Title

Extracellular ATP Neurotransmission and Nicotine Sex-Specifically Modulate Habenular Neuronal Activity in Adolescence.

Journal

Journal of Neuroscience, 43(48)

ISSN

0270-6474

Authors

Chen, Yen-Chu

Rindner, Daniel Jun

Fowler, James P

et al.

Publication Date

2023-11-29

DOI

10.1523/jneurosci.1290-23.2023

Peer reviewed

Neurobiology of Disease

Extracellular ATP Neurotransmission and Nicotine Sex-Specifically Modulate Habenular Neuronal Activity in Adolescence

Yen-Chu Chen, Daniel Jun Rindner, James P. Fowler, Valeria Lallai, Allison Mogul, Angelo Demuro, Gyorgy Lur, and Christie D. Fowler

Department of Neurobiology and Behavior, University of California, Irvine, Irvine, California 92697

The recent increase in the use of nicotine products by teenagers has revealed an urgent need to better understand the impact of nicotine on the adolescent brain. Here, we sought to examine the actions of extracellular ATP as a neurotransmitter and to investigate whether ATP and nicotinic signaling interact during adolescence. With the $GRAB_{ATP}$ (G-protein-coupled receptor activation-based ATP sensor), we first demonstrated that nicotine induces extracellular ATP release in the medial habenula, a brain region involved in nicotine aversion and withdrawal. Using patch-clamp electrophysiology, we then demonstrated that activation of the ATP receptors P2X or $P2Y_1$ increases the neuronal firing of cholinergic neurons. Surprisingly, contrasting interactive effects were observed with nicotine exposure. For the P2X receptor, activation had no observable effect on acute nicotine-mediated activity, but during abstinence after 10 d of nicotine exposure, coexposure to nicotine and the P2X agonist potentiated neuronal activity in female, but not male, neurons. For $P2Y_1$ signaling, a potentiated effect of the agonist and nicotine was observed with acute exposure, but not following extended nicotine exposure. These data reveal a complex interactive effect between nicotinic and ATP signaling in the adolescent brain and provide mechanistic insights into extracellular ATP signaling with sex-specific alterations of neuronal responses based on prior drug exposure.

Key words: **adenosine triphosphate; adolescence; habenula; nicotine withdrawal; purinergic signaling; sex difference**

Significance Statement

In these studies, it was discovered that nicotine induces extracellular ATP release in the medial habenula and subsequent activation of the ATP purinergic receptors increases habenular cholinergic neuronal firing in the adolescent brain. Interestingly, following extended nicotine exposure, nicotine was found to alter the interplay between purinergic and nicotinic signaling in a sex-specific manner. Together, these studies provide a novel understanding for the role of extracellular ATP in mediating habenular activity and reveal how nicotine exposure during adolescence alters these signaling mechanisms, which has important implications given the high incidence of e-cigarette/vape use by youth.

Introduction

Nicotine is the primary reinforcer underlying tobacco and e-cigarette dependence (Le Foll et al., 2022). While tobacco smoking has declined, this has been unfortunately paralleled by a dramatic increase in e-cigarette use, especially among adolescents (National Youth Tobacco Survey, Centers for Disease Control and Prevention; Available at: https://www.cdc.gov/tobacco/data_statistics/surveys/nyts/index.htm). Since nicotine can alter neurodevelopmental processes leading to persistent neurobiological changes (Luna et al., 2010; Siqueira, 2017; Pushkin et al., 2019; Ren and Lotfipour, 2019; Mahajan et al., 2021), it is critical to better understand how nicotine use during adolescence may induce changes in neuronal signaling. The medial habenula (MHb) projection to the interpeduncular nucleus has been shown to modulate nicotine intake, aversion, and withdrawal (Salas et al., 2004a, b, 2009; Fowler et al., 2011; Görlich et al., 2013; Dao et al., 2014; Fowler and Kenny, 2014; Shih et al., 2014; Antolin-Fontes et al., 2015). Cholinergic MHb neurons exhibit characteristic baseline spontaneous firing with a frequency of 2–10 Hz (Görlich et al., 2013; Dao et al., 2014). Nicotine acts on cholinergic MHb

Received June 26, 2023; revised Sep. 20, 2023; accepted Sep. 29, 2023.

Author contributions: Y.-C.C., V.L., G.L., and C.D.F. designed research; Y.-C.C., D.J.R., J.P.F., V.L., A.M., A.D., and C.D.F. performed research; Y.-C.C., D.J.R., J.P.F., A.D., G.L., and C.D.F. analyzed data; Y.-C.C., V.L., and C.D.F. wrote the paper.

This work was supported by National Institutes of Health National Institute on Drug Abuse Grants DP1-DA-039658 and R01-DA-051831 (to C.D.F.) and Tobacco-Related Disease Research Program Grant T31R1767 (to C.D.F.).

The authors declare no competing financial interests.

Correspondence should be addressed to Christie D. Fowler at cdfowler@uci.edu.

https://doi.org/10.1523/JNEUROSCI.1290-23.2023

neurons via the nicotinic acetylcholine receptors (nAChRs), which include α3-, α5-, and β4-containing nAChRs (Fowler et al., 2011; Görlich et al., 2013; Dao et al., 2014; Shih et al., 2014; Elayouby et al., 2021). Acute nicotine increases MHb neuronal firing frequency (Görlich et al., 2013). Interestingly, following chronic nicotine exposure, there is no difference in baseline firing frequency, but nicotine re-exposure significantly potentiates neuronal activity (Görlich et al., 2013). Thus, MHb activity appears to become altered with nicotine use, which thereby underlies the withdrawal state. Indeed, injection of a nAChR antagonist into the MHb elicits withdrawal symptoms (Salas et al., 2009), providing further evidence for the role of the MHb nAChRs in withdrawal symptomology. The MHb cholinergic neurons also express neurokinin and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which appear to interact with nicotinic signaling and can alter withdrawal symptoms (Görlich et al., 2013; Dao et al., 2014). Together, these findings demonstrate that cholinergic MHb neurons mediate nicotine withdrawal, and multiple cellular mechanisms may converge to regulate the withdrawal state.

ATP has canonically been recognized for its critical role in providing energy to support intracellular processes. However, ATP is also localized in synaptic regions and acts on purinergic P2X and P2Y receptors; moreover, interactions between purinergic receptors and nAChRs have been demonstrated at the cell membrane *in vitro*, and both receptor types are localized in MHb neurons (Edwards et al., 1992; Kanjhan et al., 1999; Morán-Jiménez and Matute, 2000; Khakh et al., 2005; Florenzano et al., 2008; Guo et al., 2008; Song et al., 2011). ATP activation leads to either influx of Na^+ and Ca^{2+} via P2X (North, 2002; Burnstock, 2018) or increased intracellular Ca^{2+} through G-protein signaling via $P2Y_1$ (Abbracchio et al., 2006; Guzman and Gerevich, 2016). Interestingly, *in vitro*, mutual inhibition has been shown between P2X receptors and $\alpha_3\beta_4$-containing nAChRs (Khakh et al., 2000; Decker and Galligan, 2009), both of which are expressed in cholinergic MHb neurons. Thus, these prior findings support the contention that purinergic signaling may be an important modulator of MHb neuronal activity.

In these studies, we sought to determine whether nicotine acts on circuit function to alter ATP signaling and whether ATP neurotransmission regulates habenular firing during the naive state and after chronic nicotine exposure. To monitor ATP release activity in the MHb, we used the G-protein-coupled receptor activation-based ATP sensor ($GRAB_{ATP}$), a modified human $P2Y_1$ receptor that elicits a florescence signal with ATP binding (Wu et al., 2022). We then used patch-clamp electrophysiology to examine the effects of activation of P2X and $P2Y_1$ receptors on MHb neuronal activity and to determine the further interaction of the receptors with nicotine, under both acute and nicotine withdrawal states, in male and female mice. Together, our studies reveal the functional relevance of extracellular ATP in the habenula and further demonstrate that purinergic signaling plays an important sex-specific role in habenular neuronal activity during nicotine abstinence in adolescence.

Materials and Methods

Animals. C57BL/6J wild-type male ($n = 39$) and female ($n = 44$) mice (RRID:IMSR_JAX:000664) were derived from six breeders in our laboratory animal facilities. Subjects were investigated during adolescence [postnatal day 21 (P21) to P50; Yuan et al., 2015]. Mice were maintained in the vivarium with a 12 h reversed light/dark schedule. Food and water were provided *ad libitum* until killed for patch-clamp recording or $GRAB_{ATP}$ imaging. All experiments were performed in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

Drugs. The P2X receptor general agonist $\alpha\beta$-methylene ATP salt (catalog #3209, Tocris Bioscience), the $P2Y_1$-specific agonist [(1*R*,2*R*,3*S*,4*R*,5*S*)−4-[6-amino-2-(methylthio)−9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid monoester trisodium salt (MRS2365; catalog #2157, Tocris Bioscience), the general P2 antagonist suramin (catalog #S2671, Sigma-Aldrich), and (–)-nicotine hydrogen tartrate salt (catalog #0215355491, MP Biomedicals) were dissolved in 0.9% sterile saline and adjusted to pH 7.4. Of note, we focused on the P2X general agonist since subtype-selective agonists are not available. Based on prior studies, the following concentrations were used: a 100 μM $\alpha\beta$-methylene adenosine 5'-triphosphate trisodium salt solution and a 30 μM suramin solution (Edwards et al., 1992; Pankratov et al., 2002). For the $P2Y_1$-specific agonist, a moderate dose of 50 μM was selected (Liston et al., 2020), and this receptor subtype was focused on, given the availability of the selective agonist and the documented expression levels in the MHb. Based on previous electrophysiology studies in the MHb (Dao et al., 2014; Shih et al., 2014; Morton et al., 2018), 1 μM nicotine was used to validate that the recordings are retrieved from cholinergic neurons. To avoid a possible ceiling effect of neuronal firing, 0.5 μM nicotine was chosen to study the interaction between cholinergic and purinergic signaling mechanisms. Of relevance, concentrations in this range are consistent with that found in human smokers (Benowitz et al., 2009).

Nicotine exposure paradigm. To investigate the role of purinergic signaling in the MHb during nicotine abstinence, adolescent mice were subject to subcutaneous nicotine injection at a concentration of 1.5 mg/kg for 10 d. The injection route and duration of this treatment was selected to provide controlled dosing over a moderate length of exposure period (Chellian et al., 2021). The 1.5 mg/kg dose was selected since mice have been previously shown to self-administer this amount in daily sessions (Fowler and Kenny, 2011). The $GRAB_{ATP}$ imaging and electrophysiology recordings were conducted 24 h after the last nicotine injection. Of note, the 24 h abstinence time point has been well established in the field as a significant point of withdrawal symptomology following nicotine exposure in animal models (Damaj et al., 2003; Matta et al., 2007). Further, humans report adverse symptoms of withdrawal after 4–24 h of cessation (Merritt et al., 2010; McLaughlin et al., 2015; Conti et al., 2020), including adolescent smokers at 24 h (Rubinstein et al., 2009), supporting the translational value of this time point.

