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Varenicline enhances dopamine release facilitation more than nicotine after long-term nicotine treatment and withdrawal

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Abstract

An important factor contributing to the high relapse rates among smokers is nicotine withdrawal symptoms. Multiple studies suggest that decreased dopamine release in nucleus accumbens plays a key role in withdrawal. However, recent reports showed that long-term nicotine exposure itself also decreases accumbal dopamine release, suggesting that additional mechanisms are involved in withdrawal. Here, we used real-time cyclic voltammetry in brain slices containing the nucleus accumbens to further elucidate the changes in dopamine release linked to nicotine withdrawal. Rats received vehicle or nicotine via the drinking water for 2–3 months. Studies assessing the expression of somatic signs in vehicle-treated, nicotine-treated, and 24-h nicotine withdrawn rats showed that nicotine withdrawal led to a significant increase in somatic signs. Subsequent voltammetry studies showed that long-term nicotine decreased single-pulse-stimulated dopamine release via an interaction at $\alpha6\beta2$* receptors. Nicotine withdrawal led to a partial recovery in $\alpha6\beta2$* receptor-mediated release. In addition, long-term nicotine treatment alone increased dopamine release paired-pulse ratios and this was partially reversed with nicotine removal. We then evaluated the effect of bath-applied nicotine and varenicline on dopamine release. Nicotine and varenicline both decreased single-pulse-stimulated release in vehicle-treated, nicotine-treated, and nicotine withdrawn rats. However, bath-applied varenicline increased paired-pulse ratios to a greater extent than nicotine during long-term nicotine treatment and after its withdrawal. Altogether these data suggest that nicotine withdrawal is associated with a partial restoration of dopamine release measures to control levels and that varenicline's differential modulation of dopamine release may contribute to its mechanism of action.

Abbreviations

ANOVA, analysis of variance; nAChRs, nicotinic acetylcholine receptors; *, the asterisk indicates the possible presence of other nicotinic subunits in the receptor complex; DHβE, dihydro-β-erythroidine; α-CtxMII, α-conotoxinMII.

Introduction

Although most smokers express a desire to quit, only about 5% of those who try are successful at remaining abstinent after 1 year (Benowitz 2010). These high relapse rates are partly due to the withdrawal symptoms that arise from the absence of nicotine, the principal addictive component in tobacco (Balfour 2009). The initiation of nicotine addiction is strongly associated with activation of the mesocorticolimbic dopaminergic reward pathway where nicotine exerts its effect via several nicotinic receptor (nAChR) subtypes (Corrigall et al. 1992; Dani and De

Biasi 2001; Balfour 2009; Benowitz 2010; Berrendero et al. 2010; De Biasi and Dani 2011; Leslie et al. 2013). Nicotine interacts with $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs leading to enhanced dopamine neuron firing in the ventral tegmental area and facilitating burst-induced dopamine release in the nucleus accumbens (Mansvelder et al. 2002; Exley and Cragg 2008; Zhang et al. 2009a; Kleijn et al. 2011; Li et al. 2011; Zhao-Shea et al. 2011; Perez et al. 2013). This effect is considered critical for the processing of reward-related behavior and reinforces the continuous use of cigarettes (Dani and De Biasi 2001; Leslie et al. 2013).

Sustained nicotine exposure results in nAChR desensitization and leads to up- or downregulation of nAChRs depending on the subtype, with consequent effects on neurotransmission in the central nervous system (CNS) (Buisson and Bertrand 2002; Nguyen et al. 2004; Nashmi et al. 2007; Perez et al. 2008; Picciotto et al. 2008; Walsh et al. 2008). Smoking cessation disrupts this altered equilibrium leading to an imbalance in numerous neuronal systems that induces a withdrawal syndrome (Epping-Jordan et al. 1998; De Biasi and Dani 2011; Bruijnzeel et al. 2012; D'Souza and Markou 2013; Li et al. 2014). The main neurochemical changes associated with early nicotine withdrawal are a decrease in dopamine levels and dopamine release in the nucleus accumbens (Rahman et al. 2004; Natividad et al. 2010; Dani et al. 2011; Zhang et al. 2011). Interestingly, recent data show that long-term nicotine exposure itself decreases dopamine release in the nucleus accumbens and dorsal striatum (Exley et al. 2013; Perez et al. 2013; Koranda et al. 2014). Thus, the observed decline in accumbal dopamine release with nicotine withdrawal may not be a direct result of nicotine removal but a consequence of long-term nicotine exposure that persists into the withdrawal stage.

Currently approved smoking cessation therapies aim to curb craving and alleviate the effects of nicotine withdrawal by counteracting the decrease in dopamine function (Fant et al. 2009; McNeil et al. 2010). Nicotine replacement therapies and varenicline are thought to do so via a direct interaction with nAChRs to enhance dopamine release, whereas bupropion's mechanism of action may relate to its capacity to block dopamine uptake and antagonize nAChRs (Fant et al. 2009; McNeil et al. 2010). Unfortunately, the success rate with these drugs alone or in combination remains low, with varenicline having the greatest efficacy (25%) (McNeil et al. 2010; Mills et al. 2012). A better understanding of the neurobiological mechanisms that contribute to nicotine dependence and withdrawal, as well as the effect that current pharmacotherapies have on dopamine function should aid in the development of more effective smoking cessation therapies.

Here, we used cyclic voltammetry in slices from vehicle and nicotine-treated rats to investigate the effect of long-term nicotine treatment and 24-h nicotine withdrawal in nucleus accumbens dopamine release. Release was examined in response to low- and high-frequency stimulation as the nAChR-mediated modulation of dopamine release and the processing of reward-related information is highly dependent on dopaminergic activity (Schultz 2002; Exley and Cragg 2008). In addition, we also assessed the effects of bath application of nicotine and varenicline to gain insight into how they modulate dopamine function after nicotine treatment and withdrawal. The results show that long-term nicotine treatment alone decreases single- and four-pulse-stimulated dopamine release. Nicotine withdrawal leads to a partial recovery of these measures. In addition, the data indicate that varenicline exerts a stronger inhibition of low-frequency-stimulated dopamine release after long-term nicotine treatment and withdrawal compared to nicotine. Such observations may contribute to varenicline's mechanism of action.

