al., 2006; Seidler et al., 1992). Within the hippocampus, nAChRs regulate  $\gamma$ -amino butyric acid (GABA) and glutamate release both in adulthood and during development (Alkondon et al., 1997; Gray et al., 1996; Maggi et al., 2001, 2003; Radcliffe et al., 1999). Thus, in addition to altering excitatory signaling within the hippocampus, we also hypothesized that chronic neonatal nicotine persistently alters the function of presynaptic nAChRs on glutamatergic and GABAergic terminals in the adult hippocampus, potentially contributing to dysregulation of hippocampal activity later in life.

Here, we show that chronic neonatal nicotine exposure disrupts the excitatory/inhibitory balance by persistently increasing AMPAR-mediated excitation without altering inhibitory activity resulting in the overall increase in excitation as previously reported within the adult male hippocampus (Damborsky et al., 2012). Furthermore, chronic neonatal nicotine treatment attenuates nicotine-induced increases in presynaptic GABA release without altering nicotine-induced increases in glutamate release in adults. Together these results demonstrate that developmental nicotine exposure differentially affects neurotransmitter systems within the hippocampus, resulting in a net increase in excitation that could be exacerbated by the differential long-term loss of nicotinic regulation of inhibitory transmission.

## 2. Materials and methods

### 2.1. Nicotine treatment

Virgin-mated pregnant Sprague–Dawley rats (Harlan, Houston, TX) arrived between gestational day 14 and 16, and were housed in accordance with the rules stipulated by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). All animal treatment protocols were approved by the Texas A&M University IACUC under the guidelines set by the NIH. The date of birth was termed postnatal day (P) 0, and on P1 litters were culled to 8–10 pups. Every day from P1–P7, pups were treated using a previously described chronic neonatal nicotine treatment paradigm (Huang et al., 2006). This developmental treatment window corresponds to a period during the third trimester of human brain development that is characterized by substantial excitatory and inhibitory synapse and circuit formation in cortical structures (de Graaf-Peters and Hadders-Algra, 2006; Tyzio et al., 1999). Pups received milk formula (Enfamil; Mead Johnson & Company, Evansville, IN) three times a day via oral gastric intubation. One half of the pups in each litter were given 2 mg/kg/dose nicotine (nicotine base) mixed in milk formula for a total of 6 mg/kg/day (nicotine group); the other pups were given milk formula only (controls). This daily dose has been used by others (Levin et al., 2006; Parameshwaran et al., 2013; Pilarski and Fregosi, 2009; Slotkin et al., 2007; Vaglenova et al., 2008) and results in blood nicotine levels comparable to those found in heavy smokers (Chen et al., 2005; Murrin et al., 1987). The intermittent application of nicotine mimics the highs and lows experienced by smokers during the day (Gritz et al., 1981) and allows desensitized nAChRs to revert back to an active state (Marks et al., 1993). At P21, rats were weaned and pair-housed.

### 2.2. Brain slice preparation

Young adult male rats (P60–P90, mean age: P75 for excitatory recordings and P73 for inhibitory recordings) were decapitated under isoflurane anesthesia, and their brains were removed and immediately placed in ice-cold cutting solution containing (in mM) 120 NaCl, 11 D-Glucose, 26 NaHCO<sub>3</sub>, 6 MgCl<sub>2</sub>, 3 KCl, 0.5 CaCl<sub>2</sub>, 5 HEPES and 0.3 kynurenic acid. Coronal slices (300  $\mu$ M) were taken through the brain using a Vibratome 3000 System. The hippocampus was dissected from each slice and transferred to artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 1.5 MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, and 10 D-Glucose bubbled with 95%O<sub>2</sub>/5%CO<sub>2</sub>. Slices were warmed to 34 °C for one hour, and then cooled to 25 °C, where they were held for the remainder of the day.

### 2.3. Electrophysiology

Slices were transferred to a recording chamber where they were continuously perfused with room temperature oxygenated ACSF at a rate of approximately 2 ml/min. Patch pipettes were pulled to a resistance of 6–10 M $\Omega$  from borosilicate glass (KG-33; King Precision Glass Inc.) using a Flaming/Brown P-87 pipette puller (Sutter Instruments). Whole cell voltage clamp recordings were performed from CA1 pyramidal cells held at –60 mV. Current recordings were digitized using Digidata 1322A, and collected using pClamp 9 software (Molecular Devices) with a Multi-clamp 700B amplifier (Axon Instruments). Data were filtered at 2 kHz, and acquired at a sampling rate of 5–10 kHz.

#### 2.3.1. Spontaneous and evoked excitatory postsynaptic currents (EPSCs)

For EPSC recordings, pipettes were filled with an internal solution containing (in mM) 130 K<sup>+</sup>-gluconate, 10 EGTA, 2 MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 HEPES, 0.1 GTP, 4 ATP, 5 QX-314; pH 7.2, mOsm 285–310. All EPSC responses were recorded in the presence of bicuculline (BIC, 10  $\mu$ M) to block GABA-A receptors (GABA<sub>A</sub>Rs), and D,L-2-amino-5-phosphonovaleric acid (AP5, 40  $\mu$ M) to block N-methyl-D-aspartic acid receptors (NMDAR) in order to isolate AMPAR-mediated responses. Evoked EPSCs were recorded from CA1 pyramidal neurons approximately 300  $\mu$ m away from a bipolar stimulating electrode (FHC, Inc. Bowdoin, ME) placed in the Schaeffer collaterals. Input/Output (I/O) curves were constructed by recording the EPSC evoked in response to the minimum voltage input capable of eliciting a response (v) and increasing voltage inputs by 2 V increments. Paired-pulse experiments were performed at 50, 100 and 200 ms inter-stimulus intervals (ISI). The paired-pulse ratio (PPR) was defined as the ratio of the amplitude of the second response, P2, to the amplitude of the first response, P1. We further recorded spontaneous EPSCs (sEPSCs) of CA1 neurons by whole-cell voltage-clamp under GABA<sub>A</sub>R inhibition to analyze the spontaneous synaptic vesicle release. First baseline spontaneous EPSCs and evoked EPSCs were recorded, then acute nicotine (nicotine hydrogen tartrate) was bath applied to the slices for approximately 3 min and sEPSCs and PPRs (P2/P1) recorded again.

#### 2.3.2. Spontaneous postsynaptic currents (sPSCs) and miniature inhibitory postsynaptic currents (mIPSCs)

sPSCs and miniature (m)IPSCs were recorded using as pipette solution containing (in mM) 124 CsCl, 20 TEA Chloride, 10 EGTA, 10 HEPES, 2 MgCl<sub>2</sub>, 0.1 GTP, 4 ATP; pH 7.2, mOsm 290. TTX-sensitive sPSCs were recorded under conditions that highly favored GABA<sub>A</sub>R-mediated currents; more than 96% of sPSCs are blocked by 10  $\mu$ M bicuculline (mean frequency decrease from  $3.79 \pm 1.07$  Hz to  $0.12 \pm 0.04$  Hz,  $n = 6$ ). Because we had determined that sPSCs recorded in these conditions are overwhelmingly GABAergic in nature, no glutamatergic receptors antagonists were applied during these experiments, allowing us to examine spontaneous GABAergic synaptic transmission while maintaining intact circuit activity within the hippocampus. For mIPSC experiments, AP5 (40  $\mu$ M) and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10  $\mu$ M) were applied to block NMDA and AMPA receptor currents, respectively, and tetrodotoxin (TTX, 0.5  $\mu$ M) was applied to block sodium channels, to isolate mIPSCs, as previously described (DuBois et al., 2013).

