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Original Article

Nicotine modulates GABAergic transmission to dopaminergic neurons in substantia nigra pars compacta

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Aim: Dopaminergic neurons in the substantia nigra pars compacta (SNc) play important roles in motor control and drug addiction. As the major afferent, GABAergic innervation controls the activity of SNc dopaminergic neurons. Although it is clear that nicotine modulates SNc dopaminergic neurons by activating subtypes of somatodendritic nicotinic acetylcholine receptors (nAChRs), the detailed mechanisms of this activation remain to be addressed.
Methods: In the current study, we recorded $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents (sIP-SCs) from dissociated SNc dopaminergic neurons that were obtained using an enzyme-free procedure. These neurons preserved some functional terminals after isolation, including those that release GABA.
Results: We found that both extra- and intra-cellular calcium modulates sIPSCs in these neurons. Furthermore, both nicotine and endogenous acetylcholine enhance the frequency of sIPSCs. Moreover, endogenous acetylcholine tonically facilitates sIPSC frequency, primarily by activating the α4β2* nAChRs on the GABAergic terminals.
Conclusion: Nicotine facilitates GABA release onto SNc dopaminergic neurons mainly via the activation of presynaptic α4β2* nAChRs.

Introduction

Nicotine is the most commonly abused drug[1]. It stimulates dopaminergic (DA) neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) by activating nicotinic acetylcholine receptors (nAChRs)[2–4] and enhances locomotor activity[5, 6], thus conferring its rewarding effect. Multiple subtypes of nAChRs are highly expressed in midbrain neurons, including DA neurons and GABAergic neurons[4, 7–10]. GABAergic neurons in the VTA and substantia nigra pars reticulata (SNr) inhibit, respectively, DA neurons in the VTA and the SNc[11–13]. Therefore, the tuning effect of nicotine on the DA neuron can be reasonably investigated by studying both the pre- and postsynaptic components in these neural circuits.

It is well documented that the activation of presynaptic

nAChRs facilitates the release of neurotransmitters, including GABA, glutamate, serotonin, and dopamine[3, 14–16]. In the VTA, nicotine stimulates glutamate release by activating α7 nAChRs and GABA release by activating α4β2* nAChRs (the asterisk indicates that there may be other subunits)[3]. Whereas the former effect appears to be persistent, the latter is transient, because the concentrations of nicotine achieved during smoking tend to rapidly desensitize α4β2* nAChRs[3, 4]. The net effect of activation of presynaptic nAChRs would therefore preferentially be excitation of VTA DA neurons.

In the substantia nigra, α4β2* nAChRs in SNr GABAergic neurons also play a pivotal role in modulating the activity of SNc DA neurons. Activation of SNr GABAergic neurons inhibits SNc DA neurons[12]. Chronic nicotine upregulates α4β2* nAChRs localized in SNr GABAergic neurons without changing α4β2* nAChR levels in SNc DA neurons[10]. Due to the tonic cholinergic innervation in the substantia nigra, the specific upregulation of α4β2* nAChR by chronic nicotine induces hyperactivity of SNr GABAergic neurons and

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hypoactivity of SNc DA neurons and attenuates excitation of SNc DA neurons by nicotine. SNc DA neurons receive GABAergic innervation from the dorsal striatum, globus pallidus, and SNr[12, 17–19]. The expression pattern of nAChRs in the SNr is different from that in the dorsal striatum and globus pallidus[2, 10, 20, 21].

In this study, we determined which subtypes of nAChRs are responsible for nicotine modulation of GABA release onto SNc DA neurons. Mechanically dissociated SNc DA neurons were used to accomplish this goal, for several reasons. First, enzyme-free dissociation keeps the presynaptic receptors intact; second, the majority of neuronal processes are severed by the dissociation, facilitating space clamping; third, the resulting preparation contains only a postsynaptic neuron and presynaptic boutons, facilitating straightforward interpretation of the results; finally, this preparation allows for fast perfusion of chemicals, which can ensure accurate drug concentrations around the recorded cell and minimize the confounding effects of desensitization[22–28].