Acute brain slicing. Mice were fully anesthetized with isoflurane, followed by transcardiac perfusion of 10 ml of 4°C *N*-methyl-D-glucamine (NMDG)-HEPES artificial CSF (aCSF) containing the following (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2 \cdot 2\ H_2O$, and 10 $MgSO_4 \cdot 7\ H_2O$, saturated with carbogen (95% O_2/5% CO_2) and a pH value adjusted to ~7.4. The transcardiac perfusion of the prechilled NMDG-HEPES aCSF solution is used to rapidly slow the metabolism of the brain to decrease neuronal death rate during slicing (Ting et al., 2018). The brain was quickly extracted, and 250 μm coronal slices of the MHb were sectioned using a MicroSlicer Zero 1N (Ted Pella). After sectioning, slices were transferred into the beaker filled with 150 ml of 32°C NMDG-HEPES aCSF, and the Na^+ spike-in procedure was performed by adding Na^+ spike-in solution (2 M NaCl in NMDG-HEPES aCSF) every 5 min. The Na^+ spike-in procedure allows the brain slices to gradually accommodate to a higher extracellular Na^+ concentration (Ting et al., 2018). This procedure can both increase neuronal survival rate and reliability of the gigaseal formation during patch clamp (Ting et al., 2018). After adding the Na^+ spike-in solution five times, the brain slices were transferred to a HEPES holding solution containing the following (in mM): 92 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 2 $CaCl_2 \cdot 2\ H_2O$, and 2 $MgSO_4 \cdot 7\ H_2O$, saturated with carbogen (95% O_2/5% CO_2) and pH value adjusted to ~7.4. The brain slices were incubated at room temperature at least 1 h before

recording with the intracellular solution osmolarity of 290 mOsmol/kg and extracellular osmolarity of 310 mOsmol/kg.

Electrophysiology patch-clamp and drug application. Brain slices were transferred to the recording chamber (SliceScope, Scientifica; RRID:SCR_018405) and submerged under 32°C recording solution containing the following (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 5 HEPES, 12.5 glucose, 2 $CaCl_2 \cdot 2\ H_2O$, and 2 $MgSO_4 \cdot 7\ H_2O$, saturated with carbogen (95% O_2/5% CO_2) and with a pH value adjusted to ~7.4. The rate of recording solution perfusing into the recording chamber was 5 ml/min. Ventral MHb neurons were visualized by infrared differential interference contrast microscopy (Olympus). Whole-cell recordings were obtained using borosilicate glass pipette electrodes (catalog #P-1000, Sutter Instrument; RRID: SCR_021042) with resistance of 3–5 MΩ. The electrodes were filled with a potassium methanesulfonate-based internal solution containing the following (in mM): 135 KMeSO3, 10 HEPES, 4 MgCl2, 4 MgCl2, 4 Na2ATP, 0.4 NaGTP, and 10 sodium creatine phosphate with a pH value adjusted to 7.3 using KOH.

Data were acquired by using an amplifier (Multiclamp 700B Amplifier, Molecular Devices; RRID:SCR_018455) and a digitizer (Digidata 1550B, Molecular Devices), and were recorded with pCLAMP 10 (Molecular Devices; RRID:SCR_011323). All signals were filtered at 5 kHz and sampled at 20 kHz. The series resistance was monitored by applying a hyperpolarizing 10 mV voltage step in voltage-clamp mode to calculate the resistance using Ohm's law. If the series resistance varied >20% across the experiment, the data were excluded because of concerns with the gigaseal state.

Ventral MHb neurons were maintained at −50 mV under current clamp to provide standardized conditions (Hsu et al., 2013; Vickstrom et al., 2020). The baseline was recorded for 5 min before drug application. The $\alpha\beta$-methylene ATP or MRS2365 was applied in 0.5 s pulses using a picospritzer (Parker Hannifin; RRID:SCR_018152). Drug action was quantified during the 5 s immediately after picospritzer application. The borosilicate glass pipettes (3–5 MΩ) containing $\alpha\beta$-methylene ATP or MRS2365 were placed ~20 µm away from recorded neurons, and the pressure meter on the picospritzer was set to 10–12 psi. To assess the interaction between cholinergic and purinergic signaling, the baseline was recorded for 2 min followed by bath application of 0.5 µM nicotine for 10 min. With the picospritzer, $\alpha\beta$-methylene ATP or MRS2365 was applied onto the recorded neuron for 0.5 s while nicotine bath application was maintained for an additional 10 min. To examine cell activity following chronic nicotine treatment, brain slices were obtained from mice injected with nicotine daily for 10 prior days, with the same tissue processing and patch-clamp procedures described above. Given our focus on nAChR-containing neurons, cells were excluded if no response was elicited with application of nicotine at the end of the study.

To examine endogenous purinergic signaling in the presence of an antagonist, neurons were held at −50 mV in current clamp. Sections were bathed in aCSF for 5 min, followed by bath application of the following: (1) 5 min of suramin (30 µM), (2) 5 min of suramin (30 µM) and nicotine (0.5 µM), and (3) 10 min of nicotine (0.5 µM), in sequential order. Neuronal activity was recorded, and firing frequency was calculated during each drug bath application. To ensure a return to baseline levels following suramin application, nicotine was bath applied for 10 min, and the neuronal activity during the last 5 min was analyzed.

Stereotaxic surgery. The adeno-associated virus (AAV)-$GRAB_{ATP}$ sensor was microinjected directly into the MHb using a stereotaxic device (Kopf). Mice were anesthetized with 1–3% of an isoflurane/oxygen mixture. The head was fixed and adjusted to flat-skull position on the Kopf stereotaxic frame. Microinjection of 0.5 µl of AAV9-hSyn-ATP1.0 (titer, $5 * 10^{13}$ vector genomes/ml; catalog #YL006001-AV9, WZ Biosciences; Wu et al., 2022) was administered bilaterally into the MHb (AP, −1.2 mm; midline, ±1.06; DV, −2.47 mm; injectors at a 20° angle toward midline) at a rate of 0.25 µl across 2 min. The injectors remained in place for an additional 3 min before moving 0.2 mm up the dorsal axis, at which time another 0.5 µl of AAV9-hSyn-ATP1.0 was administered at the same infusion rate across 2 min. The injectors remained in the injection site for 3 min before removal.

Fluorescence imaging of $GRAB_{ATP}$ in brain slices. After a period of 2.5 weeks following the AAV9-hSyn-ATP1.0 injection, acute brain slices at a 250 µm thickness were obtained from the mice, as described above. Images were acquired on the electrophysiology recording microscope with a SciCam Pro Camera (Scientifica). The frame rate of the image was set to 2 frames/s, and the binning mode was set at 2 × 2 on the Ocular software (Scientifica; RRID:SCR_021035). MHb neurons expressing $GRAB_{ATP}$ were visualized using 488 nm LED light. The baseline activity of ATP release in MHb was recorded for 2.5 min before bath applying 1 µM nicotine. After 5 min, nicotine exposure (to allow the recording chamber to be fully submerged with nicotine solution), fluorescence activity indicative of ATP release was recorded for at least 8 min.

Statistical analysis. The number of subjects for each experiment was based on the results of power analysis performed in G*Power (a priori estimated effect size: 0.6, $p < 0.05$; power, 0.95), and published guidelines for electrophysiological studies (Journal of Neuroscience, 2018). *t* tests or ANOVAs was performed to fit the experimental design. Repeated one-way ANOVAs were conducted to examine the effects of two or more treatments with a within-subject design. Following significant main and interaction effects, a Tukey's *post hoc* test was used, with correction for multiple comparisons. Data analysis was performed in GraphPad Prism 7.0 software (RRID:SCR_002798). The criterion of significance was $\alpha = 0.05$.

Results

Nicotine induces ATP release in the MHb

The expression of purinergic receptors in the MHb have been documented; however, ATP release activity in this brain region remains to be confirmed (Sperlágh et al., 1995, 1998; Kanjhan et al., 1999; Morán-Jiménez and Matute, 2000; Price et al., 2003; Florenzano et al., 2008; Guo et al., 2008; Song et al., 2011). Thus, we used $GRAB_{ATP}$ sensor imaging to directly monitor real-time ATP release, which is reflected by the change of fluorescence signal (Fig. 1*A–F*). In the MHb, we did not detect changes in $GRAB_{ATP}$ sensor fluorescence under endogenous conditions, suggesting undetectable, if any, extracellular ATP release at baseline (Fig. 1*C*). Thus, we next sought to determine whether acute nicotine would alter ATP extracellular activity. Perfusion of nicotine induced transient and localized increases in fluorescence signal, indicative of nicotine-mediated ATP release (Fig. 1*D*). Given that the MHb is highly involved in regulating nicotine withdrawal (Görlich et al., 2013; Dao et al., 2014; Fowler and Kenny, 2014; Shih et al., 2014; Antolin-Fontes et al., 2015), we next hypothesized that signaling dynamics may be altered following chronic nicotine exposure. In brain sections from mice exposed to nicotine across 10 d, we observed limited ATP release activity in the MHb at baseline, similar to that found in the naive tissue (Fig. 1*E*). Interestingly, re-exposure to nicotine during nicotine abstinence increased the occurrence of fluorescent transients across the duration of recording (Fig. 1*F*). These observations indicate that nicotine induces ATP release in the MHb.