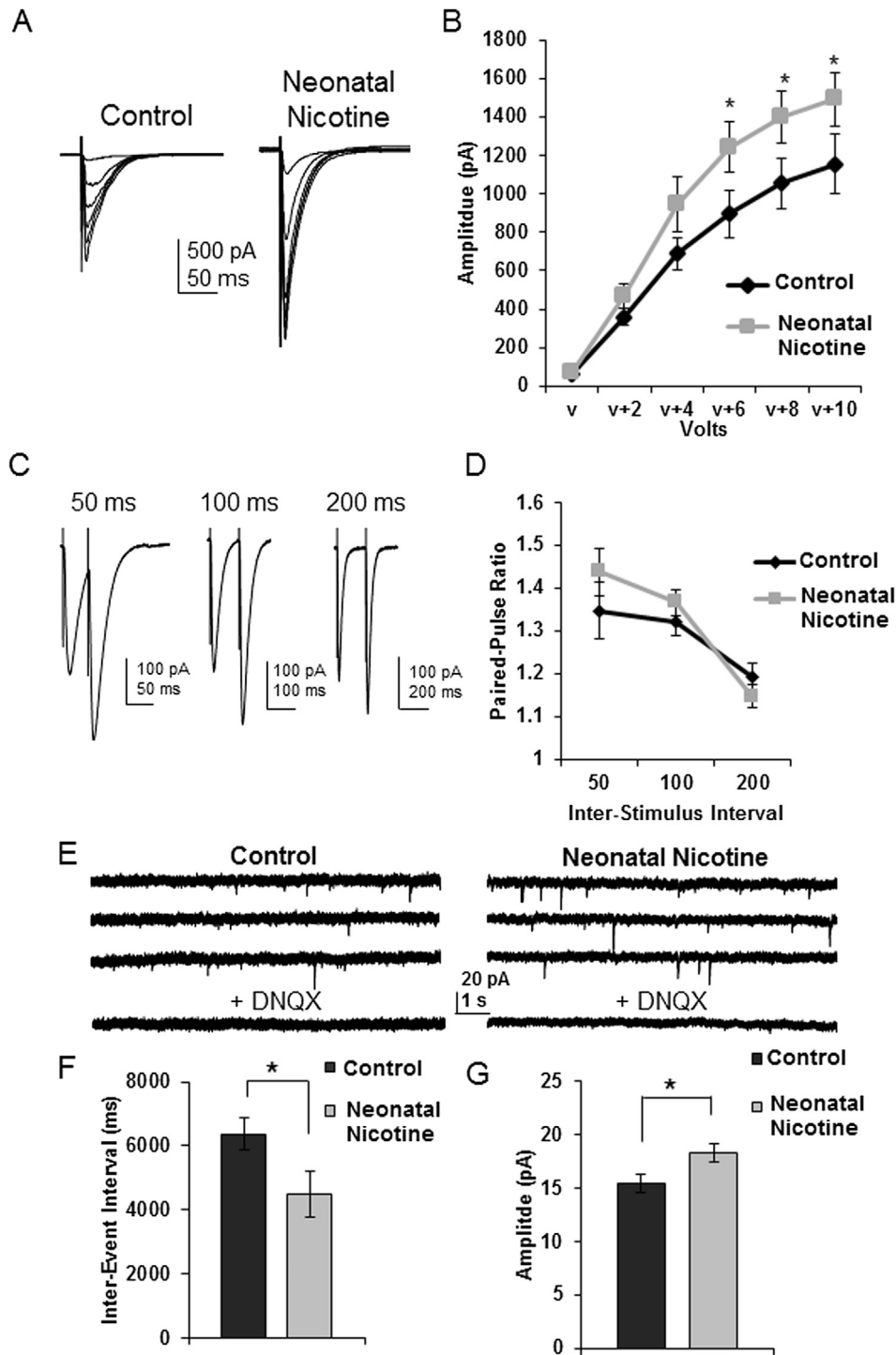
### 2.4. Data analysis

Data were acquired using Clampex 9.0 and analyzed using Clampfit 9.0 and MiniAnalysis 6.03 (Synaptosoft, Inc.). SigmaPlot 12 (Systat Software) and Mini-Analysis were used for statistical analysis. Significance of baseline differences in IPSCs and EPSCs between control- and nicotine-treated rats was assessed using Student's *t*-tests or ANOVAs, where applicable. Significance of within-group changes following acute nicotine application was determined using paired *t*-tests or a Kolmogorov–Smirnov (K–S) test to examine differences in cumulative probability distribution. Cumulative probability plots were constructed using Origin 7.5 (OriginLab). For all analyses, significance was defined as a *p* value of less than or equal to 0.05. Data are presented  $\pm$  S.E.M. All chemicals were obtained from Sigma Chemicals unless otherwise noted.

## 3. Results

### 3.1. Chronic neonatal nicotine treatment increases AMPAR-mediated excitation in the adult male hippocampus

In order to test our hypothesis that chronic neonatal nicotine treatment leads to a long-lasting enhancement of excitatory signaling through AMPARs in the CA1 hippocampus, we performed whole cell voltage clamp recording from CA1 pyramidal cells in the presence of AP5 and BIC, blocking NMDAR and GABA<sub>A</sub>R activity, respectively. Afferent stimulation of Schaeffer collaterals produced AMPAR-mediated EPSCs of varying amplitudes depending on the stimulation intensity (Fig. 1A). The threshold voltage for eliciting a synaptic response did not vary between cells from control- and nicotine-treated rats ( $4.73 \pm 1.25$  V and  $4.57 \pm 1.6$  V, respectively,  $p = 0.83$ ). I/O curves plotting EPSC amplitudes in response to 2 V incremental steps above the threshold voltage were then compared between treatment groups. Statistical analysis using a two-way repeated measures ANOVA revealed a significant treatment  $\times$  voltage interaction ( $p = 0.05$ ). Bonferroni post-hoc analysis revealed that nicotine-treated rats had larger EPSC amplitudes with increasing stimulus intensity compared to control-treated rats, with significant differences detected in response to



**Fig. 1.** Chronic neonatal nicotine treatment increases AMPAR-mediated excitation in the adult rat hippocampus. (A) Representative evoked AMPAR-mediated EPSCs recorded from CA1 pyramidal cells from control- and neonatal nicotine-treated adult (P60–90) male rats. (B) Input/Output curves constructed by recording the amplitude of EPSCs in response to the lowest voltage capable of eliciting a response ( $v$ ) and in response to increasing voltage inputs at 2 V steps above this threshold voltage.  $n = 10$  control, 14 neonatal nicotine cells. (C) Representative traces of paired-pulse facilitation of AMPAR-mediated EPSCs at 50, 100, and 200 ms inter-stimulus intervals (ISI). (D) Average paired-pulse ratios in response to different ISI in CA1 pyramidal cells from control- and neonatal nicotine-treated male rats. Stimulus artifacts in A and C were truncated for clarity.  $n = 9$  control, 12 neonatal nicotine cells for 50 ms ISI;  $n = 21$  control, 24 neonatal nicotine cells at 100 ms ISI;  $n = 9$  control, 11 neonatal nicotine cells for 200 ms interval. (E) Sample traces of sEPSCs recorded in the presence of BIC and AP5 from CA1 pyramidal cells from hippocampal slices obtained from adult control- or neonatal nicotine-treated rats. All sEPSCs activity was blocked by the AMPAR antagonist DNQX. (F) Mean sEPSC Inter-Event Interval (IEI) in pyramidal cells from control and neonatal nicotine-treated rats. (G) Mean sEPSC amplitude in pyramidal cells from control- and neonatal nicotine-treated adult rats  $n = 12$  control cells, 9 neonatal nicotine cells. \* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ .

the three highest voltage inputs (Fig. 1B,  $p = 0.039$ ,  $0.041$ ,  $0.047$ , respectively). In order to determine if this increase in AMPAR-mediated excitation in nicotine-treated rats is a result of an increase in the presynaptic release probability at Schaeffer collateral-

CA1 synapses, we compared PPRs in CA1 pyramidal neurons from control- and nicotine-treated rats (Manabe et al., 1993; Zucker, 1989). Responses of two consecutive stimuli were recorded at ISIs of 50, 100, and 200 ms (Fig. 1C, D), and the calculated PPRs were

compared between treatment groups. There was no difference in the PPRs in cells from control- versus nicotine-treated rats at 50 ( $p = 0.31$ ), 100 ( $p = 0.34$ ), or 200 ms ( $p = 0.34$ ) intervals (Fig. 1D) as determined by Student's  $t$ -test analysis, indicating that the increase in evoked EPSC amplitude responses (Fig. 1A) is not the result of increased presynaptic glutamate release probability.

In order to more closely examine long-term changes in glutamatergic transmission elicited by chronic neonatal nicotine treatment, spontaneous (s)EPSCs (TTX-sensitive) were recorded from CA1 pyramidal cells in the presence of AP5 and BIC (Fig. 1E). In nicotine-treated rats, mean IELs were significantly lower (Fig. 1F,  $p = 0.045$ ) and mean amplitudes of sEPSCs were higher (Fig. 1G,  $p = 0.034$ ) compared to controls as determined by Student's  $t$ -tests. Together, the data from evoked and spontaneous EPSCs in neurons from nicotine-treated rats point to enhanced glutamatergic transmission at Schaeffer collateral-CA1 synapses. This enhancement could be the result of increased numbers of excitatory Schaeffer collateral-CA1 synapses or increased postsynaptic AMPAR-mediated activity with little or no change in presynaptic glutamate release probability.

### 3.2. Chronic neonatal nicotine treatment has no effect on spontaneous GABA release

GABAergic inputs form local interneurons and basal forebrain projections play a critical role in regulating hippocampal excitatory activity (Freund and Antal, 1988; Miles et al., 1996). To determine if spontaneous GABA release was altered by chronic neonatal nicotine treatment sPSCs and mIPSCs were recorded from CA1 pyramidal neurons from adult males which were control- or nicotine-treated as neonates. Under our recording conditions (see Methods), more than 95% of the sPSCs were blocked by BIC indicating that the vast majority of these spontaneous events are mediated by GABA<sub>A</sub>Rs. The IEL and amplitude of primarily GABA<sub>A</sub>R-mediated sPSCs were not significantly different between control and nicotine-treated rats (Fig. 2A, B;  $p = 0.5$  for IEL,  $p = 0.87$  for amplitude) suggesting that there was no significant change in spontaneous (TTX-sensitive) GABA release. We then recorded action-potential independent asynchronous spontaneous inhibitory events (mIPSCs) in the presence of TTX (TTX-insensitive). There was also no significant difference in the mean IELs or amplitudes of mIPSCs in cells from control- versus nicotine-treated rats (Fig. 2C, D;  $p = 0.293$  for IEL,  $p = 0.459$  for amplitude). Thus, there was little or no change in TTX-sensitive or TTX-insensitive GABA release. Together, these data suggest that the increase in overall excitation observed in the hippocampus of adult males following chronic neonatal nicotine treatment (Damborsky et al., 2012) is the result of increased glutamatergic signaling rather than a decrease in inhibitory signaling.

### 3.3. Acute nicotine increases glutamate release to a similar degree in control- and chronic neonatal nicotine-treated rats

Neuronal nAChRs play an important role in the modulation of hippocampal glutamatergic transmission and can increase excitatory responses via pre- and postsynaptic mechanisms (Gray et al., 1996; Radcliffe and Dani, 1998). In order to determine if nicotinic regulation of excitatory responses was affected by neonatal nicotine treatment we bath applied nicotine to the slices at two different concentrations (0.1 and 1  $\mu$ M), and examined nicotine-induced acute effects on sEPSCs recorded from CA1 pyramidal neurons. Representative traces (Fig. 3A) and cumulative histograms of IELs and event amplitudes show the effect of 0.1  $\mu$ M nicotine (Fig. 3B, C). There was a significant decrease in sEPSC IEL in cells from both control- ( $p < 0.001$ ) and nicotine-treated rats ( $p < 0.001$ ) as demonstrated by K–S test cumulative probability analysis. In

addition, there was a significant increase in sEPSC amplitude following acute nicotine application in cells from both control- and nicotine-treated rats (control:  $p < 0.001$ , neonatal nicotine:  $p = 0.0018$ ). The same comparisons were made following acute bath application of 1  $\mu$ M nicotine (data not shown). K–S test cumulative probability analysis revealed a significant decrease in sEPSC IELs in cells from both control- ( $p < 0.001$ ) and nicotine-treated rats ( $p < 0.001$ ), but there was no significant change in sEPSC amplitude following 1  $\mu$ M nicotine application in cells from either control- or nicotine-treated rats as measured by K–S tests (control:  $p = 0.06$ ; neonatal nicotine:  $p = 0.26$ ).