Materials and Methods

Animal treatment

Adult male Sprague–Dawley rats (220–250 g) purchased from Charles River Laboratories (Gilroy, CA) were placed in a temperature-controlled room with a 12 h dark/light cycle (lights on at 7 AM) and housed 3–4 per cage. All animals had free access to food and water. After several days of acclimation, rats were given drinking water containing 1% saccharin (Sigma Chemical Co., St. Louis, MO), which was used as a vehicle to mask the bitter taste of nicotine. They were then randomly divided into three treatment groups 3 days later. One group was maintained on saccharin only. Nicotine was added to the saccharin-containing solution of the other two groups at a concentration of 25 μg/mL nicotine (free base). Nicotine treatment was given for 2–3 months, as other studies have shown that the changes that arise during such a time period may model those in long-term smokers (Zhang et al. 2011; Perez et al. 2013). The nicotine-containing drinking water was replaced with 1% saccharin 24 h before the release experiments for the third group of animals. Weights were monitored twice a week, with no significant differences between the treatment groups. The rats were killed by decapitation using a guillotine. All procedures conform to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of SRI International.

Plasma cotinine levels

Blood was drawn from the lateral saphenous vein under isofluorane anesthesia 2–3 weeks after the rats were on nicotine treatment. Plasma cotinine was determined using an

EIA kit (Orasure Technologies, Bethlehem, PA). Cotinine is a long-lasting nicotine metabolite used as an index of nicotine intake. The plasma cotinine levels in nicotine-treated rats were 275 ± 10 ng/mL (n = 20), which are comparable to those in the plasma of smokers (Matta et al. 2007). For some animals, plasma was also collected after 2 months of treatment, with cotinine values similar to samples from the first blood draw. No rats were excluded due to low cotinine levels. After 24-h nicotine withdrawal, cotinine levels were 1.2 ± 0.2 ng/mL (n = 7). There was no detectable plasma cotinine in saccharin-treated rats (n = 6).

Assessment of spontaneous somatic signs of nicotine withdrawal

Behavioral observations were performed between 9:00 and 9:30 AM in a transparent cylinder. Somatic signs were counted for 20 min for rats on vehicle treatment, nicotine treatment as well as 24 h after nicotine removal from the drinking water. The observed signs included ptosis, eye blinks, cheek tremors, chews, gasps, teeth chattering, head shakes, writhes, and body shakes as previously described (O'Dell et al. 2004; Malin and Goyarzu 2009). Each incidence of the above behaviors was marked. In regards to ptosis, if this was present continuously throughout the observation period, then it was marked as one count per minute. The total number of somatic signs reflects the summation of individual occurrences of each somatic sign type per observation period.

Tissue preparation

The brain was quickly removed and chilled in ice-cold, preoxygenated (95% O_2/5% CO_2) buffer containing: 30 mmol/L NaCl, 4.5 mmol/L KCl, 1.2 mmol/L NaH_2PO_4, 1.0 mmol/L $MgCl_2$, 10 mmol/L glucose, 26 mmol/L $NaHCO_3$, and 18 mmol/L sucrose (pH 7.4). Coronal slices containing the NAcc (350 μm thick) were cut using a vibratome (Leica, Buffalo Grove, IL, USA) in the same buffer. Slices were then incubated for 1–3 h in oxygenated physiological buffer containing: 125 mmol/L NaCl, 2.5 mmol/L KCl, 1.2 mmol/L NaH_2PO_4, 2.4 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 10 mmol/L glucose, and 26 mmol/L $NaHCO_3$ (pH 7.4) at room temperature. Each slice was transferred to a submersion-recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at 1 mL/min with 30°C oxygenated buffer, and allowed to equilibrate for 30 min before recordings started.

Cyclic voltammetry

Carbon fiber microelectrodes (7 μm in diameter; tip length ~100 μm) were constructed as previously described (Perez et al. 2008). The electrode was positioned below the surface of the slice and its potential linearly scanned every 100 msec from 0 to −400 to 1000 to −400 to 0 mV versus an Ag/AgCl reference electrode at a scan rate of 300 mV/msec. Only the carbon fiber was inserted into the slice to avoid tissue damage by the glass. Current was recorded and digitized at a frequency of 50 kHz with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Triangular wave generation and data acquisition were controlled by pClamp 9.0 software (Molecular Devices). Background current was digitally subtracted to obtain the voltammograms used for the identification of dopamine (confirmed by an oxidation peak ~500–600 mV and a reduction peak around −200 mV). Peak oxidation currents were converted into concentrations after postexperimental calibration of the electrode with fresh solutions of 0.5 μmol/L dopamine. Calibration studies showed a linear relationship between current and dopamine concentrations up to 1.0 μmol/L. The calibration factor for the electrodes used in these studies was ~15 nA/μmol/L, with responses for the vehicle-treated, nicotine-treated, and 24-h withdrawal groups of ~1.0, 0.4, and 0.75 nA, respectively. Dopamine release was measured as the maximal peak response obtained after electrical stimulation.

Electrically evoked dopamine release was measured in the dorsal half of NAcc shell. We focused on the nucleus accumbens shell as an extensive body of evidence has shown that nicotine exposure significantly increased dopamine function in this accumbal subregion, suggesting it is involved in nicotine reinforcement (Di Chiara et al. 2004; Balfour 2009; Changeux 2010). It should be noted, however, that other regions such as the nucleus accumbens core and caudate-putamen also contribute to nicotine-mediated reward and addiction (Di Chiara et al. 2004; Balfour 2009; Wise 2009; Changeux 2010). Electrical stimulation was applied using a bipolar-stimulating electrode (Plastics One, Roanoke, VA) connected to a linear stimulus isolator (WPI, Saratoga, Fl) and triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). The stimulating electrode was consistently placed so that it just touched the surface of the slice and the carbon fiber electrode was positioned ~100 μm away. Evoked release was elicited by either a single electrical pulse or a train of 2 or 4 pulses (1–4 msec in duration) at 30 Hz applied every 2.5 min. This stimulation paradigm was based on our previous studies showing similar drug effects with a 30 Hz and a 100 Hz stimulation frequency (Perez et al. 2010). In addition, previous reports show that burst firing of rat dopamine neurons in vivo occurs at ~20 Hz (Zhang et al. 2009b). Once a stable signal was obtained, control evoked release was assessed in physiological

buffer for 1–1.5 h. NAChR-modulated release was then assessed in the presence of 100 nmol/L α-conotoxinMII (α-CtxMII) to antagonize α6β2* nAChRs followed by the addition of 100 nmol/L dihydro-β-erythroidine (DHβE) to block both the α6β2* and α4β2* nAChR subtypes. Superfusion of the slice with α-CtxMII maximally decreased release within ~15 min and responses were recorded over a 1 h period. Responses in the presence of DHβE were recorded over at least a 2 h period. Slices were also exposed to nicotine (0.1–300 nmol/L) or varenicline (0.01–100 nmol/L) and signals at each dose recorded over at least a 1 h period to determine how release was altered under each treatment condition. The changes in release observed with all drugs were reversible upon washout (1–2 h) (Table 1). The reported effects represent the average of those signals obtained once a stable maximal response was established.