Antagonism of purinergic signaling demonstrates interaction with nicotine

Next, we sought to examine whether endogenous ATP signaling alters neuronal firing in the MHb at baseline. Brain sections were perfused with aCSF containing the following: (1) the P2 general receptor antagonist suramin, to block endogenous purinergic signaling; (2) nicotine alone; or (3) coperfusion of nicotine and suramin. Data analysis revealed significant differences among the groups (repeated-measures, one-way ANOVA: $F_{(3,21)} = 8.178$, $p = 0.0009$, $R^2 = 0.5388$; Fig. 2*A–C*). No significant difference in the firing frequency was found between baseline and suramin perfusion ($p = 0.9998$). This finding is consistent with

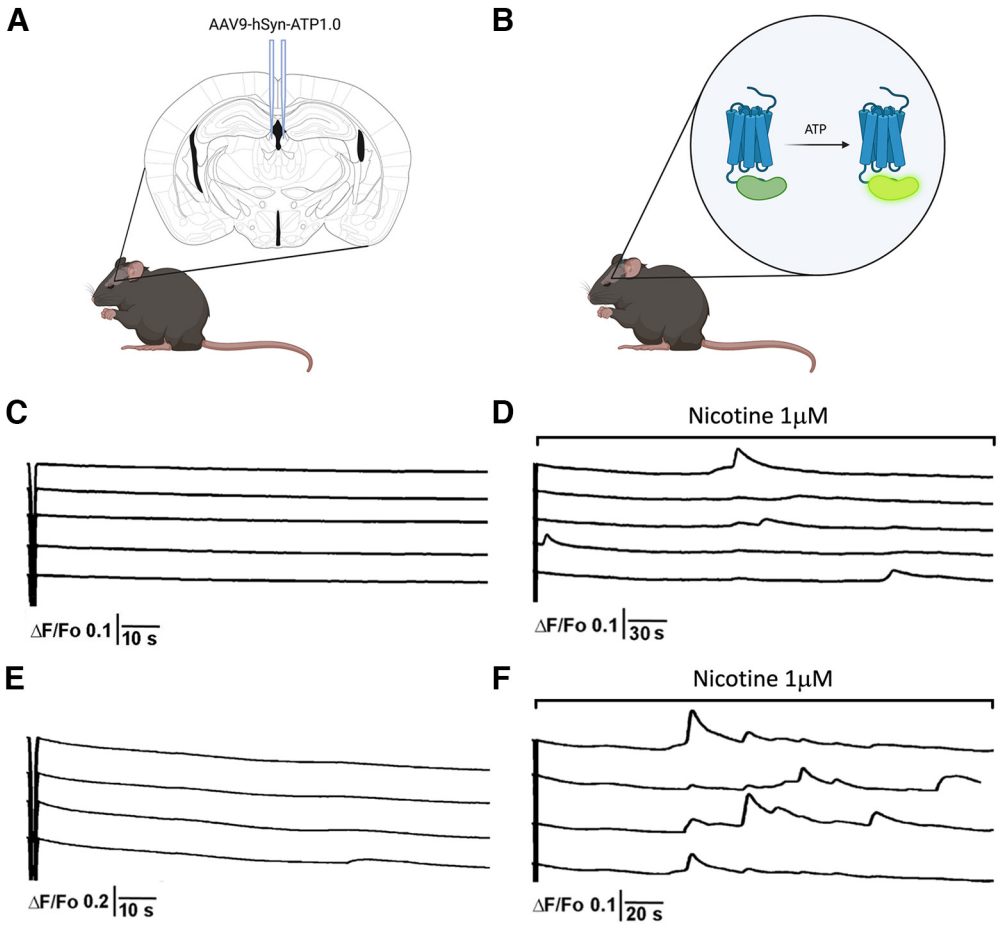

Figure 1. Nicotine exposure induces extracellular ATP release in the MHb. MHb sections expressing the $GRAB_{ATP}$ sensor were imaged to monitor ATP release activity ($n = 4$ subjects). Individual traces show fluorescence signals. ***A***, Illustration of $GRAB_{ATP}$ sensor AAV bilateral injection into the MHb. ***B***, Illustration of $GRAB_{ATP}$ sensor expressed in the MHb of the brain, in which the presence of extracellular ATP induces a change in fluorescence signal. ***C***, At baseline with aCSF perfusion, little to no changes in fluorescence signal were detected. ***D***, During 1 μM nicotine aCSF perfusion, changes in fluorescence signal were observed in the MHb. ***E***, Mice were treated with 1.5 mg/kg nicotine for 10 d, and 24 h later, brain sections were examined for $GRAB_{ATP}$ fluorescence. During abstinence with aCSF perfusion, few to no changes in fluorescence signal were detected. ***F***, During 1 μM nicotine aCSF perfusion, nicotine re-exposure induced changes in fluorescence signal in the MHb.

the results of the $GRAB_{ATP}$ sensor imaging, in which limited ATP release was observed without the presence of nicotine. Therefore, given that nicotine exposure appeared to elevate ATP signaling with the $GRAB_{ATP}$ sensor, we then investigated the effect of blocking the action of nicotine-mediated ATP release on habenular neuronal activity by coperfusing suramin and nicotine. Nicotine induced a significant increase in neuronal firing, compared with baseline levels ($p = 0.002$) and when suramin only is administered ($p = 0.0024$). Interestingly, suramin prevented the nicotine-mediated elevation in firing frequency, which was significantly lower from the level of firing found during nicotine alone ($p = 0.0082$; Fig. 2*B*,*C*). It should be noted that both males and females were used in these studies, but no significant differences were found between sexes in the effect of suramin on nicotine-mediated firing (Table 1). Moreover, one prior study suggested that higher concentrations of suramin may act on NMDA and AMPA receptors (Suzuki et al., 2004), but no statistically significant effects were found at 30 μM in their study, which was the dose used herein. Nevertheless, we conducted a control experiment to assess suramin alone, and, as expected, no differences were observed in neuronal firing [paired *t* test: $t_{(4)} = 0.5663$, $p = 0.6015$, $R^2 = 0.07421$; baseline (mean ± SEM), 2.682 ± 0.6243; suramin, 2.840 ± 0.7833]. Therefore, these findings indicate an interaction between purinergic signaling and cholinergic signaling in MHb cholinergic neurons.

ATP facilitates neuronal firing via P2X and $P2Y_1$ receptors

To determine whether ATP alone can alter neuronal activity, we next examined the effects of the P2X and $P2Y_1$ receptor agonists on firing frequency. It was previously demonstrated that activation of P2X receptors leads to influx of Na^+ and Ca^{2+} (Pankratov et al., 2009; Yang and Liang, 2012; Burnstock, 2015). Therefore, activating the P2X receptors would be expected to induce an excitatory effect on cholinergic neuronal activity in the MHb. For all of the studies, ventral MHb neurons were validated as cholinergic based on an increase in neuronal activity with nicotine application following the experimental recordings, given the known nAChR expression in this cellular population (Fowler et al., 2011; Görlich et al., 2013; Dao et al., 2014; Shih et al., 2014). As for $P2Y_1$ receptors, it has been shown that activation of these membrane receptors increases intracellular Ca^{2+} through G-protein-coupled signaling (Abbracchio et al., 2006; Guzman and Gerevich, 2016). Furthermore, in mesencephalic trigeminal neurons, ATP was demonstrated to act on $P2Y_1$ receptors to facilitate HCN channel currents (Huang et al., 2010), so such an interaction may underlie the findings observed in the current study. In sum, the current evidence from the field leads to the

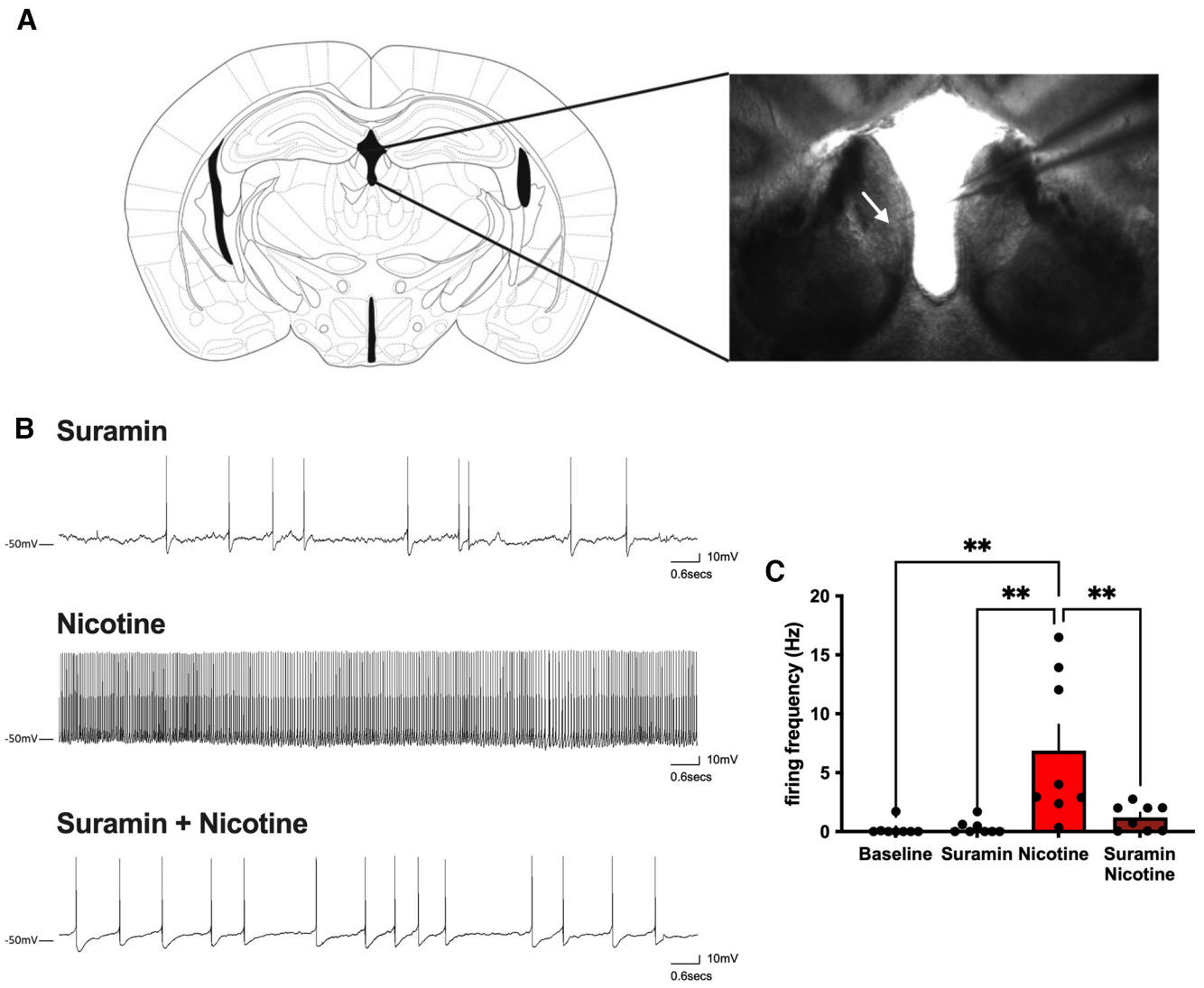

Figure 2. Perfusion of the P2 general antagonist prevented the nicotine-mediated firing frequency increase in MHb neurons. The general P2 antagonist suramin was perfused to examine the effects of endogenous ATP signaling on neuronal firing in the MHb ($n = 8$ cells from 5 subjects). ***A***, Recordings were obtained from neurons located in the ventral part of the MHb. ***B***, Representative traces during suramin and nicotine perfusion and coperfusion. ***C***, Suramin alone did not elicit any significant effect on the firing frequency, whereas nicotine substantially increased firing frequency. When suramin and nicotine were coapplied, blockade of the P2 receptors prevented the ability of nicotine to increase firing frequency in the MHb neurons. $^{**}p < 0.001$, compared with other conditions indicated. Data are presented as the mean ± SEM.