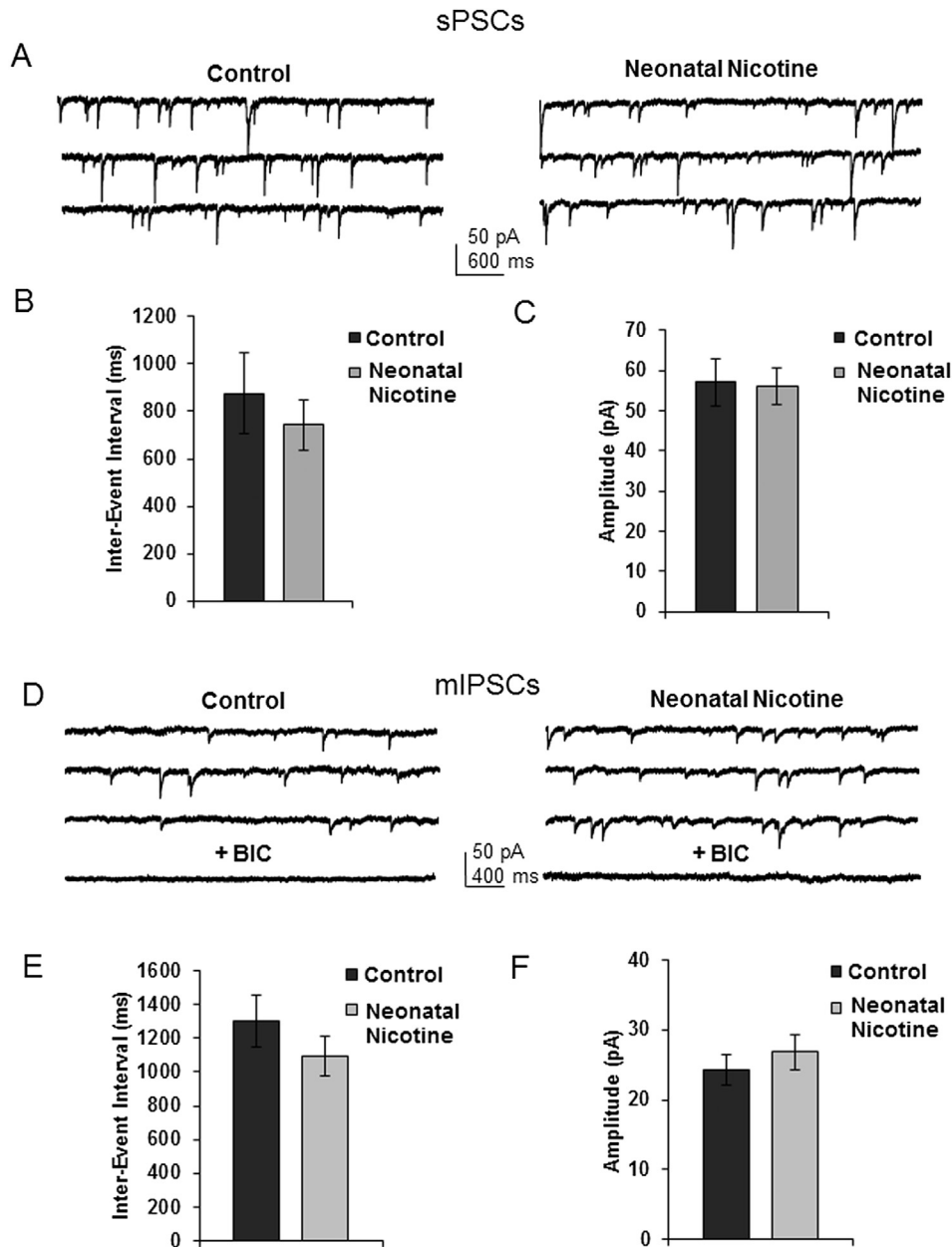
We then measured the acute effects of nicotine on evoked excitatory responses. To further determine if chronic neonatal nicotine treatment persistently altered acute nicotine-induced presynaptic glutamate release at Schaeffer collateral-CA1 synapses in adult animals, we analyzed the amplitude of the first evoked EPSC in our paired-pulse paradigm and the calculated PPRs at the 100 ms ISI before (pre-) and after (post-) acute bath application of nicotine (Fig. 3D). In neurons from both control- and nicotine-treated rats, acute 0.1  $\mu$ M nicotine, resulted in significantly increased evoked EPSC amplitude (Fig. 3E; control:  $p = 0.027$ ; neonatal nicotine:  $p = 0.008$ ), and a significantly smaller PPR (Fig. 3F; control:  $p = 0.001$ ; neonatal nicotine:  $p = 0.026$ ) as measured by paired Student's  $t$ -tests. Application of 1  $\mu$ M nicotine to the slices had similar effects in both treatment groups, and induced an increase in the amplitude of the first evoked EPSC response in cells from control- ( $330.14 \pm 22.28$  pA to  $357.30 \pm 23.55$  pA,  $p = 0.069$ ) and nicotine-treated rats ( $359.14 \pm 23.12$  pA to  $406.00 \pm 25.48$  pA,  $p = 0.024$ ). Application of 1  $\mu$ M nicotine also resulted in a significant decrease in PPRs in both groups (control:  $1.33 \pm 0.04$  to  $1.23 \pm 0.03$ ,  $p = 0.001$ ; neonatal nicotine:  $1.37 \pm 0.03$  to  $1.27 \pm 0.03$ ,  $p = 0.008$ ) indicating increased glutamate release probability in response to acute nicotine application.

Thus, acute stimulation of nAChR located on glutamatergic terminals by nicotine facilitated glutamatergic transmission and glutamate release in hippocampal slices from both control- and nicotine-treated rats. In order to determine if chronic neonatal nicotine treatment affected the magnitude of the response to an acute application of nicotine, nicotine-induced mean changes in spontaneous excitatory event frequency and amplitude following 0.1 or 1  $\mu$ M nicotine exposure were compared (Fig. 4A, B). A two-way ANOVA revealed that there was no effect of neonatal nicotine treatment for either acute nicotine-induced mean increases in sEPSC frequency ( $p = 0.98$ ) or amplitude ( $p = 0.24$ ). Furthermore, the degree of nicotine-stimulated increase in evoked EPSC amplitude and percent decrease in PPRs were also comparable between treatment groups with no significant treatment effect of chronic neonatal nicotine (Fig. 4C, D; EPSC amplitude:  $p = 0.23$ ; PPR:  $p = 0.75$ ).

Together these data indicate that chronic neonatal nicotine treatment significantly increases frequency and amplitude of excitatory glutamatergic responses in the adult hippocampus. In contrast, chronic neonatal nicotine treatment does not alter presynaptic nAChRs on glutamatergic terminals where they facilitate glutamatergic transmission; acute activation of nAChRs by nicotine enhanced excitatory transmission and facilitated glutamate release to the same degree in both treatment groups.

### 3.4. Nicotine-induced TTX-insensitive spontaneous GABA release is blunted in adult hippocampus following chronic neonatal nicotine treatment

Endogenous cholinergic activity or nicotine-induced activation of nAChRs located on GABAergic terminals can increase presynaptic