Data analyses

All statistics and curve fittings were conducted using GraphPad Prism (Graph Pad Software Co., San Diego, CA). For the behavioral studies, data were analyzed using repeated measures analysis of variance (ANOVA). For the release experiments, statistical comparisons were performed using one-way ANOVA followed by a Newman–Keuls multiple comparisons test or two-way ANOVA followed by a Bonferroni post hoc test. A value of $P \leq 0.05$ was considered significant. All values are expressed as the mean ± SEM of the indicated number of animals, with release values for each animal representing the average of 6–15 signals from 1 to 2 slices.

Table 1. Effects of nAChR drugs on dopamine release are reversible upon washout.

		Stimulated dopamine release (nmol/L)		
Drug		Vehicle-treated	Nicotine-treated	24-h withdrawal
Control		56.7 ± 1.84	30.3 ± 1.54	45.8 ± 1.52
α-CtxMII and DHβE	Present	29.3 ± 3.95	20.4 ± 1.75	18.7 ± 2.39
	Washout	59.5 ± 2.51	32.2 ± 2.06	41.2 ± 5.51
Bath-applied nicotine	Present	24.8 ± 1.99	13.6 ± 2.07	18.2 ± 2.04
	Washout	57.8 ± 0.92	35.4 ± 3.07	50.7 ± 1.47
Bath-applied varenicline	Present	21.1 ± 1.15	21.9 ± 1.02	21.6 ± 3.06
	Washout	53.1 ± 2.10	37.8 ± 4.16	50.5 ± 1.94

Single-pulse-stimulated dopamine release $[DA]_o$ was determined in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nmol/L), the general nAChR blocker DHβE (100 μmol/L), the full nAChR agonist nicotine (300 nmol/L) or the partial agonist varenicline (100 nmol/L). Dopamine signals returned to control levels after a 1–2-h washout of the nAChR drugs.

Results

Increase in somatic signs after 24-h nicotine withdrawal

Rats were treated with nicotine via the drinking water for 2–3 months. Somatic signs were evaluated during vehicle treatment, nicotine treatment, and withdrawal (24 h after nicotine removal). During vehicle and nicotine treatment, animals showed very low levels of overall somatic signs. However, nicotine withdrawal led to a significant increase in total somatic signs compared to vehicle- and nicotine-treated animals ($P < 0.001$) (Fig. 1). The most prominent somatic signs observed after 24-h withdrawal were eye blinks (32 ± 4.1; Fig. 1), chews (17 ± 1.2), ptosis (5.0 ± 1.2), and teeth chattering (3.0 ± 1.0).

Partial recovery in single- and four-pulse-stimulated dopamine release after 24-h nicotine withdrawal

We used cyclic voltammetry to measure dopamine release in rat brain slices containing the nucleus accumbens shell. Release was evoked by a single-pulse or a four-pulse stimulus train delivered at 30 Hz to mimic the tonic and phasic activity that drives dopamine release, respectively. There was a significant decline in single-pulse ($P < 0.001$)

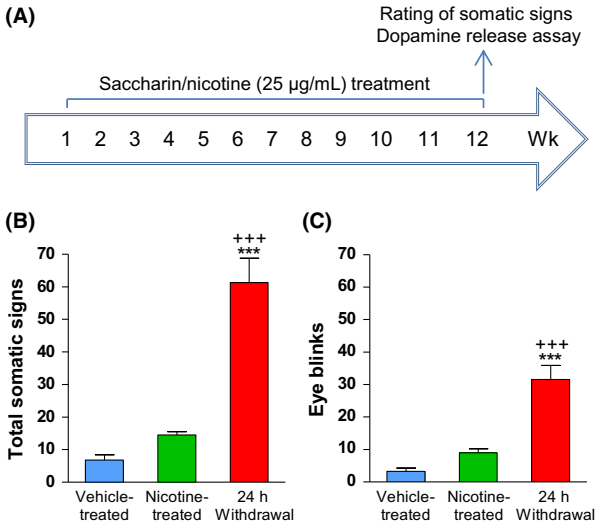

Figure 1. Increased somatic signs after 24-h nicotine withdrawal. (A) Rats were treated with 1% saccharin or nicotine (25 μg/mL) via the drinking water as shown. Somatic signs were rated in vehicle-treated, nicotine-treated, and nicotine withdrawn animals. (B) Twenty-four-hour nicotine withdrawal significantly increased total somatic signs (bottom left), with a prominent increase in eye blinks (C). Values represent the mean ± SEM of eight rats. Significance of difference from vehicle-treated rats, $^{***}P < 0.001$; significance of difference from nicotine-treated rats, $^{+++}P < 0.001$.

and four-pulse-stimulated ($P < 0.01$) dopamine release in slices from animals on nicotine treatment (Fig. 2), in agreement with previous studies (Exley et al. 2013; Perez et al. 2013).

A significant decline in single ($P < 0.001$) and four-pulse-stimulated ($P < 0.05$) release was also observed in slices from animals that had gone through 24-h nicotine withdrawal (Fig. 2). However, there was a significant 20% recovery in release compared to nicotine-treated animals ($P < 0.001$ and $P < 0.05$ for single- and four-pulse-stimulated release, respectively).