Table 1. Statistical analyses of the change from baseline values for each treatment, comparing between males and females

Figure	Statistical test	*p*-value
Figure 2*C*, Suramin	Unpaired *t* test, $t_{(6)} = 1.390$, $R^2 = 0.2436$	0.2139
Figure 3*B*, Vehicle	Unpaired *t* test, $t_{(3)} = 1.44$, $R^2 = 0.4087$	0.2455
Figure 3*D*, $\alpha\beta$-methylene ATP nicotine naive	Unpaired *t* test, $t_{(12)} = 1.674$, $R^2 = 0.1893$	0.1200
Figure 3*F*, MRS2365 nicotine naive	Unpaired *t* test, $t_{(11)} = 0.9556$, $R^2 = 0.07665$	0.3598
Figure 4*B*, $\alpha\beta$-methylene ATP nicotine abstinence (no nicotine applied)	Unpaired *t* test, $t_{(11)} = 0.1073$, $R^2 = 0.00104$	0.9165
Figure 4*D*, MRS2365 nicotine abstinence (no nicotine applied)	Unpaired *t* test, $t_{(5)} = 0.2358$, $R^2 = 0.01100$	0.8229
Figure 5*B*, nicotine + $\alpha\beta$-methylene ATP acute nicotine	Unpaired *t* test, $t_{(11)} = 1.126$, $R^2 = 0.1033$	0.2843
Figure 5*D*, nicotine + MRS2365 acute nicotine	Unpaired *t* test, $t_{(9)} = 0.7889$, $R^2 = 0.0646$	0.4505
Figure 6*G*, nicotine + MRS2365 nicotine re-exposure during abstinence	Unpaired *t* test, $t_{(11)} = 0.8077$, $R^2 = 0.05599$	0.4364

hypothesis that activation of both P2X and $P2Y_1$ receptors would result in an elevation of habenular neuronal firing frequency. As a first control measure to validate the approach, we applied vehicle (0.9% saline) with picospritzer application, and no significant change was found in the firing frequency (paired *t* test: $t_{(4)} = 1.730$, $p = 0.1587$, $R^2 = 0.4280$; Fig. 3*A*,*B*). No differences were observed between males and females in this assessment (Table 1). Next, to investigate the effects of the P2X receptor, we applied the general agonist, $\alpha\beta$-methylene ATP and found a significant increase in firing frequency with the P2X receptor activation in the habenular cholinergic neurons (paired *t* test: $t_{(13)} = 3.886$, $p = 0.0019$, $R^2 = 0.5374$; Fig. 3*C*,*D*). Thereafter, we examined the

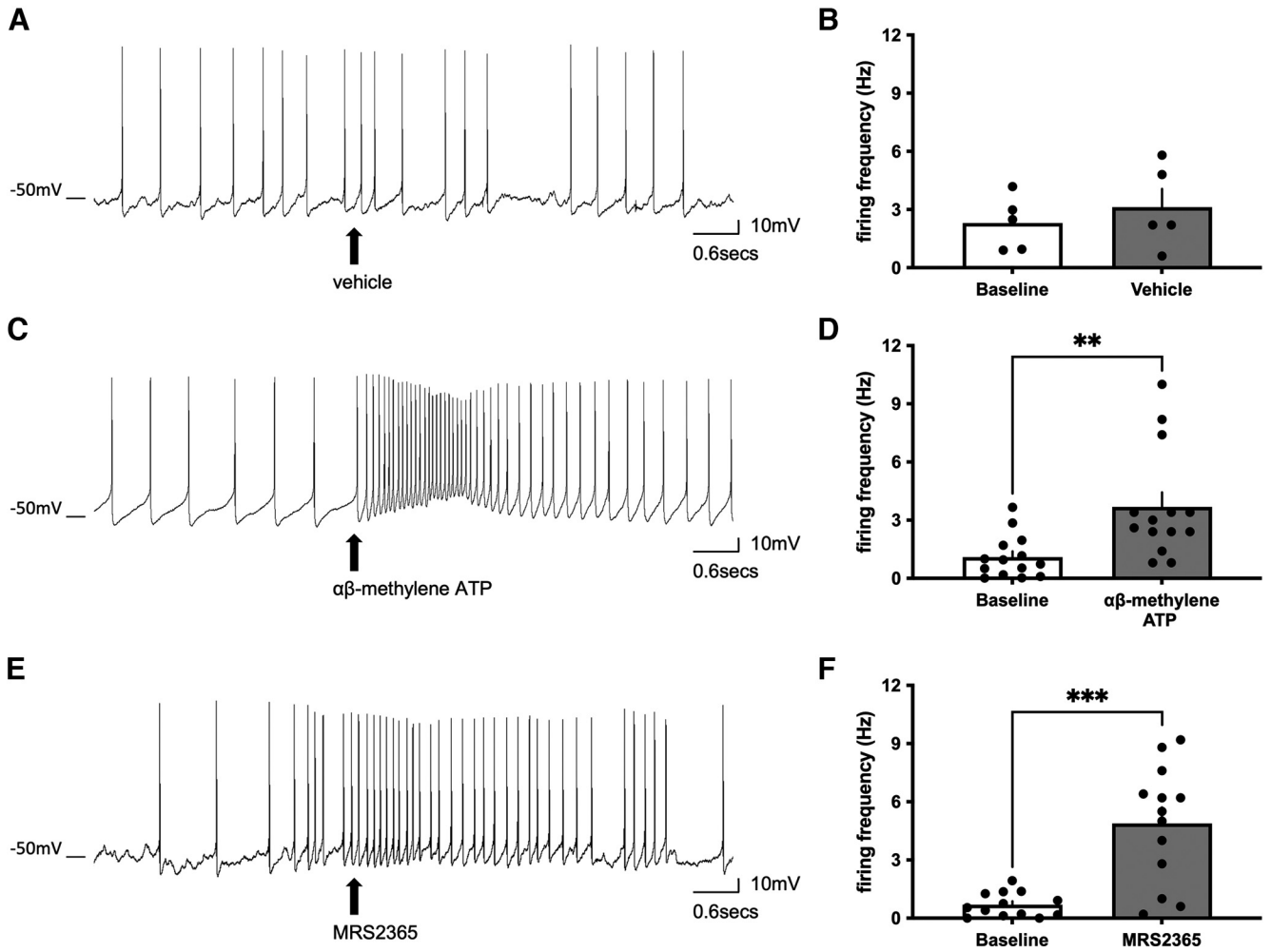

Figure 3. Activation of the P2X or $P2Y_1$ receptors increases firing frequency in cholinergic neurons. Ventral MHb neuron activity was recorded, and then the agonists for the P2X receptor and $P2Y_1$ receptor were applied to examine changes in neuronal firing. ***A***, Representative trace of neuronal activity during vehicle application. The arrow indicates the time point of vehicle application. ***B***, Vehicle application did not alter firing frequency across time ($n = 5$ cells from 4 subjects). ***C***, Representative trace of the firing frequency change with P2X agonist application. The arrow indicates the time point of $\alpha\beta$-methylene ATP application. ***D***, The P2X receptor agonist significantly increased firing frequency in neurons ($n = 14$ cells from 8 subjects). ***E***, Representative trace of the firing frequency change during $P2Y_1$ agonist application. The arrow indicates the time point of MRS2365 application. ***F***, The activation of $P2Y_1$ receptors significantly increased firing frequency ($n = 13$ cells from 10 subjects). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with baseline. Data are presented as the mean ± SEM.

function of the $P2Y_1$ receptor with the specific agonist MRS2365 and observed a significant increase in firing frequency in the cholinergic neurons (paired t test: $t_{(12)} = 5.545$, $p = 0.0001$, $R^2 = 0.7193$; Fig. 3*E*,*F*). Together, these data indicate that activation of either the P2X or $P2Y_1$ receptors can induce an increase in MHb activity independent of the presence of nicotine.

To further investigate whether ATP differentially affects neuronal activity during nicotine abstinence, we then examined the effects of the agonists in mice treated with nicotine across 10 consecutive days prior (nicotine abstinence condition). Specifically, brain sections were examined at 24 h after the last nicotine treatment, in the absence of nicotine. Application of the P2X receptor agonist (paired t test: $t_{(11)} = 4.377$, $p = 0.0011$, $R^2 = 0.6352$; Fig. 4*A*,*B*) or the $P2Y_1$ receptor agonist (paired t test: $t_{(6)} = 3.679$, $p = 0.0103$, $R^2 = 0.6929$; Fig. 4*C*,*D*) significantly increased firing frequencies in cholinergic neurons during nicotine abstinence. Of note, no difference was found in these studies between males and females for both the effects of P2X or $P2Y_1$ agonists under naive or nicotine abstinence conditions (Table 1). To further investigate whether the effects were greater with P2X or $P2Y_1$ agonist application, we then derived the change in neuronal firing from baseline and examined it across datasets, but no statistically significant differences were found (mean ± SEM; nicotine naive state: P2X agonist, 2.586 ± 0.6656; $P2Y_1$ agonist, 3.888 ± 0.7603; nicotine abstinence state: P2X agonist, 2.835 ± 0.6477; $P2Y_1$ agonist, 3.644 ± 0.9903; one way ANOVA: $F_{(3,43)} = 0.7546$, $p = 0.5258$, $R^2 = 0.05{,}001$).

Interaction between nicotinic and ATP signaling during acute nicotine exposure

A prior study reported a potential mutual inhibition between P2X receptors and cholinergic signaling in HEK-293 cells and cultured myenteric neurons (Decker and Galligan, 2009). Specifically, application of ATP or acetylcholine induced independent effects on inward cell currents, but when both were applied together, a summative effect was not found in the cellular response (Decker and Galligan, 2009), leading to the conclusion of mutual inhibition. Given this and our findings above, we hypothesized that the mutual inhibition between P2X and cholinergic signaling would limit the effects of the P2X agonist on neuronal activity. Consistent with this expectation, application of $\alpha\beta$-methylene ATP did not induce a significant change in firing frequency during nicotine perfusion