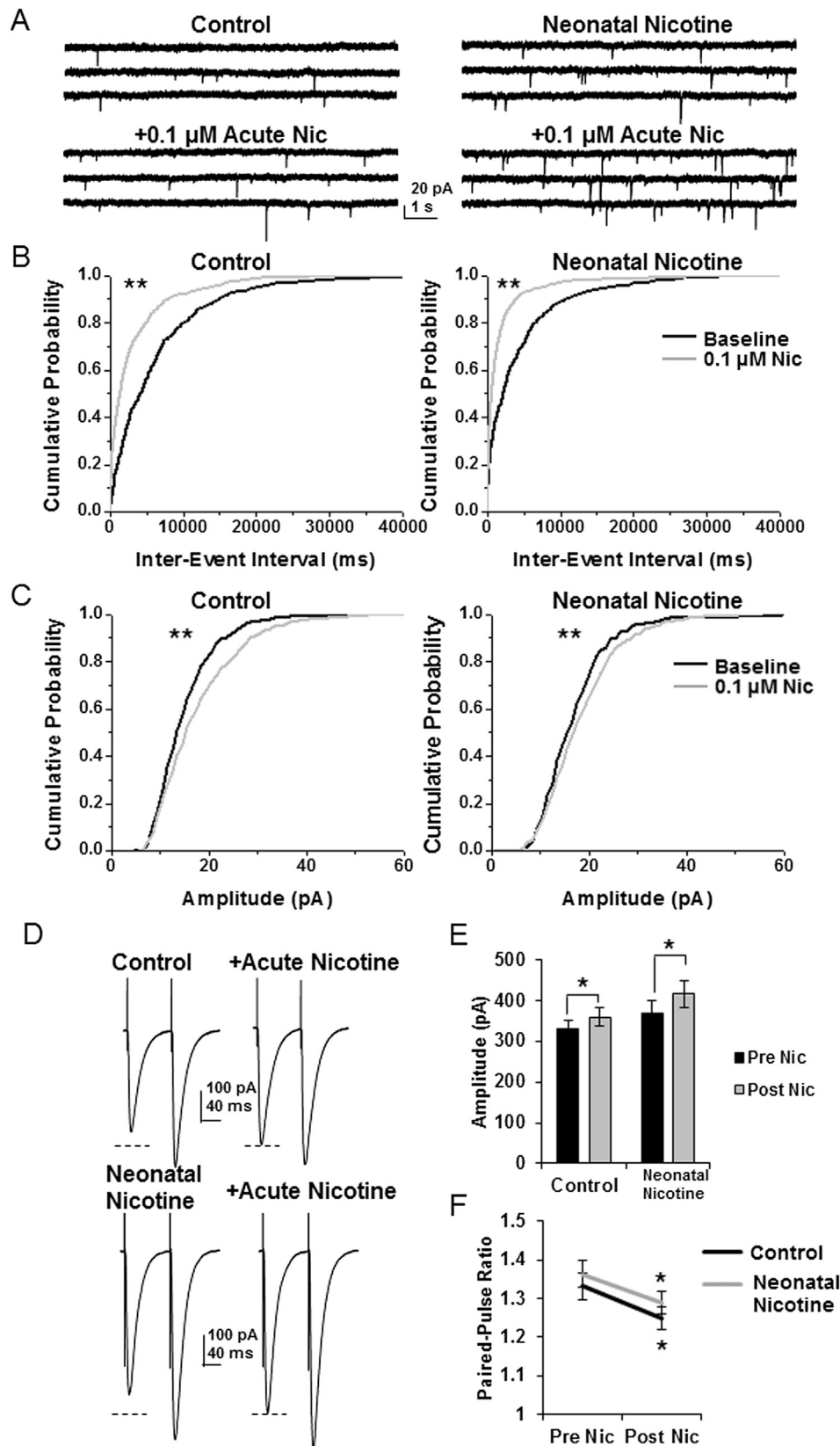


**Fig. 2.** Chronic neonatal nicotine treatment does not affect spontaneous GABA release in the adult hippocampus. (A) Representative traces showing primarily GABA<sub>A</sub>-mediated sPSCs from CA1 pyramidal cells from Control- and neonatal nicotine-treated young adult male rats. (B) Mean sPSC IEI in cells from Control- and neonatal nicotine-treated rats. (C) Mean sPSC amplitude in cells from control- and neonatal nicotine-treated rats.  $n = 16$  control, 18 neonatal nicotine cells. (D) Representative traces of mIPSCs recorded in the presence of AP5 and DNQX from CA1 pyramidal neurons from hippocampal slices obtained from control- and neonatal nicotine-treated young adult rats. All mIPSC activity was blocked after bath application of the GABA<sub>A</sub>R antagonist bicuculline (BIC). (E) Mean mIPSC IEI in cells from control- and neonatal nicotine-treated rats. (F) Mean mIPSC amplitude in cells from control- and neonatal nicotine-treated rats.  $n = 23$  control, 27 neonatal nicotine cells.

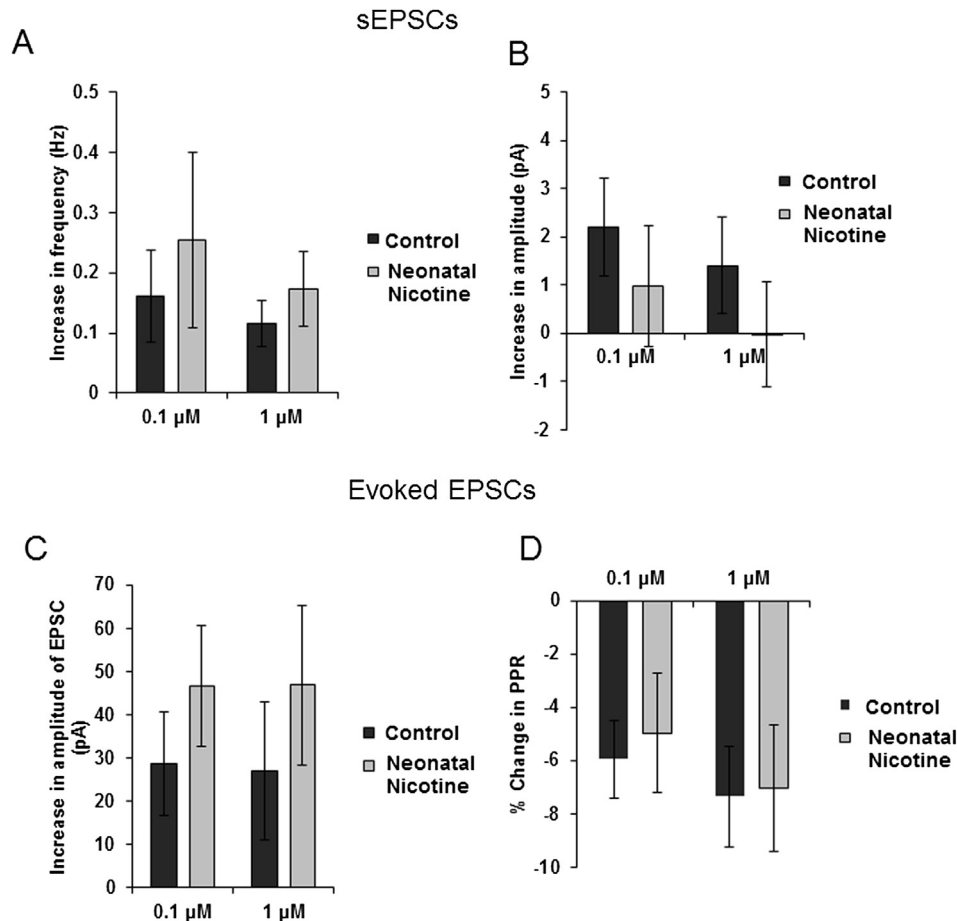
GABA release and thereby modulate the activity of pyramidal neurons (Alkondon et al., 1997; DuBois et al., 2013; Proctor et al., 2011). In order to determine whether acute nicotine-induced GABA release was affected by chronic neonatal nicotine treatment, we acutely bath applied nicotine at the same two concentrations (0.1 and 1  $\mu$ M) and recorded action potential independent (TTX-insensitive) mIPSCs from CA1 pyramidal cells in slices from adult animals. Representative traces (Fig. 5A, D) and cumulative histograms of IEIs (Fig. 5B, E) and event amplitudes (Fig. 5C, F) show the effect of acute nicotine on mIPSCs. Application of 0.1  $\mu$ M nicotine resulted in a significant decrease in mIPSC IEIs in neurons from both control- ( $p < 0.001$ ) and neonatal nicotine-treated rats ( $p < 0.001$ ) as measured by K–S tests. There was also a significant

increase in mIPSC amplitude following acute nicotine application in cells from control- ( $p < 0.001$ ) and nicotine-treated rats ( $p = 0.037$ ), although the effect was much smaller in neonatal nicotine-treated rats (Fig. 5C). In contrast, acute application of 1  $\mu$ M nicotine significantly decreased mIPSC IEI in cells from control- ( $p < 0.001$ ) but not nicotine-treated rats ( $p = 0.186$ ) (Fig. 5E), and significantly increased mIPSC amplitude in control- ( $p < 0.001$ ) but not nicotine-treated rats ( $p = 0.15$ ) (Fig. 5F).