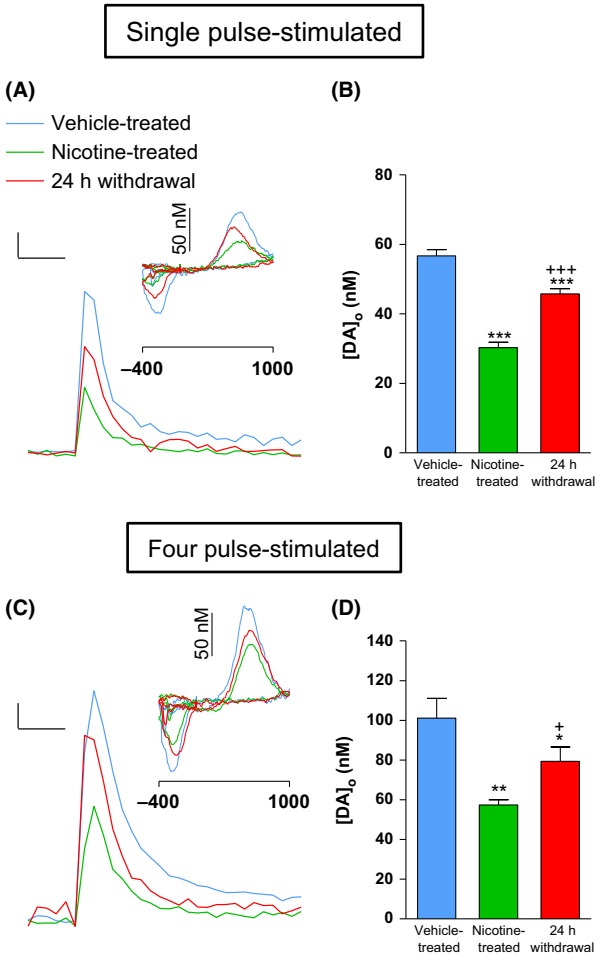

Figure 2. Partial recovery of single-pulse-stimulated dopamine release after 24-h nicotine withdrawal. Dopamine release $[DA]_o$ was measured in the dorsal portion of the accumbens shell in response to a single- (A, B) or four-pulse stimulus (C, D). Representative traces of $[DA]_o$ are shown from slices of animals on vehicle treatment, nicotine treatment or 24 h after nicotine withdrawal. Scale bar represents 10 nmol/L and 0.5 sec. Typical voltammograms for dopamine with an oxidation peak at 500–600 mV and a reduction peak around −200 mV are shown in the upper right panels (A, C). Quantitative analyses show that long-term nicotine treatment significantly decreased $[DA]_o$ regardless of the stimulation paradigm (B, D). Although $[DA]_o$ was still decreased after 24-h nicotine withdrawal, a significant recovery in $[DA]_o$ was observed compared to that in the nicotine treatment group. The values represent the mean ± SEM of 7–8 rats. Significance of difference from vehicle-treated rats, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; significance of difference from nicotine-treated rats, $^{+++}P < 0.001$, $^{+}P < 0.05$.

α6β2* nAChR-mediated dopamine release is partially restored after 24-h nicotine withdrawal

We used the α6β2* nAChR antagonist, α-CtxMII, as well as the β2* nAChR antagonist, DHβE, to discern the specific contributions of α6β2* and α4β2* nAChRs to the functional changes observed after nicotine withdrawal. Both antagonists decreased dopamine release by the same extent (~50%) in vehicle-treated animals, in line with previous observations that only the α6β2* nAChR subtype regulates accumbal dopamine release (Exley et al. 2008; Perez et al. 2012, 2013). In addition, we observed a loss in the α6β2* nAChR regulation of dopamine release as evident by the lack of effect of either α-CtxMII or DHβE (Fig. 3), in agreement with previous reports (Perez et al. 2012, 2013). Electrically evoked release was still decreased after a 24-h nicotine withdrawal period, albeit to a lesser extent than with nicotine treatment. However, α-CtxMII and DHβE equally decreased dopamine release by ~45%, indicating a recovery of α6β2* nAChR function with nicotine withdrawal (Fig. 3).

We also evaluated the effects of nAChR blockade on dopamine release evoked via a four-pulse stimulus train at 30 Hz. Exposure to either α-CtxMII or DHβE did not modify four-pulse-stimulated release in any of the treatments groups compared to burst release in the absence of the drug (Fig. 3). These findings agree with previous studies showing that nAChR antagonism or desensitization facilitates or does not change electrically evoked dopamine release stimulated by high frequencies (Zhang and Sulzer 2004; Exley and Cragg 2008; Perez et al. 2008; Zhang et al. 2009a; Exley et al. 2011). Four-pulse-stimulated release was significantly decreased under all conditions in slices from animals on nicotine treatment and animals after 24-h withdrawal compared to vehicle-treated animals (Fig. 3). In addition, burst-induced release was significantly higher in slices from animals after 24-h withdrawal compared to nicotine treated.

Enhanced paired-pulse ratios after long-term nicotine are partially attenuated after withdrawal

As an approach to further understand how nicotine withdrawal further modifies nAChR-mediated dopamine release, paired-pulse stimulation studies were done.