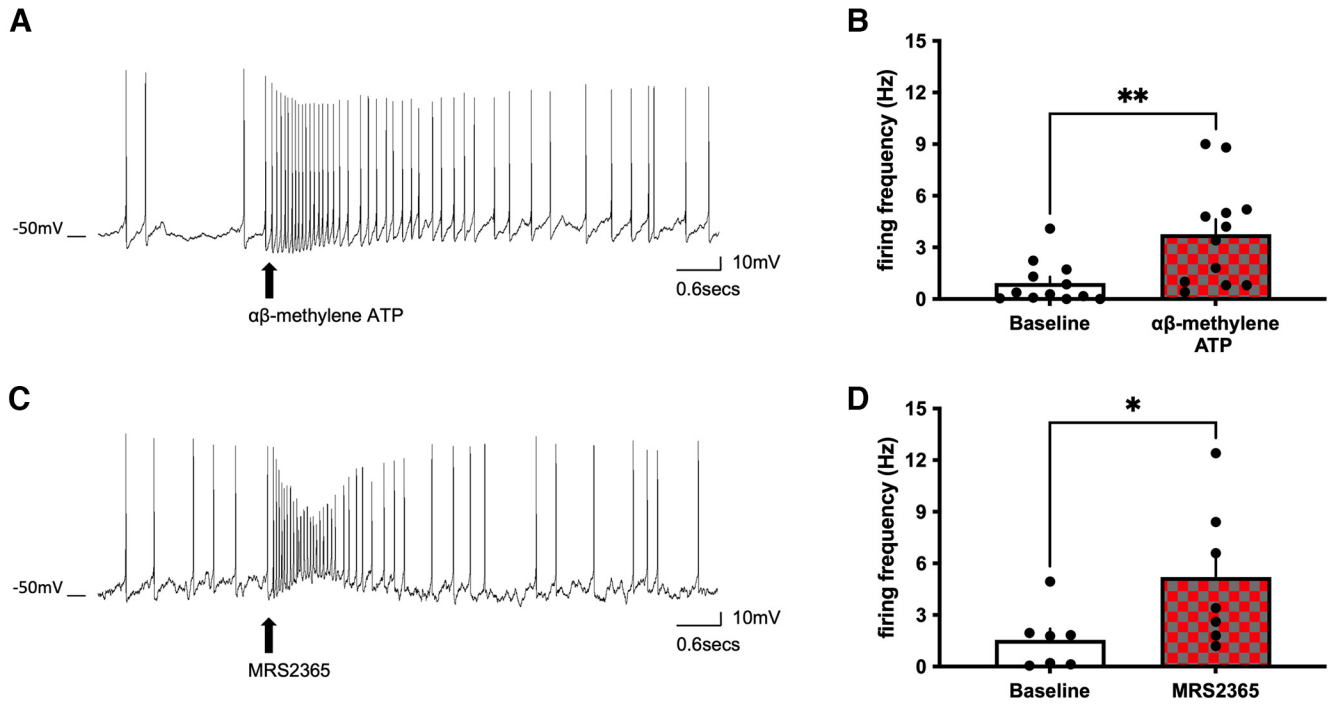

Figure 4. Activation of the P2X or $P2Y_1$ receptors during nicotine abstinence increases firing frequency. MHb neuronal activity was recorded 24 h after the last nicotine injection, and the agonists of the P2X or $P2Y_1$ receptors were applied. ***A***, Representative trace of the firing frequency change during P2X agonist application. The arrow indicates the time point of $\alpha\beta$-methylene ATP application. ***B***, The P2X receptor agonist significantly increased firing frequency during nicotine abstinence ($n = 12$ cells from 8 subjects). ***C***, Representative trace of the firing frequency change during $P2Y_1$ agonist application. The arrow indicates the time point of MRS2365 application. ***D***, The $P2Y_1$ receptor agonist significantly increased firing frequency during nicotine abstinence ($n = 7$ cells from 7 subjects). $^*p < 0.05$, $^{**}p < 0.01$, compared with baseline. Data are presented as the mean ± SEM.

(repeated-measures, one-way ANOVA: $F_{(2,24)} = 14.29$, $p < 0.0001$, $R^2 = 0.3191$; Fig. 5*A*,*B*). *Post hoc* test results indicate that while nicotine perfusion increased firing frequency above baseline levels (baseline vs nicotine, $p = 0.0091$; baseline vs nicotine with $\alpha\beta$-methylene ATP, $p < 0.0001$), no difference was found between nicotine and coexposure to nicotine and $\alpha\beta$-methylene ATP ($p = 0.4550$). Furthermore, no significance differences were found between males and females during nicotine perfusion with the P2X agonist (Table 1). We next examined whether a similar effect could be found between cholinergic and $P2Y_1$ receptor mediated signaling, and significant differences were found among the treatment groups (repeated-measures, one-way ANOVA: $F_{(2,20)} = 21.14$, $p < 0.0001$, $R^2 = 0.6789$; Fig. 5*C*,*D*). The *post hoc* analysis revealed a significant increase in firing frequency during nicotine exposure ($p = 0.0446$), and a further statistically significant increase with application of the $P2Y_1$ agonist MRS2365 during nicotine perfusion ($p = 0.0026$). The cotreatment group also differed from baseline firing frequency levels ($p < 0.0001$). No sex differences were found with coadministration of the $P2Y_1$ receptor and nicotine (Table 1). Together, these data support the notion of mutual inhibition between the P2X receptor and nicotinic receptors, but not the $P2Y_1$ receptor.

Chronic nicotine exposure alters the interactive effects of purinergic signaling and nicotine

Given the importance of cholinergic MHb neurons in mediating nicotine withdrawal and previously documented changes in cellular activity following chronic nicotine exposure (Görlich et al., 2013; Dao et al., 2014), we sought to determine whether the interaction between purinergic and cholinergic signaling mediates the altered state of circuit function. Following 10 d of nicotine exposure, the effects of P2X agonist were examined with coapplication of nicotine and $\alpha\beta$-methylene ATP (repeated-measures, one-way ANOVA: $F_{(2,28)} = 39.11$, $p = 0.0151$, $R^2 = 0.2555$). Nicotine increased the firing frequency from baseline (mean ± SEM; baseline, 0.330 ± 0.162; nicotine, 3.79 ± 0.615; $p = 0.0020$), and this effect was further potentiated in the presence of $\alpha\beta$-methylene ATP and nicotine (baseline, 0.331 ± 0.162; nicotine and $\alpha\beta$-methylene ATP, 8.35 ± 1.22; $p < 0.0001$). Coexposure of nicotine and $\alpha\beta$-methylene ATP was also significantly different from baseline (nicotine, 3.791 ± 0.615; nicotine and $\alpha\beta$-methylene ATP, 8.347 ± 1.22; $p < 0.0001$). However, when these data were separated based on sex, we observed relevant sex-dependent differences (Fig. 6). Specifically, the potentiated firing frequency in the presence of nicotine and $\alpha\beta$-methylene ATP was strikingly observed in females (repeated-measures, one-way ANOVA: $F_{(2,14)} = 34.03$, $p < 0.0001$, $R^2 = 0.8302$; *post hoc*, baseline vs nicotine, $p = 0.0494$; baseline vs nicotine and $\alpha\beta$-methylene ATP, $p < 0.0001$; nicotine vs nicotine and $\alpha\beta$-methylene ATP, $p = 0.0002$; Fig. 6*A*,*B*). However, a significant potentiated effect was not found in males (repeated-measures, one-way ANOVA: $F_{(2,12)} = 13.04$, $p = 0.0001$, $R^2 = 0.6848$; *post hoc*, baseline vs nicotine, $p = 0.0232$; baseline vs nicotine plus $\alpha\beta$-methylene ATP, $p = 0.0008$; nicotine vs nicotine plus $\alpha\beta$-methylene ATP, $p = 0.1636$; Fig. 6*C*, *D*). Thus, we next compared the change in firing frequency by subtracting the values for nicotine and $\alpha\beta$-methylene ATP from the values for nicotine alone. The change in firing frequency was significantly higher in females compared with males (unpaired *t* test: $t_{(13)} = 3.422$, $p = 0.0045$, $R^2 = 0.4739$; Fig. 6*E*), thereby demonstrating a notable sex difference in cholinergic neuron response. Finally, the effects of the $P2Y_1$ receptor agonist were also examined with nicotine re-exposure during abstinence (repeated-measures, one-way ANOVA: $F_{(2,24)} = 9.927$, $p = 0.0007$, $R^2 = 0.4527$; Fig. 6*F*,*G*). The *post hoc* analysis revealed a significant increase in neuronal firing frequency with nicotine ($p = 0.0054$) and coexposure of nicotine and MRS2365 ($p = 0.0010$). However, there was no difference in

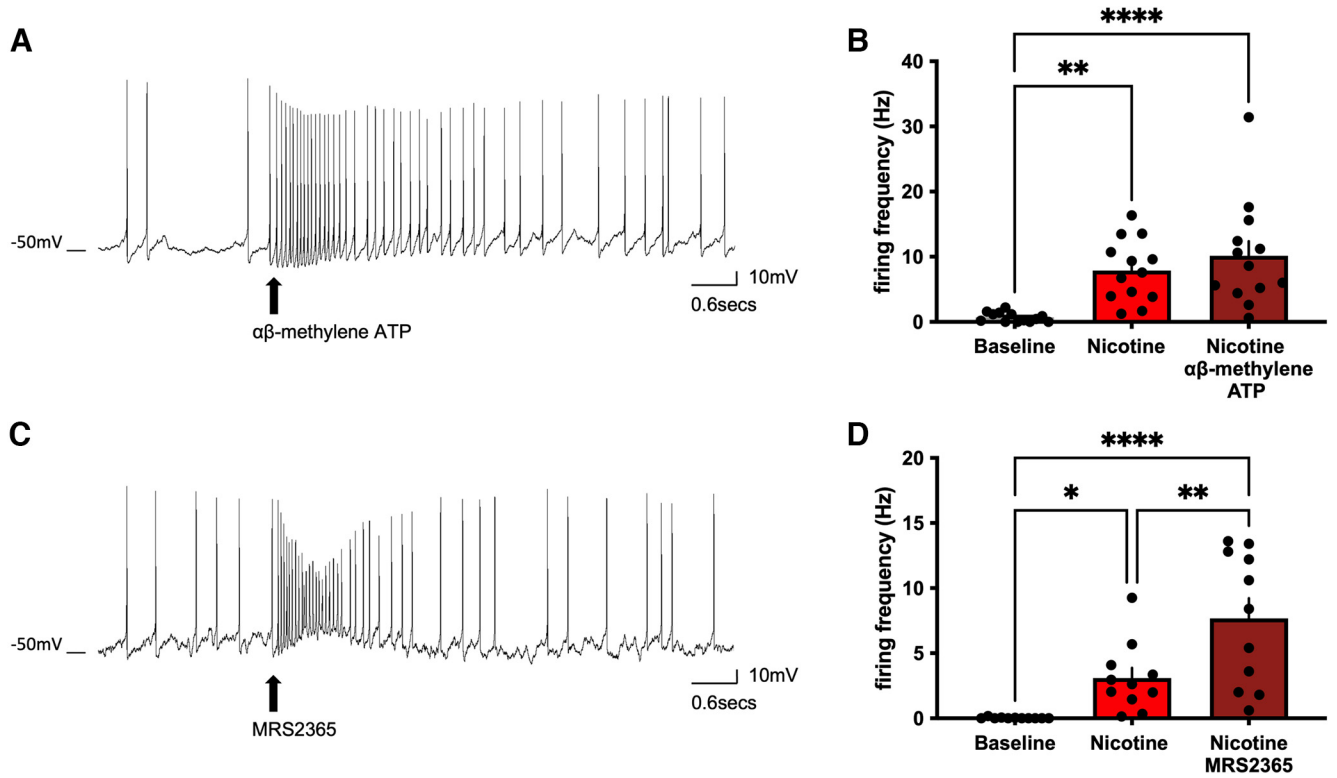

Figure 5. Coexposure to acute nicotine and the $P2Y_1$ receptor agonist potentiates neuronal firing in cholinergic MHb neurons. The interaction between purinergic signaling and nicotinic signaling was examined by applying the purinergic receptor agonists to MHb cholinergic neurons during nicotine perfusion. ***A***, Representative trace of neuronal firing when the P2X agonist was applied during nicotine perfusion. The arrow indicates the time point of $\alpha\beta$-methylene ATP application. ***B***, The P2X receptor agonist did not further elevate the firing frequency during nicotine perfusion ($n = 13$ cells from 11 subjects). ***C***, Representative trace of the neuronal firing change with the $P2Y_1$ agonist application during nicotine perfusion. The arrow indicates the time point of MRS2365 application. ***D***, $P2Y_1$ receptor agonist application further elevated the firing frequency during nicotine perfusion ($n = 11$ cells from 5 subjects). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with other conditions indicated. Data are presented as the mean ± SEM.

the response to nicotine, either with or without MRS2365 ($p = 0.7789$). Further, no significant difference was found between males and females during abstinence with the $P2Y_1$ receptor agonist (Table 1). Together, these results reveal an ATP receptor and sex-specific interactive effect with nicotinic signaling in the MHb.