Materials and methods

Slice preparation The care and use of animals and the experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. Midbrain slices were prepared as described previously[27, 29]. In brief, rats aged 6 to 17 postnatal days were decapitated and the brains were quickly excised and coronally sliced (300 μm) using a VF-200 Slicer (Precisionary Instruments, Greenville, NC). This was done while the brains were immersed in ice-cold artificial cerebrospinal fluid saturated with 95% O_2–5% CO_2 (carbogen) containing 126 mmol/L NaCl, 1.6 mmol/L KCl, 1.25 mmol/L NaH_2PO_4, 1.5 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, and 10 mmol/L glucose. Midbrain slices were kept in carbogen-saturated artificial cerebrospinal fluid at room temperature (22–24 °C) for at least 1 h before use.

Preparation of mechanically dissociated SNc neurons Neurons with functional terminals were obtained by mechanical dissociation as described previously[22, 27, 28]. In brief, one midbrain slice was transferred to a 35-mm culture dish (Falcon, Rutherford, NJ) filled with a standard external solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose (320 mOsm, pH adjusted to 7.3 with Tris base). The region of SNc was identified with an inverted microscope (Nikon, Tokyo, Japan). A heavily fire-polished glass pipette with a 50-μm diameter tip was fixed on

a homemade device. Using a manipulator, the pipette was then positioned so as to slightly touch the surface of the SNc region. Neurons close to the surface of the tissue were dissociated by horizontal vibration at a frequency of 15 to 20 Hz with a range from 0.1 to 0.3 mm for 2 to 5 min. The slice was then removed. Within 20 min, the isolated neurons adhered to the bottom of the dish and were ready for electrophysiological recordings. These mechanically dissociated neurons differed from neurons dissociated using enzymes. Whereas the latter lost most, if not all, of the nerve terminals during the dissociation process, the former often preserved some functional nerve terminals[22, 27, 28, 30].

Electrophysiological recording The whole-cell patch clamp technique was used to record action potential and currents using pClamp 9.2 software (MDS Analytical Technologies, Sunnyvale, CA, USA) through a Digidata 1322A analog-to-digital converter (MDS Analytical Technologies) connected to an Axopatch 200B amplifier (MDS Analytical Technologies). The signals were filtered at 1 kHz and sampled at 5 kHz. The junction potential between the pipette and the bath solution was nullified just before the giga-seal was formed.

The patch electrodes had a resistance of 3 to 5 MΩ when filled with pipette solution containing 140 mmol/L CsCl or KCl, 2 mmol/L $MgCl_2$, 4 mmol/L EGTA, 0.4 mmol/L $CaCl_2$, 10 mmol/L HEPES, 2 mmol/L Mg-ATP, and 0.1 mmol/L GTP. The pH was adjusted to 7.2 with Tris-base, and the osmolarity was adjusted to 280 to 300 mOsm with sucrose. Electrophysiological recordings were performed at room temperature (22–24 °C).

Chemicals and applications Most of the chemicals used in this study, including bicuculline methiodide, DL-2-amino-5-phosphono-valeric acid (APV), 6,7-dinitroquinoxaline-2,3-dione (DNQX), tetrodotoxin (TTX), dopamine, mecamylamine hydrochloride (MEC), dihydro-β-erythroidine hydrobromide (DHβE), α-bungarotoxin (α-BTX), RJR-2403, and choline chloride were purchased from Sigma-Aldrich (St Louis, MO, USA). All solutions were freshly prepared on the day of use. Chemicals were applied to dissociated neurons with a Y-tube; this exchanged the external solution surrounding the neurons within 40 ms[28, 30].

Data analyses Spontaneous inhibitory postsynaptic currents (sIPSCs) were analyzed with Clampfit 9.2 software (MDS Analytical Technologies) as described previously[25, 28]. In brief, the sIPSCs were screened automatically using a template with an amplitude threshold of 5 pA. These were visually accepted or rejected based upon rise and decay times. More than 95% of the sIPSCs that were visually accepted were screened using a suitable template. The frequency and

amplitude of sIPSCs in different conditions were measured. Differences in amplitude and frequency were tested by Student's paired two-tailed *t* test using the raw data. Numerical values are presented as mean±SEM. Values of *P*<0.05 were considered significant.