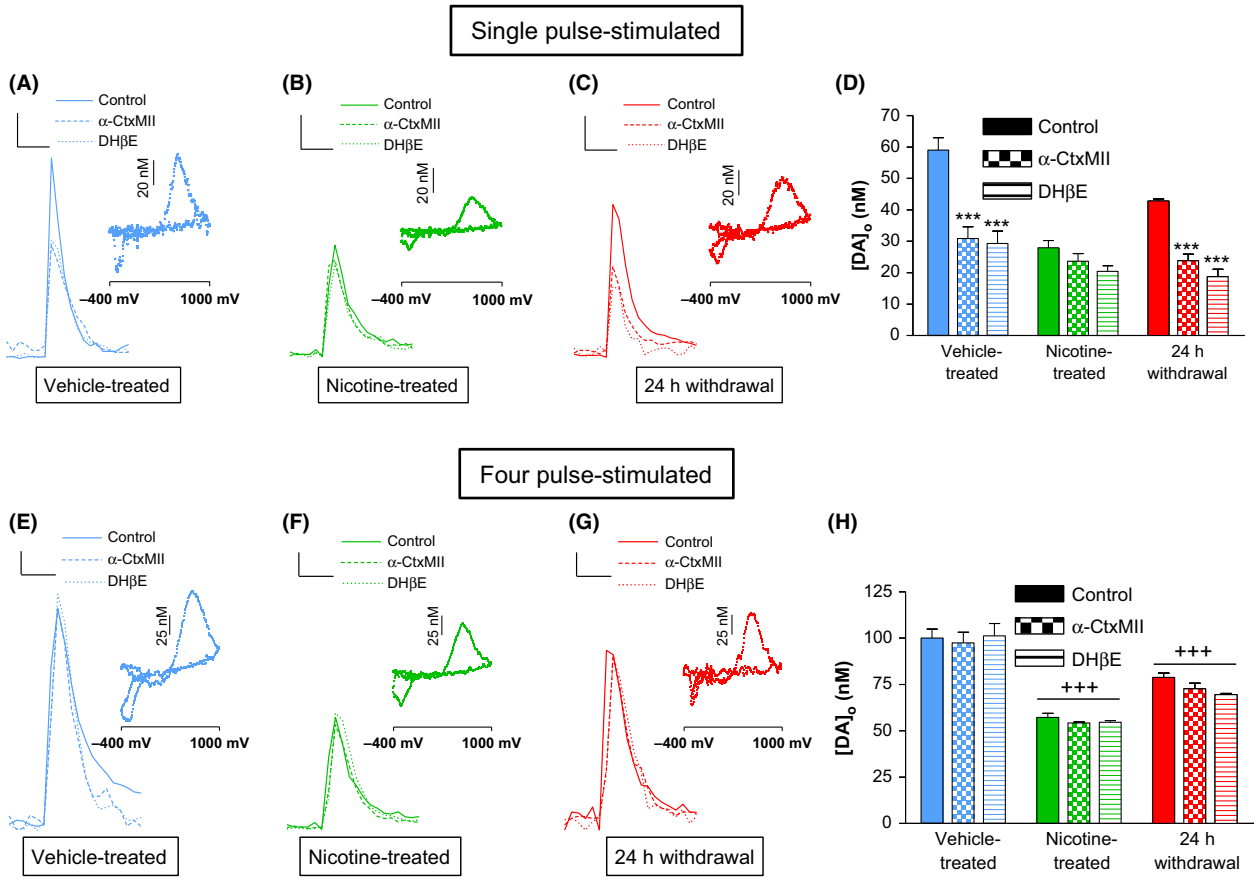

Figure 3. α6β2* nAChR-mediated dopamine release is partially restored after 24-h nicotine withdrawal. Single- and four-pulse-stimulated dopamine release $[DA]_o$ was measured in the dorsal portion of the accumbens shell in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nmol/L) or the β2* nAChR antagonist DHβE (100 nmol/L). Representative traces of single-pulse (A–C) and four-pulse-stimulated (E–G) $[DA]_o$ are shown from slices of animals on vehicle treatment, on nicotine treatment or 24 h after nicotine withdrawal. Scale bar represents 10 nmol/L and 0.5 sec. Typical voltammograms for dopamine with an oxidation peak at 500–600 mV and a reduction peak around −200 mV are shown in the upper right panels. (D) Quantitative analyses show that α-CtxMII and DHβE similarly affected single-pulse-stimulated release in vehicle-treated animals, indicating that nAChR-modulated $[DA]_o$ occurs through α6β2* nAChRs. Single-pulse-stimulated release was not further decreased in the presence of any of the nAChR antagonists in slices from animals on nicotine treatment. In contrast, both α-CtxMII and DHβE decreased release by the same extent in slices from animals subjected to 24-h nicotine withdrawal, suggesting a restoration of α6β2* nAChR-mediated $[DA]_o$. (H) Additionally, four-pulse-stimulated $[DA]_o$ was not affected by any of the nicotinic receptor blockers. The values represent the mean ± SEM of 3–7 rats. Significance of difference from control release, $^{***}P < 0.001$; significant main effect of treatment compared to vehicle-treated, $^{+++}P < 0.001$.

Paired-pulse ratios, defined as the response of a second stimulus within a train divided by that of the first, provide a measure of dopamine release probability. Interventions that decrease the probability of release with the first pulse and facilitate release by a second pulse increase paired-pulse ratios and are interpreted as conditions leading to facilitation of burst-induced release (Cragg 2003).

Representative traces and voltammograms of dopamine release elicited by 1 or 2 pulses at 30 Hz for vehicle-treated, nicotine-treated, and 24-h withdrawal animals are shown in Figure 4. Quantitative analyses showed that paired-pulse ratios under control conditions are significantly increased twofold ($P < 0.01$) in slices from animals on nicotine treatment compared to slices from vehicle-treated animals (Fig. 4). Notably, paired-pulse ratios in slices from nicotine withdrawn animals were only increased ($P < 0.05$) by 50% compared to slices from vehicle-treated animals (Fig. 4); with paired-pulse ratios in slices from animals after 24-h withdrawal significantly lower ($P < 0.05$) than those for slices of animals on nicotine treatment. This decrease in paired-pulse ratios is more likely related to the enhanced single-pulse-stimulated release observed after nicotine withdrawal.

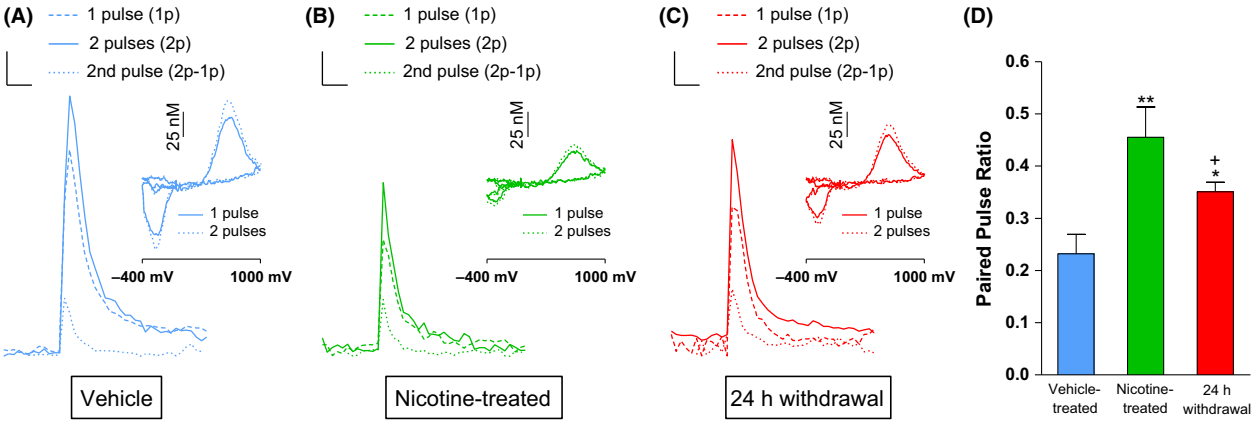

Figure 4. Enhanced dopamine release paired-pulse ratio after long-term nicotine treatment is partially attenuated after 24-h nicotine withdrawal. (A–C) Dopamine release $[DA]_o$ was stimulated by one or two pulses at 30 Hz as shown in the representative traces. Scale bar represents 10 nmol/L and 0.5 sec. Release stimulated by one pulse (1p) was subtracted from that evoked by two pulses (2p) to determine release by the second pulse (2p–1p). Paired-pulse ratios were calculated by dividing release stimulated with the second pulse by that stimulated with a single pulse. Typical voltammograms for dopamine with an oxidation peak at 500–600 mV and a reduction peak around −200 mV are shown in the upper right panels. (D) Quantitative analyses show that there is a significant increase in paired-pulse ratios after long-term nicotine treatment. Twenty-four-hour nicotine withdrawal significantly attenuated paired-pulse ratios. The values represent the mean ± SEM of 7–15 rats. Significance of difference from vehicle-treated, $^{**}P < 0.01$, $^{*}P < 0.05$; significance of difference from nicotine-treated, $^{+}P < 0.05$.

Bath-applied nicotine and varenicline decrease single-pulse-stimulated dopamine release

Nicotine replacement therapy and varenicline are among the most commonly used smoking cessation aids to date (Cahill et al., 2013; Mills et al. 2012). Therefore, we bath-applied nicotine or varenicline to slices from animals on vehicle treatment, nicotine treatment, and after withdrawal to gain a better understanding of how these drugs modulate dopamine release under these conditions. First, we compared the functional effect of nicotine (0.1–300 nmol/L) and varenicline (0.01–100 nmol/L) on single-pulse-stimulated release in vehicle-treated animals (Fig. 5). Both drugs decreased release to the same extent (~65%) indicating that nicotine and varenicline equally modulate $\alpha6\beta2^*$ nAChR-mediated release. However, varenicline more potently inhibited dopamine release than nicotine with IC_{50} values of 3.7 ± 1.1 and 20 ± 5.3 nmol/L, respectively.