Discussion

These studies reveal that purinergic signaling selectively modulates neuronal activity in the MHb and signaling dynamics become dysregulated with chronic nicotine exposure in the adolescent brain. The effects nicotine appear to be interactive with purinergic signaling in MHb cholinergic neurons. Both the P2X and $P2Y_1$ receptors independently increased cholinergic neuron firing during a naive or nicotine abstinence state. However, in the presence of nicotine, a dramatically different effect was observed, which was based on sex and a history of drug exposure. Specifically, for the P2X receptor, the agonist had no observable effect on nicotine-mediated activity, but during nicotine abstinence, exposure to nicotine and the P2X receptor agonist induced a potentiated effect, but only in adolescent females. In contrast, with activation of the $P2Y_1$ receptor, a potentiated effect of the agonist and nicotine was found in nicotine-naive subjects, but not following chronic nicotine exposure. This suggests that mutual inhibition between purinergic and nicotinic signaling may occur during the first exposure to nicotine with P2X receptor activation and following chronic nicotine exposure with the $P2Y_1$ receptor. Together, these data provide a complex understanding of the interactions between purinergic and nicotinic signaling in habenular cholinergic neurons during adolescence and further reveal the impact of nicotine on extracellular ATP signaling and subsequent altered circuit function.

Independent effects of P2X and $P2Y_1$ receptors in the MHb

Activation of P2X receptors induced an excitatory effect on cholinergic neuronal activity in the MHb in naive subjects. These studies are the first to reveal this functional role of purinergic signaling in the MHb on action potential firing. Previous studies have investigated P2X receptors in synaptic transmission in other brain regions. For instance, in the hippocampus, a P2X receptor agonist inhibited NMDA receptor-mediated excitatory postsynaptic currents, whereas a P2 antagonist facilitated the formation of long-term potentiation (Pankratov et al., 2002). It has been proposed that the influx of Ca^{2+} through the P2X channel leads to calcium-dependent NMDA inactivation/desensitization (Legendre et al., 1993; Zhang et al., 1998). Of relevance, recent cell-specific investigations have identified functional habenular NDMA receptors (Otsu et al., 2018), suggesting a potential link. However, in our studies, we examined the immediate effects of P2X receptor activation on cellular firing, which would have occurred in a more discrete time frame than that required for NMDA receptor modulation through downstream intracellular signaling processes. Furthermore, we observed an increase in neuronal activity with the agonist, not a decrease, as found in the prior study (Pankratov et al., 2002), thereby suggesting cell type-specific effects. Next, activation of presynaptic P2X receptors could have facilitated glutamate release, as demonstrated in the hippocampus (Sperlágh et al., 2002; Khakh

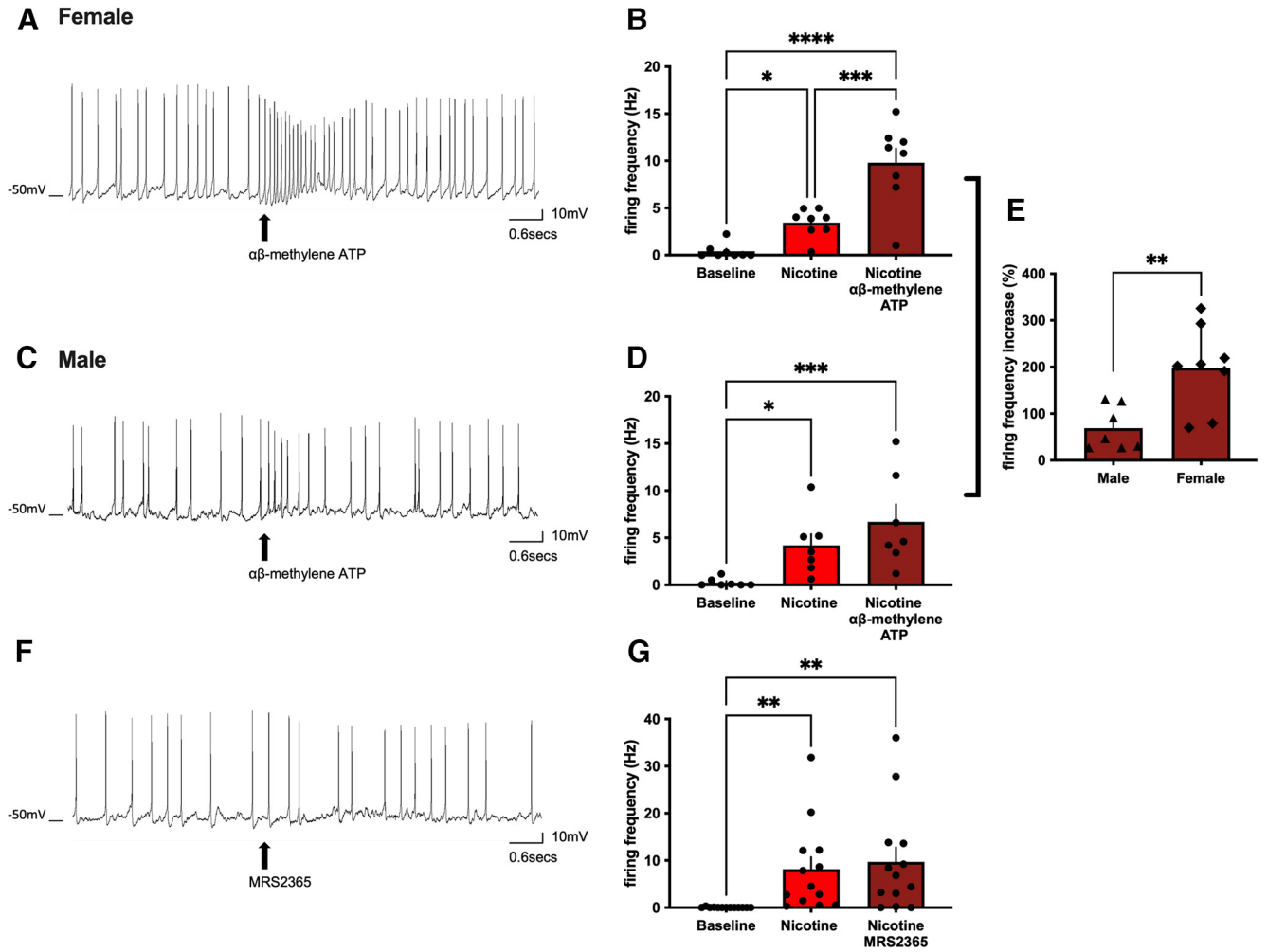

Figure 6. During nicotine abstinence, coexposure to nicotine and the P2X receptor agonist potentiates neuronal firing in female, but not male, cholinergic MHb neurons. The interaction between purinergic signaling and cholinergic signaling during nicotine abstinence (24 h post-nicotine exposure) was examined by applying purinergic receptor agonists during nicotine perfusion. ***A***, Representative trace of neuronal firing change in females when the P2X agonist was applied during nicotine perfusion. The arrow indicates the time point of $\alpha\beta$-methylene ATP application. ***B***, In females, the P2X receptor agonist significantly potentiated the firing frequency during nicotine perfusion ($n = 8$ cells from 6 subjects). ***C***, Representative trace of neuronal firing in males when the P2X agonist was applied during nicotine perfusion. The arrow indicates the time point of $\alpha\beta$-methylene ATP application. ***D***, In males, application of the P2X receptor agonist did not significantly elevate the firing frequency during nicotine perfusion ($n = 7$ cells from 6 subjects). ***E***, When comparing the P2X agonist effects with nicotine during abstinence in the male and female adolescent mice, the females exhibited a significantly higher firing frequency elevation than that found in males. ***F***, Representative trace of the neuronal firing when the $P2Y_1$ receptor agonist was applied during nicotine perfusion. The arrow indicates the time point of MRS2365. ***G***, Application of $P2Y_1$ receptor agonist did not elevate the firing frequency during nicotine perfusion in males and females ($n = 13$ cells from 12 subjects). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{***}p < 0.0001$, compared with other conditions indicated. Data are presented as the mean ± SEM.

et al., 2003; Rodrigues et al., 2005), which would thereby increase AMPA and/or NMDA receptor activation. However, activation of presynaptic $P2Y_1$ receptors has been shown to dampen glutamatergic transmission in the hippocampus (Rodrigues et al., 2005), whereas activation of postsynaptic $P2Y_1$ receptors inhibited NMDA receptor-mediated currents in the prefrontal and parietal cortex (Luthardt et al., 2003). Given these opposing effects, one would expect that each agonist for the P2X or P2Y receptors would have opposite effects on habenular activity. However, we found increased neuronal activity with either the P2X or $P2Y_1$ agonist, suggesting that different mechanisms likely predominate in the MHb. ATP has been shown to act on $P2Y_1$ receptors to facilitate HCN channel currents in mesencephalic trigeminal neurons (Huang et al., 2010). Given the function of HCN channels in maintaining spontaneous MHb neuronal activity (Görlich et al., 2013), the increased neuronal firing evidenced with activation of the $P2Y_1$ receptor could involve interaction with habenular HCN channel signaling via downstream calcium-mediated mechanisms, a possible interaction that will need to be tested further.