Because there was a significant increase in mIPSC frequency and amplitude following acute application of 0.1 but not 1  $\mu$ M nicotine in neurons from neonatal nicotine-treated rats it is possible that the developmental nicotine exposure altered nAChR properties located on GABAergic presynaptic terminals, and thereby differentially



**Fig. 3.** Acute application of nicotine to hippocampal slice from neonatally control- or nicotine-treated adult animals increases presynaptic glutamate release. (A) Sample traces of sEPSCs recorded from CA1 pyramidal cells from slices from control- and neonatal nicotine-treated adult rats before and after bath application of 0.1  $\mu$ M nicotine. (B) Cumulative probability plots of the distribution of sEPSC IELs before and after nicotine application in pyramidal neurons from control and neonatal nicotine-treated rats. (C) Cumulative probability plots of the distribution of sEPSC amplitudes before and after acute nicotine application in pyramidal neurons from control and neonatal nicotine-treated rats. (D) Sample traces of paired-pulse ratios (PPRs) from control- and neonatal nicotine-treated rats before and after bath application of nicotine. (E) Amplitude of EPSC elicited by first stimulus in the paired pulse paradigm before (pre) and after (post) nicotine application in cells from control- and neonatal nicotine-treated rats. (F) PPRs before and after nicotine



**Fig. 4.** Chronic neonatal nicotine treatment does not affect acute nicotine-stimulated glutamate release in adult hippocampus. (A) Mean increase in frequency, and (B) amplitude of sEPSCs following application of 0.1 or 1  $\mu$ M nicotine in CA1 pyramidal cells from control- and neonatal nicotine-treated rats. (C) Mean increases in amplitude of evoked EPSCs following application of 0.1 or 1  $\mu$ M nicotine in cells from control- and neonatal nicotine-treated rats. (D) Percent change in PPRs following 0.1 or 1  $\mu$ M nicotine application in cells from control- and neonatal nicotine-treated rats.  $n = 10$ –11 control and 7–8 neonatal nicotine cells for sEPSCs;  $n = 19$  control and 19–22 neonatal nicotine cells for evoked EPSCs. Data analyzed using two-way ANOVAs.

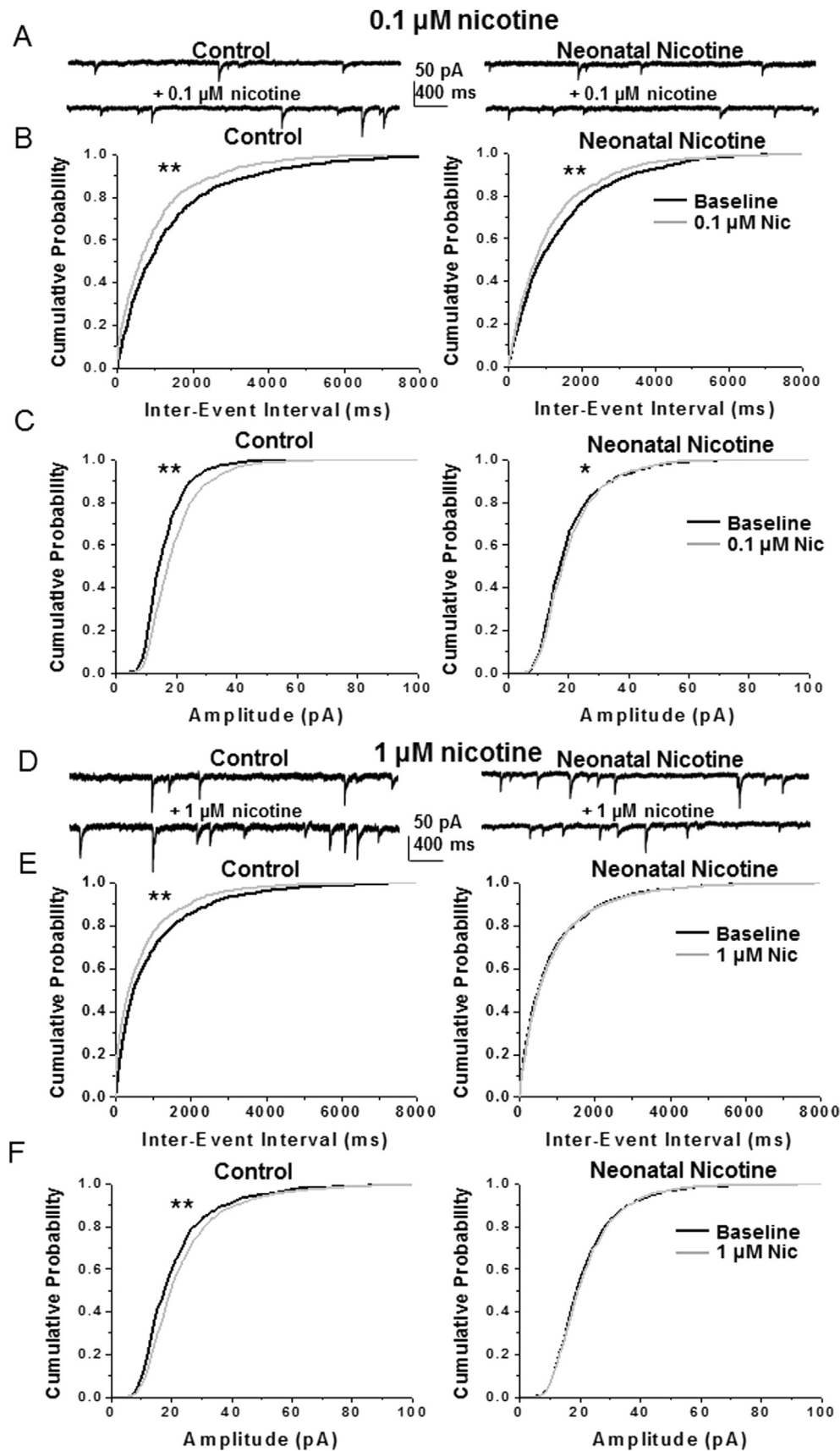
affected the response to varying concentrations of acute nicotine in adults. In order to test this, we examined two additional concentrations of nicotine: 0.01 and 10  $\mu$ M. Cumulative probability distribution analysis of IEL and amplitude in cells from control-treated rats revealed that mIPSC frequency and amplitude were also increased in response to 0.01 and 10  $\mu$ M nicotine (K–S test;  $p < 0.001$  for both). Conversely, when the same concentrations of nicotine were applied to hippocampal slices from nicotine-treated rats, there was no potentiation of mIPSC frequency in CA1 pyramidal cells at either nicotine concentration (K–S test:  $p = 0.152$  for 0.01  $\mu$ M,  $p = 0.258$  for 10  $\mu$ M), and the only other concentration resulting in an increase in amplitude was the 10  $\mu$ M concentration (K–S test;  $p < 0.001$ ).

When the magnitude of the mean changes in frequency and amplitude induced by all 4 concentrations of nicotine were compared we observed a typical inverted U-shaped dose–response relationship in cells from control-treated animals, but a much narrowed responsivity to nicotine in cells from neonatal nicotine-treated rats (Fig. 6A, B). Statistical analysis using a two-way ANOVA revealed an overall chronic neonatal nicotine treatment effect for the acute response of nicotine-induced increases in frequency ( $p = 0.009$ ) but not amplitude ( $p = 0.132$ ), suggesting an

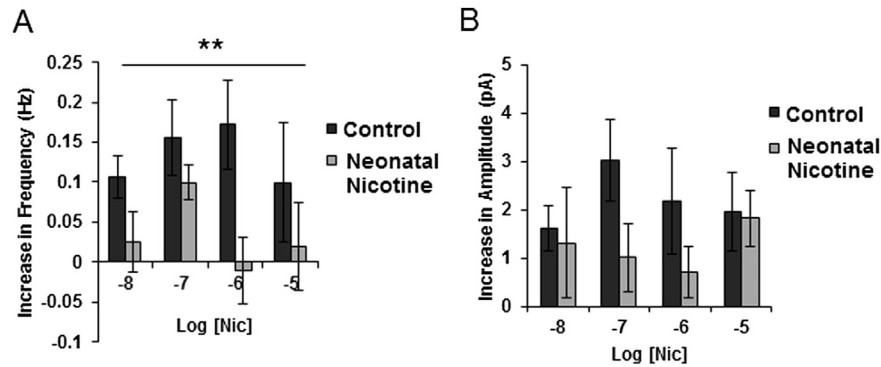
overall decrease in nicotine-induced presynaptic GABA release in hippocampal slices from chronic neonatal nicotine-treated rats.