We also tested the effect of various concentrations of bath-applied nicotine (0.1–300 nmol/L) and varenicline (0.01–100 nmol/L) after long-term nicotine treatment and withdrawal. Neither treatment affected the IC_{50} values for either drug. Both bath-applied nicotine and varenicline still decreased dopamine release in slices from animals on nicotine treatment and after 24-h withdrawal (Fig. 6). Bath-applied nicotine decreased dopamine release by ~65% in all groups (Fig. 6). Interestingly, bath-applied varenicline decreased release by only 25% in slices from nicotine-treated animals whereas decreasing release by 65% and 50% in

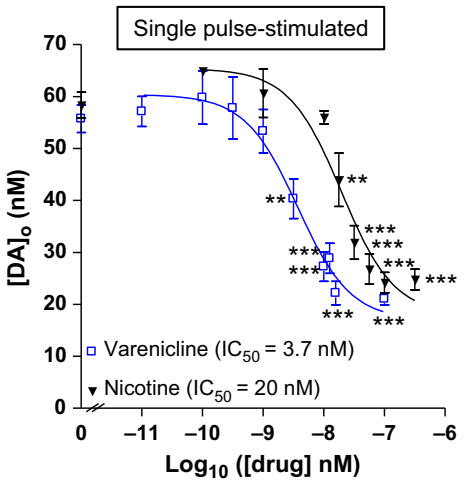

Figure 5. Nicotine and varenicline decrease single-pulse-stimulated dopamine release in vehicle-treated animals. Dopamine release $[DA]_o$ was determined in the absence (control) and presence of varenicline (0.1–100 nmol/L) or nicotine (0.1–300 nmol/L). Dose–response curves are shown for vehicle-treated animals. Curve fits were sigmoidal with $R = 0.8–0.9$. The values represent the mean ± SEM of 3–5 rats. Significance of difference from control (zero drug), $^{***}P < 0.001$, $^{**}P < 0.01$.

slices from vehicle-treated and 24-h withdrawal animals, respectively. Statistical analyses of the varenicline-induced decrease in dopamine release concentration in vehicle-treated (34.7 ± 3.80 nmol/L), nicotine-treated (7.21 ± 3.4 nmol/L), and 24-h withdrawn (22.1 ± 5.09 nmol/L) animals revealed a significant difference between the effect

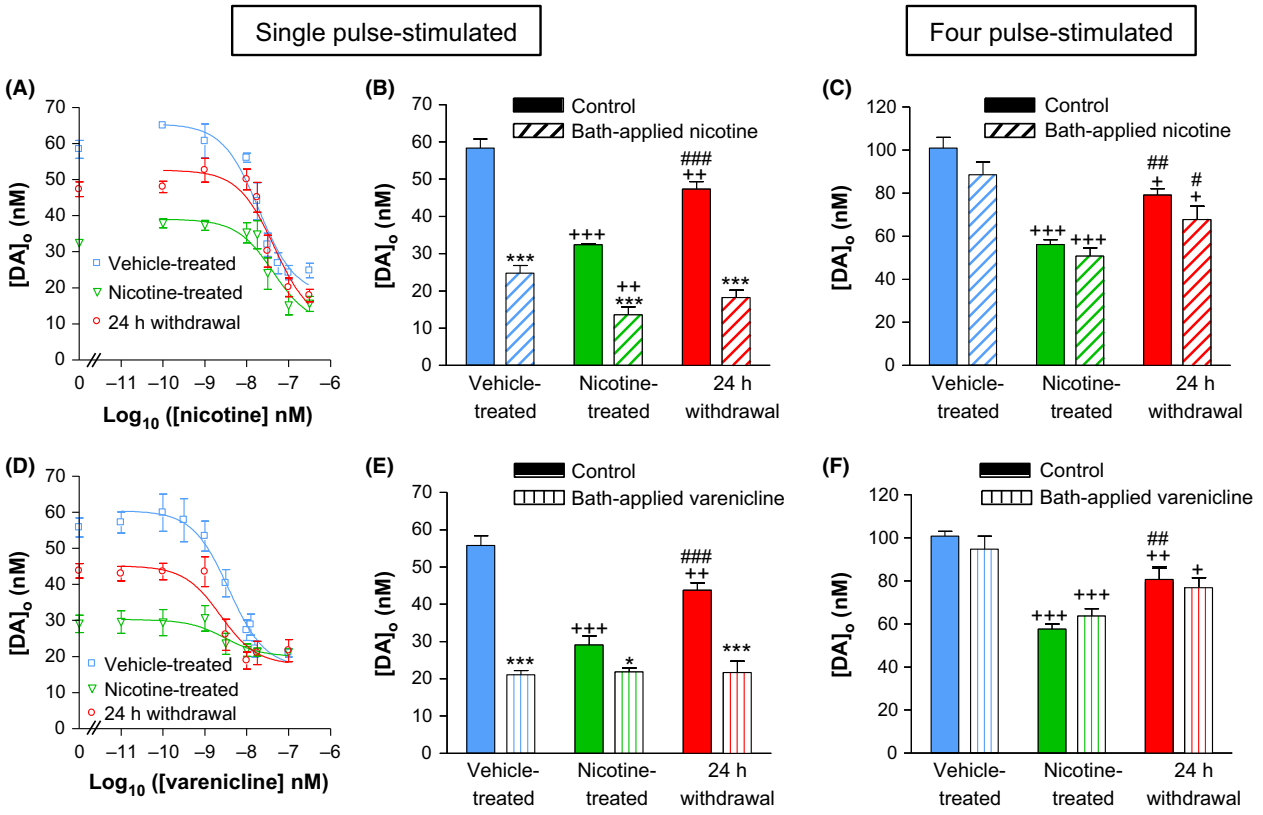

Figure 6. Bath-applied nicotine and varenicline decrease single-pulse-stimulated dopamine release after long-term nicotine treatment and after withdrawal. Dopamine release $[DA]_o$ was determined in the absence (control) and presence of varenicline (0.1–100 nmol/L) or nicotine (0.1–300 nmol/L). Dose–response curves for nicotine (A) and varenicline (D) are shown for single-pulse-stimulated dopamine release from slices of animals on vehicle treatment, on nicotine treatment and after 24-h nicotine withdrawal. Curve fits were sigmoidal with $R = 0.8–0.9$. Quantitative analyses show that bath-applied nicotine (B) and varenicline (E) significantly decreased release in all treatment groups at the maximal effective concentration (100 nmol/L for varenicline and 300 nmol/L for nicotine). In contrast, four-pulse-stimulated $[DA]_o$ was not affected by bath application of nicotine (C) or varenicline (F) at any of the concentrations tested. The values represent the mean ± SEM of 3–5 rats. Significance of difference from own control condition (zero drug), $^{***}P < 0.001$, $^{*}P < 0.05$; significance of difference from vehicle-treated under the same condition, $^{+++}P < 0.001$, $^{++}P < 0.01$, $^{+}P < 0.05$; significance of difference from nicotine-treated under the same condition, $^{\#\#\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$.

of varenicline in nicotine-treated compared to vehicle-treated ($P < 0.01$) and 24-h withdrawn ($P < 0.05$) animals.