Results

Identification of SNc DA neurons The SNc was first identified in coronal slices with reference to the rat brain atlas[31] using an inverted microscope and the DA neuron was then confirmed early in the recording session based on previously described criteria[10, 32]: (1) 1−3 Hz spontaneous firing (Figure 1A), (2) inhibition of spontaneous firing during exposure to 0.2 μmol/L quinpirole (Figure 1A), and (3) expression of a hyperpolarization-induced current (H-current; in voltage-clamp mode; Figure 1B).
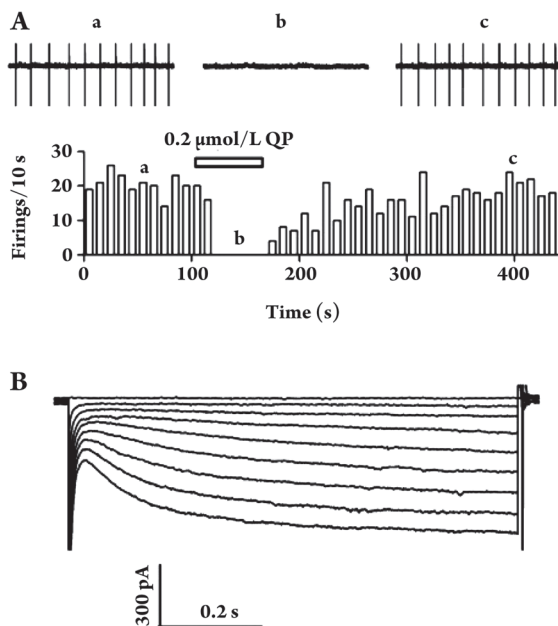

Figure 1. SNc DA neurons have distinct electrophysiological and pharmacological properties. A, spontaneous firing of a dissociated putative SNc DA neuron, which was reversibly depressed by 0.2 μmol/L quinpirole(QP) (Upper panels: typical traces; Lower panel: time course). B, under voltage clamp mode, an I_h current was induced using a series of incremental 10 mV hyperpolarizing steps from a holding potential of -60 mV.

Properties of sIPSCs in mechanically dissociated SNc DA neuronal preparations We were able to record spontaneous postsynaptic currents (sPSCs) in >80% of SNc DA neurons in mechanically dissociated neuronal preparations

from SNc from midbrain slices. We isolated sIPSCs in voltage-clamped SNc DA neurons (V_H=-55 mV) by including 50 μmol/L APV and 20 μmol/L DNQX in the perfusate. The reversible blockade by 10 μmol/L bicuculline indicates that these sIPSCs were mediated by $GABA_A$ receptors (Figure 2A). Consistent with our previous reports, the preparation, although simpler than brain slices in terms of synaptic connections, does contain the principal machinery for modulating neurotransmitter release. For instance, 1 μmol/L TTX (Figure 2B), 50 μmol/L $CdCl_2$ (Figure 2C), or replacement of extracellular Ca^{2+} with Mg^{2+} (data not shown) reduced both the frequency and the amplitude of sIPSCs. The magnitude of these effects was as follows: frequency (illustrated in the upper panel of Figure 2D): TTX, by 62%±10% (from 1.12±0.19 to 0.46±0.17 Hz, *n*=6, *P*<0.01); Cd^{2+}, by 79%±3% (from 1.51±0.06 to 0.32±0.03 Hz, *n*=5, *P*<0.01); 0 Ca^{2+}, by 65%±5% (from 1.33±0.24 to 0.51±0.13 Hz, *n*=7, *P*<0.01); and amplitude (illustrated in the lower panel of Figure 2D): TTX, by 33%±8% (from 65.5±18.5 to 44.7±12.4 pA, *n*=6, *P*<0.01); Cd^{2+}, by 38%±9% (from 54.5±7.6 to 34.4±8.5 pA, *n*=5, *P*<0.01); 0 Ca^{2+}, by 27%±8% (from 31.7±5.6 to 21.3±2.1 pA, *n*=7, *P*<0.01). Moreover, incubation of the preparation in 30 μmol/L BAPTA-AM for 60–80 min eliminated almost all sIPSCs (data not shown). These results indicate that GABAergic synaptic transmission onto mechanically isolated SNc DA neurons is controlled by presynaptic voltage-gated sodium and calcium channels and also by extra- and intra-terminal Ca^{2+}.