Interaction between purinergic signaling and acute nicotine

While the P2X receptor agonist and nicotine independently increased neuronal firing, we did not observe an additive effect on firing frequency during coexposure. This finding could potentially be because of either a ceiling-related effect with cellular activity, or mutual inhibition. Given that higher firing frequencies have been observed in habenular neurons with other experiments (e.g., during nicotine abstinence herein and as shown previously; Görlich et al., 2013), a putative ceiling-related effect can be excluded. Interestingly, mutual inhibition between P2X and cholinergic signaling has been suggested (Khakh et al., 2000; Decker and Galligan, 2009). Coapplication of ATP and acetylcholine led to a significantly lower current than the predictive summation when ATP or acetylcholine were individually applied, as demonstrated in HEK293 cells and *Xenopus* oocytes expressing

$\alpha3\beta4$ nAChRs and P2X receptors (Khakh et al., 2000; Decker and Galligan, 2009). Another study in acutely dissociated sympathetic neurons also demonstrated mutual inhibition between P2X receptors and nAChR-mediated signaling (Searl et al., 1998). However, it should be noted that such inhibitory effects have not been documented in all cell types that express both purinergic and nicotinic receptors, including visceral sensory ganglion (Reyes et al., 2006) and cultured sympathetic neurons (Rogers et al., 1997). Such discrepancies are likely because of the different expression profile of P2X receptor and nAChR subtypes across cellular populations. Since $\alpha3\beta4$ nAChRs and $P2X_2$ receptors are both expressed in the MHb (Zoli et al., 1995; Kanjhan et al., 1999), consistent with the cellular populations demonstrating mutual inhibition (Khakh et al., 2000; Decker and Galligan, 2009), these findings support the conclusion of mutual inhibition for the effects observed in our study.

Interaction between purinergic signaling and nicotine following chronic nicotine treatment

The P2X agonist induced a potentiated effect on neuronal firing during re-exposure to nicotine in female subjects undergoing nicotine abstinence. One of the possible mechanisms for this finding is the interaction between purinergic and neurokinin signaling. Neurokinin receptor activation can modulate nicotine-induced firing frequency in MHb neurons and has been linked to nicotine withdrawal (Dao et al., 2014). Further, neurokinin has been shown to potentiate ATP-mediated currents in dorsal root ganglion neurons (Wang et al., 2001). However, while this prior study included both males and females (Dao et al., 2014), potential sex differences were not examined, and as such, this would not explain why we only observed a potentiated response in females. Moreover, it should be noted that neurokinin would be expected to be present during the acute nicotine application, for which we did not find a potentiated effect. Thus, it remains to be determined whether chronic nicotine induces changes in neurokinin signaling that could explain the differences found during the abstinence state, or whether such nicotine exposure induces sex-dependent effects on the expression of ATP and/or nicotinic signaling mechanisms independently. For instance, it has been demonstrated that, in the interpeduncular nucleus during nicotine withdrawal, females exhibit an increase in expression of the $\alpha5$ nAChR subunit, while males have an increase in the expression of the $\alpha2$ and $\alpha3$ nAChR subunits (Correa et al., 2019). Females also exhibit greater withdrawal-induced cellular activation in the central region of the interpeduncular nucleus (Correa et al., 2019; Matos-Ocasio et al., 2021) and differ in the response to the aversive properties of nicotine (Torres et al., 2009). Thus, these changes may lead to differences in circuit function, thereby altering nicotine dependence susceptibility. Along these lines, it will be interesting in future studies to examine whether purinergic signaling is also involved in directly mediating nicotine self-administration. In humans, women suffer more from adverse symptoms during nicotine withdrawal and are more likely to relapse during quit attempts (Swan et al., 1993; Bjornson et al., 1995; Rojas et al., 1998). Thus, the current findings provide mechanistic insight into a putative sensitized state in the female brain during nicotine withdrawal, in which nicotine re-exposure can lead to altered neuronal activity to thereby promote relapse.

Interestingly, for $P2Y_1$ receptor-mediated signaling, we did not observe any potentiation in neuronal activity with nicotine and the agonist during nicotine withdrawal, contrary to that observed during acute nicotine exposure. This provides evidence that chronic nicotine exposure altered the cellular response, leading to an effect that is reminiscent of the mutual inhibition observed with the P2X receptor in naive subjects. However, given that the P2X is an ionotropic receptor and $P2Y_1$ is a metabotropic receptor, the molecular interactions resulting in the effect on neuronal firing likely differ. Thus, it will be of interest in future studies to examine downstream intracellular signaling mechanisms.

Considerations

In these studies, we reveal a sex difference in potentiated firing frequency with the activation of both the nAChRs and P2X receptors, which was significantly higher in females compared with males. Given that a complex experimental design to investigate sex differences across treatment groups would require high power demands to reach statistical significance with reasonable sample sizes (e.g., for two-way or three-way ANOVAs), we used a normalization approach to allow us to directly compare between sexes. Specifically, the values for nicotine and $\alpha\beta$-methylene ATP were subtracted from the values of nicotine alone to derive the potentiated difference in firing frequency. These values were then statistically examined with a t test, allowing for direct comparison to determine a sex difference. These studies also examined one dose of nicotine to induce withdrawal with controlled, subcutaneous injections. It will be informative in future studies to examine across a variety of nicotine doses to determine dose-dependent effects, as well as examine with other routes of nicotine exposure, such as e-cigarette vape inhalation.

Conclusions

The findings reported herein have multiple implications beyond that discussed above. For instance, the endogenous neurotransmitter acetylcholine acts on nAChRs in the MHb, and, as such, activation of nAChRs with the receptor agonist nicotine provides further potential insight into endogenous circuit function. Second, humans consume nicotine recreationally, and, as such, these findings add an important mechanistic understanding of the effects of nicotine on brain circuit activity. We focused our studies in adolescent mice since increased e-cigarette use in teenagers has become prevalent. Adolescents are more prone to develop nicotine dependence, and the impact of nicotine on the adolescent developing brain may lead to long-term changes in neural function and mental health (Luna et al., 2010; National Center for Chronic Disease Prevention and Health Promotion, 2016; Siqueira, 2017; Grant et al., 2019; Ren and Lotfipour, 2019; Mahajan et al., 2021). It will be interesting in future studies to examine whether these effects of nicotine on extracellular ATP signaling mechanisms persist into adulthood. Together, these findings reveal an interaction between extracellular ATP signaling and the actions of nicotine and further identify specific purinergic receptors that could be targeted for future therapeutic development with receptor subtype-selective drugs.

References

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 58:281–341.

Antolin-Fontes B, Ables JL, Görlich A, Ibanez-Tallon I (2015) The habenulo-interpeduncular pathway in nicotine aversion and withdrawal. Neuropharmacology 96:213–222.

Benowitz NL, Hukkanen J, Jacob P 3rd (2009) Nicotine chemistry, metabolism, kinetics and biomarkers. Handb Exp Pharmacol (129):29–60.

Bjornson W, Rand C, Connett JE, Lindgren P, Nides M, Pope F, Buist AS, Hoppe-Ryan C, O'Hara P (1995) Gender differences in smoking cessation after 3 years in the Lung Health Study. Am J Public Health 85:223–230.

Burnstock G (2015) Physiopathological roles of P2X receptors in the central nervous system. Curr Med Chem 22:819–844.

Burnstock G (2018) Purine and purinergic receptors. Brain Neurosci Adv 2:2398212818817494.

Chellian R, Behnood-Rod A, Bruijnzeel DM, Wilson R, Pandy V, Bruijnzeel AW (2021) Rodent models for nicotine withdrawal. J Psychopharmacol 35:1169–1187.

Conti AA, Tolomeo S, Steele JD, Baldacchino AM (2020) Severity of negative mood and anxiety symptoms occurring during acute abstinence from tobacco: a systematic review and meta-analysis. Neurosci Biobehav Rev 115:48–63.

Correa VL, Flores RJ, Carcoba LM, Arreguin MC, O'Dell LE (2019) Sex differences in cholinergic systems in the interpeduncular nucleus following nicotine exposure and withdrawal. Neuropharmacology 158:107714.

Damaj MI, Kao W, Martin BR (2003) Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. J Pharmacol Exp Ther 307:526–534.

Dao DQ, Perez EE, Teng Y, Dani JA, De Biasi M (2014) Nicotine enhances excitability of medial habenular neurons via facilitation of neurokinin signaling. J Neurosci 34:4273–4284.

Decker DA, Galligan JJ (2009) Cross-inhibition between nicotinic acetylcholine receptors and P2X receptors in myenteric neurons and HEK-293 cells. Am J Physiol Gastrointest Liver Physiol 296:G1267–1276.

Edwards FA, Gibb AJ, Colquhoun D (1992) ATP receptor-mediated synaptic currents in the central nervous system. Nature 359:144–147.

Elayouby KS, Ishikawa M, Dukes AJ, Smith AC, Lu Q, Fowler CD, Kenny PJ (2021) α3* nicotinic acetylcholine receptors in the habenula-interpeduncular nucleus circuit regulate nicotine intake. J Neurosci 41:1779–1787.

Florenzano F, Carrive P, Viscomi MT, Ferrari F, Latini L, Conversi D, Cabib S, Bagni C, Molinari M (2008) Cortical and subcortical distribution of ionotropic purinergic receptor subunit type 1 (P2X(1)R) immunoreactive neurons in the rat forebrain. Neuroscience 151:791–801.

Fowler CD, Kenny PJ (2011) Intravenous nicotine self-administration and cue-induced reinstatement in mice: effects of nicotine dose, rate of drug infusion and prior instrumental training. Neuropharmacology 61:687–698.

Fowler CD, Kenny PJ (2014) Nicotine aversion: neurobiological mechanisms and relevance to tobacco dependence vulnerability. Neuropharmacology 76:533–544.

Fowler CD, Lu Q, Johnson PM, Marks MJ, Kenny PJ (2011) Habenular α5 nicotinic receptor subunit signalling controls nicotine intake. Nature 471:597–601.

Görlich A, Antolin-Fontes B, Ables JL, Frahm S, Slimak MA, Dougherty JD, Ibañez-Tallon I (2013) Reexposure to nicotine during withdrawal increases the pacemaking activity of cholinergic habenular neurons. Proc Natl Acad Sci U S A 110:17077–17082.

Grant JE, Lust K, Fridberg DJ, King AC, Chamberlain SR (2019) E-cigarette use (vaping) is associated with illicit drug use, mental health problems, and impulsivity in university students. Ann Clin Psychiatry 31:27–35.

Guo W, Xu X, Gao X, Burnstock G, He C, Xiang Z (2008) Expression of P2X5 receptors in the mouse CNS. Neuroscience 156:673–692.

Guzman SJ, Gerevich Z (2016) P2Y receptors in synaptic transmission and plasticity: therapeutic potential in cognitive dysfunction. Neural Plast 2016:1207393.

Hsu YW, Tempest L, Quina LA, Wei AD, Zeng H, Turner EE (2013) Medial habenula output circuit mediated by α5 nicotinic receptor-expressing GABAergic neurons in the interpeduncular nucleus. J Neurosci 33:18022–18035.