We also determined the effect of bath-applied nicotine and bath-applied varenicline on dopamine release evoked via a four-pulse stimulus train at 30 Hz. Neither agonist modified four-pulse-stimulated release in any of the treatments groups compared to burst release in the absence of the drug (control) (Fig. 6). Four pulse-stimulated release was significantly decreased in slices from animals on nicotine treatment and after 24-h withdrawal compared to vehicle-treated animals (Fig. 6). In addition, burst-induced release was significantly higher in slices from animals after 24-h withdrawal compared to nicotine-treated with bath-applied nicotine ($P < 0.05$), but was similar in the presence of bath-applied varenicline.

Varenicline enhances dopamine release paired-pulse ratios to a greater extent than nicotine with long-term nicotine treatment and after withdrawal

We also assessed the effect of bath-applied nicotine and varenicline on paired-pulse ratios as changes in release probability may contribute to the mechanisms by which these drugs further modulate function after long-term nicotine and withdrawal. In slices from vehicle-treated animals, bath-applied nicotine and varenicline similarly increased paired-pulse ratios (Fig. 7). Conversely, in slices of nicotine-treated and 24-h withdrawal animals, paired-pulse ratios were significantly higher after bath application of varenicline than that of nicotine (Fig. 7).

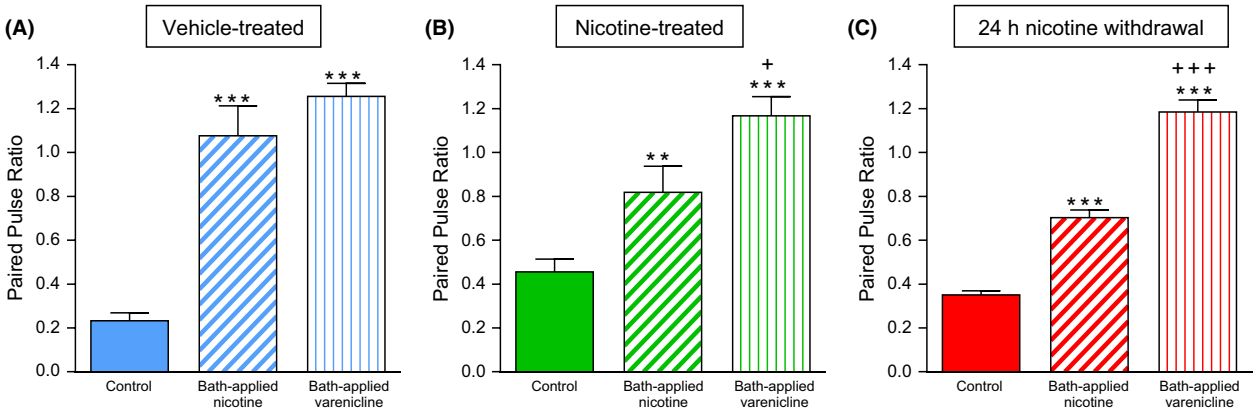

Figure 7. Varenicline enhances dopamine release paired-pulse ratios to a greater extent than nicotine with long-term nicotine treatment and after withdrawal. Dopamine release $[DA]_o$ was stimulated by one (1p) or two pulses (2p) at 30 Hz in the absence (control) and presence of the full nAChR agonist nicotine (300 nmol/L) or the partial agonist varenicline (100 nmol/L). Release stimulated by one pulse was subtracted from that evoked by two pulses to determine release by the second pulse. Paired-pulse ratios were calculated by dividing release stimulated with the second pulse by that stimulated with a single pulse. Quantitative analyses show that bath application of nicotine or varenicline similarly increases paired-pulse ratios in vehicle-treated animals (A). In contrast, varenicline leads to a greater enhancement in paired-pulse ratios than bath-applied nicotine with long-term nicotine treatment (B) and after withdrawal (C). The values represent the mean ± SEM of 7–15 rats. Significance of difference from control condition, $^{***}P < 0.001$, $^{**}P < 0.01$; significance of difference from nicotine, $^{+++}P < 0.001$, $^{+}P < 0.05$.

Discussion

The present results are the first to show that 24-h withdrawal is associated with a partial reversal of the nicotine treatment-induced decline in single- and four-pulse-stimulated dopamine release in accumbal slices. The data further demonstrate that bath application of maximally effective doses of varenicline (100 nmol/L) or nicotine (300 nmol/L) decreased single-pulse-stimulated dopamine release under all treatment conditions. Notably, acute varenicline increased release paired-pulse ratios to a greater extent than acute nicotine after long-term nicotine treatment and withdrawal. This differential effect of varenicline on dopamine release dynamics may relate to its mechanism of action as a partial agonist.

In this study, nicotine was administered via the drinking water. This approach models long-term, intermittent nicotine exposure and induces widespread neurochemical changes similar to that with smoking (Malin and Goyarzu 2009; Dani et al. 2011; Picciotto and Mineur 2014). In addition, subsequent removal of nicotine from the drinking water results in withdrawal signs (Jackson et al. 2009; Malin and Goyarzu 2009; Locklear et al. 2012). However, although this approach is well documented in mouse models, nicotine withdrawal signs after this route of administration have not yet been assessed in rats. Our results show that nicotine removal from the drinking water increased somatic signs in rats, with the rate of eye blinks reflecting the observed pattern of the total somatic signs. These findings are in line with previous studies administering nicotine via subcutaneous osmotic minipumps (Skjei and Markou 2003; O'Dell et al. 2004).