Nicotinic modulation of sIPSCs on SNc DA neurons Next, we examined the effect of nicotine on sIPSCs in SNc DA neurons. As illustrated in Figure 3C, nicotine significantly increased sIPSC frequency. On average, 1 μmol/L nicotine increased sIPSC frequency by 69%±13% (from 1.2±0.2 Hz to 1.9±0.2 Hz, *n*=5, *P*<0.01, Figure 3F). Furthermore, 1 μmol/L nicotine enhanced sIPSC amplitude by 42%±16% (from 29±4 pA to 39±2 pA, *n*=5, *P*<0.05, Figure 3F). In order to determine the subunit composition of the nAChRs that mediated nicotinic modulation of sIPSCs, we tested the modulation of sIPSCs by subtype-selective nAChR agonists, including choline for α7 nAChRs (Figure 3B), and RJR-2403 for α4β2* nAChRs (Figure 3D). While choline (10 mmol/L) had no significant effect on sIPSCs (frequency: 98%±9% of control; amplitude: 95%±9% of control, *n*=8, *P*>0.05, Figure 3E), RJR-2403 (100 μmol/L) increased both the frequency (by 137%±27%, *n*=7, *P*<0.001) and the amplitude (by 47%±20%, *n*=7, *P*<0.001) (Figure 3G) of sIPSCs. These results suggest that α4β2* nAChRs are the principal subtype of nAChR that mediates nicotine enhancement of sIPSCs.