Huang W, Xiu Y, Yan JA, He WJ, Zhao YD, Hu ZA, Ruan HZ (2010) Facilitation of Ih channels by P2Y1 receptors activation in mesencephalic trigeminal neurons. Neurosci Lett 482:156–159.

Journal of Neuroscience (2018) Recommendations for the design and analysis of *in vivo* electrophysiology studies [editorial]. J Neurosci 38:5837–5839.

Kanjhan R, Housley GD, Burton LD, Christie DL, Kippenberger A, Thorne PR, Luo L, Ryan AF (1999) Distribution of the P2X2 receptor subunit of the ATP-gated ion channels in the rat central nervous system. J Comp Neurol 407:11–32.

Khakh BS, Zhou X, Sydes J, Galligan JJ, Lester HA (2000) State-dependent cross-inhibition between transmitter-gated cation channels. Nature 406:405–410.

Khakh BS, Gittermann D, Cockayne DA, Jones A (2003) ATP modulation of excitatory synapses onto interneurons. J Neurosci 23:7426–7437.

Khakh BS, Fisher JA, Nashmi R, Bowser DN, Lester HA (2005) An angstrom scale interaction between plasma membrane ATP-gated P2X2 and alpha4beta2 nicotinic channels measured with fluorescence resonance energy transfer and total internal reflection fluorescence microscopy. J Neurosci 25:6911–6920.

Le Foll B, Piper ME, Fowler CD, Tonstad S, Bierut L, Lu L, Jha P, Hall WD (2022) Tobacco and nicotine use. Nat Rev Dis Primers 8:19.

Legendre P, Rosenmund C, Westbrook GL (1993) Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. J Neurosci 13:674–684.

Liston TE, Hinz S, Müller CE, Holstein DM, Wendling J, Melton RJ, Campbell M, Korinek WS, Suresh RR, Sethre-Hofstad DA, Gao ZG, Tosh DK, Jacobson KA, Lechleiter JD (2020) Nucleotide P2Y1 receptor agonists are in vitro and in vivo prodrugs of A1/A3 adenosine receptor agonists: implications for roles of P2Y1 and A1/A3 receptors in physiology and pathology. Purinergic Signal 16:543–559.

Luna B, Padmanabhan A, O'Hearn K (2010) What has fMRI told us about the development of cognitive control through adolescence? Brain Cogn 72:101–113.

Luthardt J, Borvendeg SJ, Sperlagh B, Poelchen W, Wirkner K, Illes P (2003) P2Y(1) receptor activation inhibits NMDA receptor-channels in layer V pyramidal neurons of the rat prefrontal and parietal cortex. Neurochem Int 42:161–172.

Mahajan SD, Homish GG, Quisenberry A (2021) Multifactorial etiology of adolescent nicotine addiction: a review of the neurobiology of nicotine addiction and its implications for smoking cessation pharmacotherapy. Front Public Health 9:664748.

Matos-Ocasio F, Espinoza VE, Correa-Alfonzo P, Khan AM, O'Dell LE (2021) Female rats display greater nicotine withdrawal-induced cellular activation of a central portion of the interpeduncular nucleus versus males: a study of Fos immunoreactivity within provisionally assigned interpeduncular subnuclei. Drug Alcohol Depend 221:108640.

Matta SG, et al. (2007) Guidelines on nicotine dose selection for in vivo research. Psychopharmacology (Berl) 190:269–319.

McLaughlin I, Dani JA, De Biasi M (2015) Nicotine withdrawal. Curr Top Behav Neurosci 24:99–123.

Merritt PS, Cobb AR, Moissinac L, Hirshman E (2010) Evidence that episodic memory impairment during tobacco abstinence is independent of attentional mechanisms. J Gen Psychol 137:331–342.

Morán-Jiménez MJ, Matute C (2000) Immunohistochemical localization of the P2Y(1) purinergic receptor in neurons and glial cells of the central nervous system. Brain Res Mol Brain Res 78:50–58.

Morton G, Nasirova N, Sparks DW, Brodsky M, Sivakumaran S, Lambe EK, Turner EE (2018) Chrna5-expressing neurons in the interpeduncular nucleus mediate aversion primed by prior stimulation or nicotine exposure. J Neurosci 38:6900–6920.

National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health (2016) E-cigarette use among youth and young adults: a report of the surgeon general. Atlanta, GA: Centers for Disease Control and Prevention.

North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013–1067.

Otsu Y, Lecca S, Pietrajtis K, Rousseau CV, Marcaggi P, Dugué GP, Mailhes-Hamon C, Mameli M, Diana MA (2018) Functional principles of posterior septal inputs to the medial habenula. Cell Rep 22:693–705.

Pankratov Y, Lalo U, Krishtal OA, Verkhratsky A (2009) P2X receptors and synaptic plasticity. Neuroscience 158:137–148.

Pankratov YV, Lalo UV, Krishtal OA (2002) Role for P2X receptors in long-term potentiation. J Neurosci 22:8363–8369.

Price GD, Robertson SJ, Edwards FA (2003) Long-term potentiation of glutamatergic synaptic transmission induced by activation of presynaptic P2Y receptors in the rat medial habenula nucleus. Eur J Neurosci 17:844–850.

Pushkin AN, Eugene AJ, Lallai V, Torres-Mendoza A, Fowler JP, Chen E, Fowler CD (2019) Cannabinoid and nicotine exposure during adolescence induces sex-specific effects on anxiety- and reward-related behaviors during adulthood. PLoS One 14:e0211346.

Ren M, Lotfipour S (2019) Nicotine gateway effects on adolescent substance use. West J Emerg Med 20:696–709.

Reyes EP, Alcayaga J, Zapata P (2006) Are there interactions between acetylcholine- and ATP-induced responses at the level of a visceral sensory ganglion? Brain Res 1107:97–103.

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, Cunha RA (2005) Dual presynaptic control by ATP of glutamate release via facilitatory P2X1, P2X2/3, and P2X3 and inhibitory P2Y1, P2Y2, and/or P2Y4 receptors in the rat hippocampus. J Neurosci 25:6286–6295.

Rogers M, Colquhoun LM, Patrick JW, Dani JA (1997) Calcium flux through predominantly independent purinergic ATP and nicotinic acetylcholine receptors. J Neurophysiol 77:1407–1417.

Rojas NL, Killen JD, Haydel KF, Robinson TN (1998) Nicotine dependence among adolescent smokers. Arch Pediatr Adolesc Med 152:151–156.

Rubinstein ML, Benowitz NL, Auerback GM, Moscicki AB (2009) Withdrawal in adolescent light smokers following 24-hour abstinence. Nicotine Tob Res 11:185–189.

Salas R, Cook KD, Bassetto L, De Biasi M (2004a) The alpha3 and beta4 nicotinic acetylcholine receptor subunits are necessary for nicotine-induced seizures and hypolocomotion in mice. Neuropharmacology 47:401–407.

Salas R, Pieri F, De Biasi M (2004b) Decreased signs of nicotine withdrawal in mice null for the beta4 nicotinic acetylcholine receptor subunit. J Neurosci 24:10035–10039.

Salas R, Sturm R, Boulter J, De Biasi M (2009) Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. J Neurosci 29:3014–3018.

Searl TJ, Redman RS, Silinsky EM (1998) Mutual occlusion of P2X ATP receptors and nicotinic receptors on sympathetic neurons of the guinea-pig. J Physiol 510:783–791.

Shih PY, Engle SE, Oh G, Deshpande P, Puskar NL, Lester HA, Drenan RM (2014) Differential expression and function of nicotinic acetylcholine receptors in subdivisions of medial habenula. J Neurosci 34:9789–9802.

Siqueira LM (2017) Nicotine and tobacco as substances of abuse in children and adolescents. Pediatrics 139:e20163436.

Song X, Guo W, Yu Q, Liu X, Xiang Z, He C, Burnstock G (2011) Regional expression of P2Y(4) receptors in the rat central nervous system. Purinergic Signal 7:469–488.

Sperlágh B, Kittel A, Lajtha A, Vizi ES (1995) ATP acts as fast neurotransmitter in rat habenula: neurochemical and enzymecytochemical evidence. Neuroscience 66:915–920.

Sperlágh B, Maglóczky Z, Vizi ES, Freund TF (1998) The triangular septal nucleus as the major source of ATP release in the rat habenula: a combined neurochemical and morphological study. Neuroscience 86:1195–1207.

Sperlágh B, Köfalvi A, Deuchars J, Atkinson L, Milligan CJ, Buckley NJ, Vizi ES (2002) Involvement of P2X7 receptors in the regulation of neurotransmitter release in the rat hippocampus. J Neurochem 81:1196–1211.

Suzuki E, Kessler M, Montgomery K, Arai AC (2004) Divergent effects of the purinoceptor antagonists suramin and pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS) on alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Mol Pharmacol 66:1738–1747.

Swan GE, Ward MM, Carmelli D, Jack LM (1993) Differential rates of relapse in subgroups of male and female smokers. J Clin Epidemiol 46:1041–1053.

Ting JT, Lee BR, Chong P, Soler-Llavina G, Cobbs C, Koch C, Zeng H, Lein E (2018) Preparation of acute brain slices using an optimized *N*-methyl-D-glucamine protective recovery method. J Vis Exp (132):53825.

Torres OV, Natividad LA, Tejeda HA, Van Weelden SA, O'Dell LE (2009) Female rats display dose-dependent differences to the rewarding and aversive effects of nicotine in an age-, hormone-, and sex-dependent manner. Psychopharmacology (Berl) 206:303–312.

Vickstrom CR, Liu X, Zhang Y, Mu L, Kelly TJ, Yan X, Hu MM, Snarrenberg ST, Liu QS (2020) T-type calcium channels contribute to burst firing in a subpopulation of medial habenula neurons. eNeuro 7:ENEURO.0201-20.2020.

Wang MJ, Xiong SH, Li ZW (2001) Neurokinin B potentiates ATP-activated currents in rat DRG neurons. Brain Res 923:157–162.

Wu Z, He K, Chen Y, Li H, Pan S, Li B, Liu T, Xi F, Deng F, Wang H, Du J, Jing M, Li Y (2022) A sensitive GRAB sensor for detecting extracellular ATP in vitro and in vivo. Neuron 110:770–782.e5.

Yang R, Liang BT (2012) Cardiac P2X(4) receptors: targets in ischemia and heart failure? Circ Res 111:397–401.

Yuan M, Cross SJ, Loughlin SE, Leslie FM (2015) Nicotine and the adolescent brain. J Physiol 593:3397–3412.

Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL (1998) Calmodulin mediates calcium-dependent inactivation of N-methyl-D-aspartate receptors. Neuron 21:443–453.

Zoli M, Le Novère N, Hill JA Jr, Changeux JP (1995) Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. J Neurosci 15:1912–1939.