We next examined the effect of nicotine treatment and withdrawal on accumbal dopamine release using voltammetry in brain slices. Although one limitation of this approach is that much of the brain circuitry is removed as inputs to the accumbens are severed, cholinergic interneurons remain tonically active in slice preparations (Exley and Cragg 2008; Rice et al. 2011). Ex vivo voltammetry thus enables us to isolate specific brain regions and explore local circuitry control of dopamine release dynamics. We focused on the accumbens shell because dopamine release in this region has been linked to the rewarding or reinforcing properties of nicotine, whereas release in the accumbens core and striatum are more highly associated with the presentation of a conditioned stimulus (Sellings et al. 2008; Balfour 2009; Wise 2009). Our studies show a decrease in electrically evoked release in nicotine withdrawn rats, consistent with previous work (Rahman et al. 2004; Natividad et al. 2010; Dani et al. 2011; Zhang et al. 2011). Our data extend these findings by showing that nicotine withdrawal is actually associated with a partial recovery from the decreased dopamine release that occurs with long-term nicotine treatment. To determine the receptors contributing to the recovery, we used nAChR subtype selective antagonists. The results show a partial restoration of $\alpha6\beta2^*$ nAChR-mediated release after 24-h nicotine withdrawal, with little contribution from $\alpha4\beta2^*$ nAChRs. Previous studies had shown that in vivo $\alpha6\beta2^*$ nAChR antagonism decreases nicotine

withdrawal signs (Pons et al. 2008; Jackson et al. 2009; Brunzell 2012). The present data would thus suggest that the appearance of withdrawal signs is associated with a recovery of $\alpha6\beta2$* nAChR-mediated dopamine release after nicotine withdrawal.

Multiple studies have shown that a mechanism through which nicotine regulates neuronal function is via nAChR desensitization (Buisson and Bertrand 2002; Benowitz 2010; De Biasi and Dani 2011). To investigate whether long-term nicotine treatment modulated dopamine release dynamics via such mechanism, we looked at paired-pulse ratios. Long-term nicotine decreased single-pulse-stimulated release to a greater extent than release by a second pulse, leading to a greater paired-pulse ratio. Such disproportionate inhibition of single-pulse-stimulated release also occurs with dampened nAChR function via short-term desensitization after bath-applied nicotine or nAChR antagonists (Rice and Cragg 2004; Zhang and Sulzer 2004; Exley et al. 2008; Zhang et al. 2011). Thus, the altered dopamine release dynamics observed after long-term nicotine treatment could arise from nAChR desensitization. As accumbal dopamine release is mediated primarily via $\alpha6\beta2$* nAChRs, the observed changes are most likely due to $\alpha6\beta2$* nAChRs desensitization. As expected, partial recovery of nAChR function after nicotine withdrawal was associated with a smaller increase in paired-pulse ratio compared to that after nicotine treatment.

Despite the enhanced paired-pulse ratios with nicotine treatment and withdrawal, four-pulse-stimulated release continued to show depression under all conditions. These observations suggest that long-term nicotine treatment induces alterations in function that prevent the system from overcoming the reduced dopamine release during bursting activity. A similar effect of long-term nicotine has been reported both in vitro and in vivo at stimulation frequencies similar to those used in our studies (Zhang et al. 2011; Exley et al. 2013; Koranda et al. 2014). These studies suggested that long-term nicotine decreases the range of activity-dependent dopamine release (Koranda et al. 2014).

We assessed the effects of bath-applied nicotine and varenicline on dopamine release with nicotine treatment and after withdrawal to mimic the in vivo use of these drugs as smoking cessation therapies. Both nicotine and varenicline significantly decreased single-pulse-stimulated dopamine release by ~78% in vehicle-treated animals. Electrically-evoked dopamine release in brain slices is modulated by both cholinergic interneuron and dopamine terminal activity, with nAChRs only partly regulating dopamine release (Zhou et al. 2001; Threlfell et al. 2012; Wang et al. 2014). The incomplete block of dopamine release by exogenous agonist application agrees with studies showing that nAChRs predominantly mediate release induced via cholinergic stimulation without affecting release arising from dopamine terminal activation (Wang et al. 2014).

We also tested the effects of bath-applied nicotine and varenicline on single-pulse-stimulated release after nicotine treatment and withdrawal. Varenicline decreased release to a lesser extent than nicotine in slices from nicotine-treated animals. In addition, varenicline increased paired-pulse ratios to a greater extent than nicotine both after nicotine treatment and withdrawal. A possible explanation is that long-term nicotine treatment decreases the effect of acute nicotine exposure on dopamine release dynamics without altering that of varenicline (Rahman et al. 2004). Alternately, varenicline could deplete dopamine vesicles to a lesser extent than nicotine (Turner 2004; Wang et al. 2014). In fact, only varenicline elicited similar amounts of four-pulse-stimulated dopamine release in slices from nicotine-treated and nicotine withdrawn animals, although release was still decreased compared to vehicle-treated animals. The greater enhancement of paired-pulse ratios in the presence of varenicline with no overall change in burst-induced release suggests that short-term varenicline exposure may not be sufficient to overcome the generalized loss of function observed with nicotine treatment.

The observation that nAChR drugs affect single-pulse-evoked but not burst-evoked (30 Hz) dopamine release is consistent with numerous other voltammetry studies (Exley and Cragg 2008; Zhang et al. 2009b, 2011; Perez et al. 2012, 2013; Exley et al. 2013; Wang et al. 2014). The apparent lack of effect in dopamine release with burst firing may relate to the ability of these drugs to reduce cholinergic interneuron activity and suppress single-pulse-evoked dopamine release (Wang et al. 2014). This results in a smaller depletion of vesicular dopamine and allows for higher dopamine release with subsequent pulses within a burst that results on unaffected or even increased burst-induced release (Rice and Cragg 2004; Zhang and Sulzer 2004; Exley and Cragg 2008; Zhang et al. 2009b, 2011; Perez et al. 2012, 2013; Exley et al. 2013; Wang et al. 2014).

Both nicotine and varenicline interact equally well with $\alpha4\beta2$* and $\alpha6\beta2$* nAChRs, with varenicline acting as a partial agonist with higher potency than nicotine at these nAChR subtypes (Grady et al. 2010; Bordia et al. 2012). Likewise, both agonists are potent full agonists at $\alpha7$ nAChRs (Grady et al. 2010). In contrast, the antagonists used in these experiments show greater specificity for $\beta2$* nAChRs. Thus the differential effect of the agonists on dopamine release may relate to their ability to interact with $\alpha7$ nAChRs. Additionally, previous studies have shown that long-term nicotine treatment reduces striatal acetylcholine levels (Yu and Wecker 1994; Perez et al.

2013; Falasca et al. 2014). Thus, a decrease in acetylcholine levels could also alter the action of antagonists but not agonists or vice versa.

In summary, our findings show that nicotine withdrawal is associated with a partial recovery of $\alpha6\beta2$* nAChR function. Moreover, our data show a primary role for $\alpha6\beta2$* nAChRs in nicotine withdrawal, consistent with previous work (Pons et al. 2008; Jackson et al. 2009; Brunzell 2012). Thus, drugs targeting the $\alpha6\beta2$* nAChR population may represent a beneficial smoking cessation pharmacotherapy.

Author Contributions

Perez and Quik participated in research design. Perez and Khroyan conducted experiments. McIntosh contributed new agents or analytical tools. Perez performed data analysis. Perez, Khroyan, McIntosh and Quik wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

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