#### 4. Discussion

In this study we demonstrate that neonatal exposure to nicotine results in long-lasting changes to hippocampal function; causing an increase in baseline excitatory signaling with no change in inhibitory signaling. Therefore, during development, nicotine is an environmental factor capable of persistently altering the excitatory/inhibitory ratio (E/I ratio). Furthermore, presynaptic nAChR function, as determined by nicotine administration to the slices, is specifically reduced on presynaptic GABAergic but not glutamatergic terminals innervating CA1 pyramidal cells. This differential change in nicotinic responses would exacerbate the overall increase in excitation within the hippocampus because of the diminished regulation of inhibitory but not excitatory transmission via presynaptic nAChRs. Together, these lasting changes could contribute to the long-term behavioral deficits observed in animals developmentally exposed to nicotine, and these findings might extend to humans exposed to nicotine in utero via maternal smoking (Shea and Steiner, 2008; Winzer-Serhan, 2008).



**Fig. 5.** Chronic neonatal nicotine treatment alters nicotine-induced GABA release in adulthood. (A) Sample traces of mIPSCs recorded from CA1 pyramidal neurons in slices from control- and neonatal nicotine-treated adult rats before and after bath application of 0.1  $\mu$ M nicotine. (B) Cumulative probability plots of the distribution of mIPSC IEIs before and after 0.1  $\mu$ M nicotine application in cells from control and neonatal nicotine-treated rats. (C) Cumulative probability plots of the distribution of mIPSC amplitudes before and after



**Fig. 6.** Chronic neonatal nicotine treatment significantly reduces acute nicotine-stimulated GABA release in the adult hippocampus (A) Mean changes in mIPSC frequency following bath application of nicotine (0.01–10  $\mu$ M) in CA1 pyramidal cells from control- and neonatal nicotine-treated rats. (B) Mean changes in mIPSC amplitude following bath application of nicotine (0.01–10  $\mu$ M) in CA1 pyramidal cells from control- and neonatal nicotine-treated rats.  $n = 8$ –19 cells. \*\* indicates significant chronic neonatal nicotine treatment effect as determined by two-way ANOVA.

#### 4.1. Neonatal nicotine exposure affects excitatory transmission in the adult hippocampus

We have previously demonstrated increased dendritic excitation, as measured by field excitatory postsynaptic potentials (EPSPs), in the CA1 region of chronic neonatally nicotine-treated adult males compared to controls (Damborsky et al., 2012). Here, we expand upon those findings by showing that this increase in excitation is accompanied by increases in glutamatergic signaling through AMPARs in whole-cell voltage clamp recordings from CA1 pyramidal neurons. In neonatally nicotine-treated adult males, evoked AMPAR-mediated EPSCs were significantly larger than those in controls suggesting increased excitatory transmission. We used a paired-pulse paradigm and found that PPRs were unchanged in nicotine-treated animals. These data suggest that postsynaptic mechanisms may be responsible for the increased AMPAR-mediated response rather than a presynaptic effect of an increase in glutamate release probability (Manabe et al., 1993; Zucker, 1989). We also found an increase in postsynaptic AMPAR-mediated amplitude in spontaneous EPSCs which is consistent with enhanced postsynaptic AMPAR responses. The mechanism(s) responsible for augmented postsynaptic AMPAR-mediated events may involve a number of different mechanisms, and to elucidate them will require further studies.

Adults, neonatally treated with nicotine, also had increased frequency of spontaneous AMPAR-mediated EPSCs suggesting an increase in excitatory synapse numbers. An increase in glutamatergic synaptic connections is in line with recent evidence suggesting a critical role for nAChRs in glutamatergic synapse formation (Lozada et al., 2012a, 2012b). While we show here that persistent nicotine-induced activation of nAChRs during early postnatal development leads to an increase in glutamatergic signaling, the studies by Lozada and colleagues show that a loss of endogenous nicotinic signaling can impair glutamatergic synapse development. Both findings suggest that nAChRs influence the numbers of excitatory synapses. In addition, there is also an increase in postsynaptic amplitude suggesting an enhanced postsynaptic excitatory response.

Our observations demonstrate for the first time that developmental nicotine exposure enhances hippocampal excitatory

synaptic transmission in adulthood, potentially by strengthening excitatory synapses and augmenting postsynaptic AMPAR-mediated responses. Together, these results point out a vital developmental role for nAChRs in shaping cortical excitatory networks.

Although postnatal nicotine exposure leads to an increase in hippocampal AMPAR-mediated excitation (this study), prenatal exposure causes a long-lasting decrease in AMPAR function and expression, reducing excitatory signaling in the hippocampus (Parameshwaran et al., 2012; Vaglenova et al., 2008; Wang et al., 2011). These apparent contradictory findings might be explained by the temporal maturation of the hippocampus. For example, nicotine's effect on excitatory synapse formation is also seen in the brainstem where prenatal nicotine exposure results in an increase in AMPAR-mediated excitation (Pilarski and Fregosi, 2009). The medulla develops and matures prenatally whereas the hippocampus mostly postnatally (Bayer et al., 1993). Additionally, the divergent outcomes in the hippocampus following pre- versus postnatal nicotine exposure may be mediated by the opposing effects of nicotine-facilitated glutamate release on high- and low-probability synapses (Maggi et al., 2003, 2004), and the declining proportion of functionally silent glutamatergic synapses during postnatal development (Durand and Konnerth, 1996; Hsia et al., 1998). Thus, the timing of nicotine exposure relative to developmental processes taking place in different brain regions examined might be a critical determinant of how excitatory signaling is affected. It would therefore be important to study different exposure periods to determine critical periods with increased sensitivity to the developmental effects of nicotine.

Although the effects of developmental nicotine exposure on glutamatergic signaling later in life may vary depending on the timing of exposure, it is clear that appropriate nAChR signaling is essential for mediating normal glutamatergic synapse formation within the hippocampus.

#### 4.2. Neonatal nicotine exposure has little effect on baseline inhibitory transmission in the adult hippocampus

In contrast to the robust increase in excitatory signaling observed in the adult hippocampus following chronic neonatal

0.1  $\mu$ M nicotine application in cells from control and neonatal nicotine-treated rats.  $n = 9$  control, 9 neonatal nicotine cells for 0.1  $\mu$ M nicotine. (D) Sample traces of mIPSCs recorded from CA1 pyramidal neurons in slices from control- and neonatal nicotine-treated adult rats before and after bath application of 1  $\mu$ M nicotine. (E) Cumulative probability plots of the distribution of mIPSC IELs before and after 1  $\mu$ M nicotine application in cells from control and neonatal nicotine-treated rats. (F) Cumulative probability plots of the distribution of mIPSC amplitudes before and after 1  $\mu$ M nicotine application in cells from control and neonatal nicotine-treated rats.  $n = 14$  control, 19 neonatal nicotine cells for 1  $\mu$ M nicotine.

nicotine treatment, we found no significant change in spontaneous GABA release as measured by sPSCs and mIPSCs. Thus, any nicotine effect on spontaneous GABA release or GABA<sub>A</sub>R function is relatively minor, and unlikely to compensate for the drastic increase in excitatory signaling. This is in agreement with our data showing that excitation is still increased in adult males neonatally treated with nicotine, even when GABAergic signaling is present (Damborsky et al., 2012). This suggests that inhibitory and excitatory synapses are influenced by different mechanisms. This is in agreement with other studies showing that endogenous nicotinic signaling during development may be more important for excitatory synapse development than for inhibitory synapse development (Lozada et al., 2012b). It is possible that by affecting the expression of neurotrophic factors in the neonatal hippocampus (Damborsky and Winzer-Serhan, 2012; Son and Winzer-Serhan, 2009) nicotine may differentially favor the formation of excitatory synapses during postnatal development. Whatever the mechanisms, our results indicate that neonatal nicotine exposure differentially affects glutamatergic and GABAergic synapse formation resulting in an altered E/I ratio of synaptic inputs to CA1 pyramidal neurons.