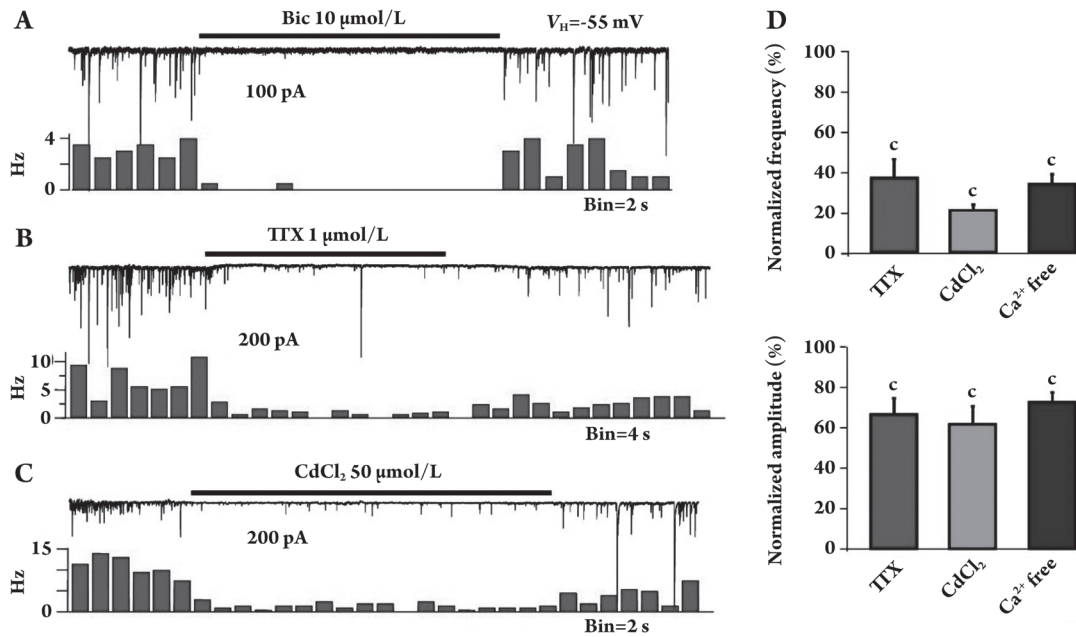
Figure 2. Spontaneous GABA$_A$ receptor-mediated IPSCs (sIPSCs) in DA neurons mechanically dissociated from the SNc. In the presence of 50 μmol/L APV and 20 μmol/L DNQX and at a holding potential (V_H) of -55 mV, sIPSCs were completely but reversibly abolished by bicuculline (Bic, 10 μmol/L) and were reversibly and significantly inhibited by TTX (1 μmol/L) and by CdCl$_2$ (50 μmol/L). Summary data (means±SEM) shows the effects of TTX, CdCl$_2$ and calcium free (Ca^{2+} free) on the frequency (upper panel) and amplitude of sIPSCs (lower panel). $^c P<0.01$ compared to control.
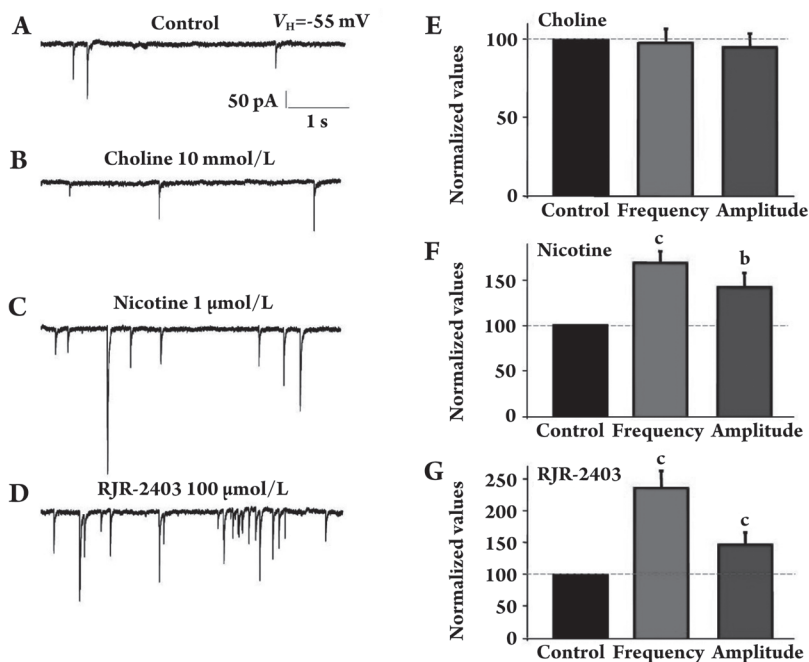
Figure 3. Effects of nicotinic agonists on sIPSCs in SNc DA neurons. Typical traces of sIPSCs recorded under the control condition (A) and in the presence of 10 mmol/L choline (B), 1 μmol/L nicotine (C), and 100 μmol/L RJR-2403 (D). All traces were from the same SNc DA neuron (V_H=-55 mV). E–G: Summary of the effects of choline (E), nicotine (F), and RJR-2403 (G) on both frequency and amplitude of sIPSCs. $^b P<0.05$, $^c P<0.01$ compared to control.