#### 4.3. Neonatal nicotine exposure differentially affects nicotinic modulation of excitatory and inhibitory transmission in the adult hippocampus

Nicotinic receptors located on excitatory and inhibitory presynaptic terminals facilitate glutamate and GABA transmitter release, respectively (Alkondon et al., 1997; Gray et al., 1996; Radcliffe and Dani, 1998). Furthermore, glutamatergic and GABAergic synaptic transmission in CA1 pyramidal neurons is in part regulated by basal activity of nAChRs which are activated by endogenous levels of acetylcholine (Banerjee et al., 2012a, 2012b). Recent studies have also shown that nAChRs can modulate hippocampal synaptic plasticity (Gu and Yakel, 2011; Nakauchi and Sumikawa, 2012). Thus, nAChRs play a critical role in the regulation of excitatory and inhibitory transmission onto CA1 pyramidal cells. It is therefore possible that a persistent alteration of nAChR function resulting from neonatal exposure to nicotine could contribute to increased excitatory hippocampal transmission.

Previous studies have shown that developmental nicotine exposure can permanently decrease presynaptic nAChR function, affecting nAChR-facilitated transmitter release long after chronic nicotine exposure had stopped (Britton et al., 2007; Gold et al., 2009; Kane et al., 2004; Seidler et al., 1992). In this study we report that developmental nicotine exposure selectively reduces nicotine-induced GABA but not glutamate release within the adult hippocampus. The reasons for this differential effect of developmental nicotine exposure are not known. We have shown that chronic neonatal nicotine transiently increases the expression of heteromeric but not homomeric nAChRs, but the functional changes described here are most likely not caused by changes in nAChR numbers which return to control levels after the end of nicotine treatment (Huang and Winzer-Serhan, 2006). It is possible that the developmental exposure to nicotine causes long-lasting functional changes to specific presynaptic nAChR populations perhaps by locking the receptors in a permanently desensitized state, or by altering the activation and desensitization kinetics. An alteration in channel kinetics would be in line with our data revealing a lack of nicotine-induced GABA release in response to both low (0.01  $\mu$ M) and high (1 and 10  $\mu$ M) concentrations of nicotine in cells from adult neonatally nicotine-treated rats. Further studies would need to be conducted to determine specifically how nAChR function on GABAergic terminals is altered and if lower doses of nicotine during the developmental treatment

period, have similar long lasting consequences. It also remains unknown why chronic neonatal nicotine alters nAChRs on GABAergic but not glutamatergic terminals. Expression of nAChRs in the hippocampus is complex (Son and Winzer-Serhan, 2008), and it is possible that nicotine exposure selectively changes the properties of presynaptic nAChR subtypes differentially expressed on transmitter terminals. Heteromeric  $\alpha 4\beta 2^*$  nAChRs are widely expressed on GABAergic terminals (Alkondon et al., 1999; McClure-Begley et al., 2009), and could be persistently altered by chronic neonatal nicotine treatment. In contrast, homomeric  $\alpha 7$  nAChRs predominantly regulate nicotine-stimulated glutamate release on glutamatergic terminals in the hippocampus (Gray et al., 1996; Radcliffe and Dani, 1998), although there are reports that activation of the  $\alpha 4\beta 2$ -type nAChRs could also modulate glutamate release (Garduno et al., 2012; Lambe et al., 2003). Thus, nAChR subtype specific effects might explain the differential long-term outcomes of chronic neonatal nicotine on nicotine-facilitated transmitter release in the adult hippocampus.

In the hippocampus, heteromeric nAChRs are also located on catecholaminergic axon terminals where they regulate transmitter release (Cao et al., 2005; Leslie et al., 2002). It remains to be determined if neonatal nicotine treatment also depresses nAChR-facilitated hippocampal release of dopamine and norepinephrine as has been reported for other brain areas (Britton et al., 2007; Gold et al., 2009; Kane et al., 2004; Seidler et al., 1992). Differentially diminished responsiveness of nAChRs to endogenous activation on afferent axonal terminals could have profound consequences for hippocampal function, which is not only determined by glutamatergic and GABAergic synaptic transmission but is also modulated by catecholamines (Penzes et al., 2013).

#### 4.4. Possible consequences of neonatal nicotine exposure on hippocampal function in adults

Several neurodevelopmental disorders including schizophrenia, ADHD and autism have been associated with nAChRs (Adams and Stevens, 2007; Heath and Picciotto, 2009; Lee et al., 2002). A unifying principle of these complex neurological disorders seems to be an altered E/I ratio, perhaps caused by interactions between genetic and environmental variables during development (Bakhtiari et al., 2012; Eichler and Meier, 2008; Lewis and Hashimoto, 2007; Rubenstein and Merzenich, 2003). The data presented here strongly implicate developmental nicotine exposure as an environmental factor capable of affecting the excitatory/inhibitory ratio, and suggest that derailed nAChR function during development may contribute to the disease etiology of some neurological disorders.

Nicotinic cholinergic signaling also plays a critical role in cognition and anxiety in adults (Levin et al., 2006; Picciotto et al., 2002). Thus, the alteration to nAChR function described here could contribute to behavioral deficits in adults. Furthermore, because of the specific effects on nAChRs on GABAergic terminals, nicotinic regulation of GABA release is diminished which could intensify the imbalance between excitation and inhibition, and exacerbate any changes to hippocampal function and behavior caused by the increased E/I ratio.

#### 4.5. Potential long-term consequences of maternal smoking during pregnancy

Maternal smoking during pregnancy is often associated with behavior problems which include an increased risk for ADHD, anxiety, conduct disorders, depression and drug addiction suggesting that the limbic system is especially vulnerable to prenatal tobacco smoke exposure (Abbott and Winzer-Serhan, 2012). Animal studies have demonstrated that developmental nicotine

exposure increases anxiety- and depression-like behavior (Eppolito et al., 2010; Huang et al., 2007; Vaglenova et al., 2004), increases locomotor activity (Zhu et al., 2014), and alters responses to drugs of abuse (Levin et al., 2006) suggesting that the behavioral teratogenic effect of smoking could be caused by nicotine's effects on the developing limbic system. In this study we show that nicotine exposure, even during a narrow treatment window, increased excitatory electrophysiological responses in the hippocampus, a structure that is an integral part of the limbic system (Drevets et al., 2008; Price and Drevets, 2010). It is not known at this point, if other limbic areas are affected as well, however, altered hippocampal transmission alone could potentially have far-reaching implications for hippocampal-mediated behaviors, including those that are attributed to the limbic system. It would be important to determine in future studies if different exposure periods would result in similar changes and if other brain structures are similarly affected or have different sensitive periods.

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