SNc DA neurons receive cholinergic innervation from lateral dorsal tegmentum and peduncular pontine tegmentum[2]. Acetylcholinesterase (AChE) is densely expressed in SNc[33]. In order to determine whether our preparations exhibit tonic ACh release and AChE presence, we tested the effect of eserine (an AChE inhibitor) on sIP-

SCs in the presence of 0.5 μmol/L atropine, an antagonist of muscarinic AChRs. Under these experimental conditions, eserine (3 μmol/L) significantly increased sIPSC frequency, by 70%±17% (from 0.83±0.21 Hz to 1.28±0.25 Hz, *n*=5, *P*=0.004) but did not significantly alter sIPSC amplitude (by −11%±7%, from 58±19 pA to 51±12 pA, *n*=5, *P*>0.05) (Figure 4A, 4B). This result supports the idea that the mechanically dissociated SNc DA neurons contain attached cholinergic nerve terminals. Furthermore, these cholinergic terminals appear to tonically release ACh, which modulates GABA release onto SNc neurons.

Subtypes of nAChRs in GABAergic terminals synapsing onto SNc DA neurons In order to address the question of whether tonically released ACh in our preparations can activate presynaptic nAChRs, we determined the effects of nAChR antagonists on sIPSCs. Perfusion of α-bungarotoxin (α-BTX, 300 nmol/L, Figure 5B), a specific α7 nAChR antagonist, did not significantly alter sIPSC frequency (95%±6% of control, from 0.77±0.17 Hz to 0.67±0.14 Hz, *n*=7, *P*=0.21, Figure 5F, 5G), whereas a 5-min incubation with mecamylamine hydrochloride (MEC, 10 μmol/L, Figure 5D), a broad spectrum nAChR antagonist, significantly depressed sIPSC frequency (by 42%±5%, from 1.53±0.40 Hz to 0.90±0.27 Hz, *n*=6, *P*=0.02, Figure 5F, 5G). Similarly, the application of dihydro-β-erythroidine hydrobromide (DHβE, 100 nmol/L, Figure 5C), an antagonist for α4β2* nAChRs, decreased sIPSC frequency (by 39%±4%, from 1.79±0.40 Hz to 1.06±0.22 Hz, *n*=6, *P*=0.006, Figure 5F,

5G). These data indicate that the principal subtypes of nAChRs that mediate the endogenous ACh modulation of sIPSCs contain α4β2 subunits.

Discussion

Mechanically dissociated neuronal preparations from many brain regions have been used to study the modulation of spontaneous postsynaptic events in hippocampus, VTA, SNc, periaqueductal gray, and other areas of the brain[22–28, 30]. Although these preparation give the appearance of single neurons, they appear to contain attached synaptic boutons, where this interpretation is supported by the existence of vesicles and pre- and post-synaptic structures that are revealed, respectively, by FM1-43 staining and electron microscopy[22, 27]. Interestingly, this preparation retains the machinery that controls neurotransmitter release, including voltage-gated sodium and calcium channels, many types of presynaptic receptors, and the potential to undergo calcium elevation[22–28, 30]. Consistent with these findings, we observed that TTX, Cd^{2+}, and elimination of extracellular calcium reduce sIPSC frequency in mechanically dissociated SNc DA neurons, and that chelation of calcium in pre- and post-synaptic structures using BAPTA-AM eliminates the sIPSCs. It is noteworthy that the amplitude of sIPSCs recorded in mechanically dissociated DA neurons usually reaches tens to 200 pA, a range of values that is many times larger than single vesicle GABA release-induced $GABA_A$
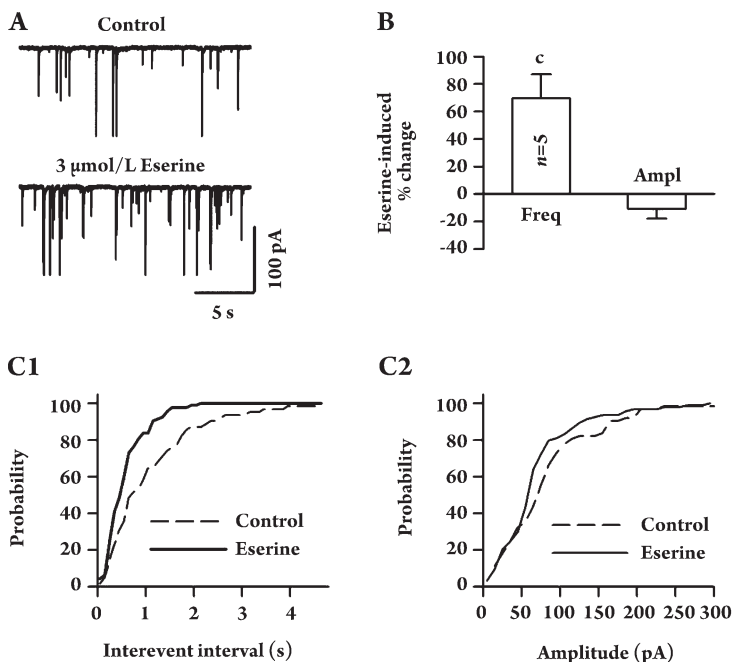

Figure 4. Eserine enhances sIPSCs in SNc DA neurons. A-B, Eserine increases the frequency (Freq), but not amplitude (Ampl) of sIPSCs (A, typical traces; B, summary). C1 and C2, Eserine causes a leftward shift of the cumulative probability of interevent intervals of two consecutive sIPSCs (C1, K–S test, *P*<0.001), but not that of sIPSC amplitude (C2, K–S test, *P*= 0.13).
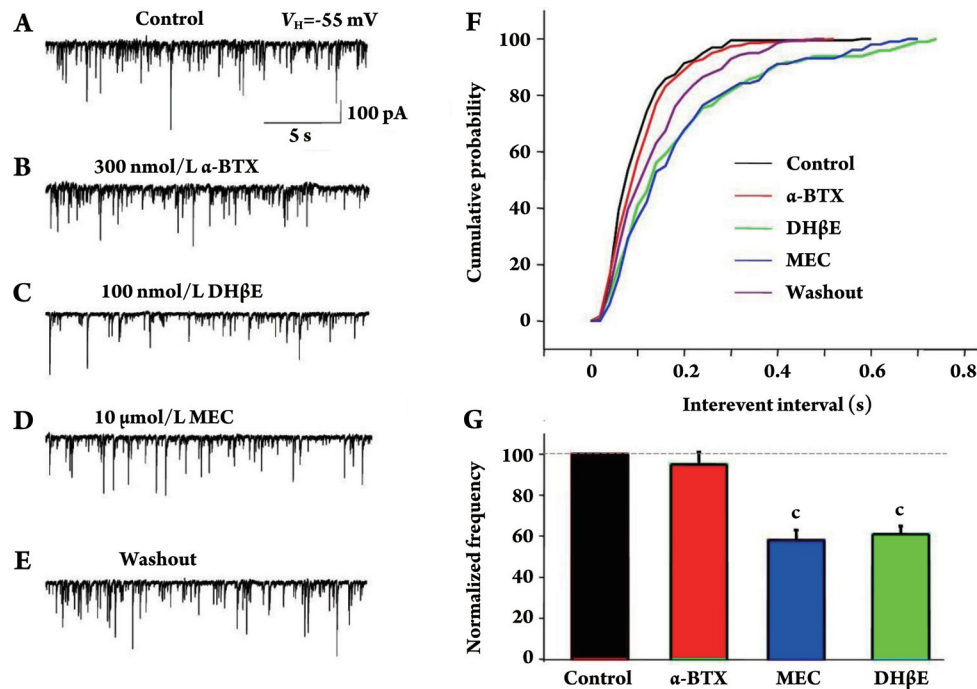
Figure 5. Effects of nicotinic antagonists on sIPSCs in SNc DA neurons. Representative traces of sIPSCs recorded under control conditions (A), in the presence of 300 nmol/L α-BTX (B), 100 nmol/L DHβE (C), 10 μmol/L MEC (D), and after 10 min washout (E) with the standard external solution. All traces were from the same DA neuron (V_H=-55 mV). F: Cumulative probability plots show that DHβE and MEC significantly increased incidence of long sIPSC interevent intervals (K–S test, $P<0.001$), but that α-BTX did not significantly alter the distribution of sIPSC interevent intervals (K–S test, $P>0.05$). G: Summary of the effects of α-BTX, DHβE, MEC on the frequency of sIPSCs. $^c P<0.01$ compared to control.

receptor current, which has a magnitude of several pA. This suggests that synchronized release of GABA is common in GABAergic terminals which make synapses onto SNc DA neurons. This interpretation is supported by the fact that TTX, Cd^{2+}, and elimination of extracellular calcium all reduced sIPSC amplitude.

We showed that nicotine enhanced both the frequency and amplitude of sIPSCs in SNc DA neurons. The enhancement of sIPSCs by nicotine in our preparation suggests that the GABAergic terminals innervating the SNc DA neurons possess presynaptic nAChRs and that activation of these receptors can increase both the frequency and synchrony of GABA release. Further experiments examining nicotine's effects on the desynchronized GABA release are warranted in order to clarify this possibility. Surprisingly, the elevation of endogenous ACh by 3 μmol/L eserine enhanced sIPSC frequency as much as did 1 μmol/L nicotine (Figure 3F and Figure 4B), while it did not change sIPSC amplitude. This suggests that nicotine, rather than ACh, which predominantly activates nAChRs in the presence of 0.5 μmol/L atropine, increases the probability of synchronization in GABA release. It is worth noting that, unlike nicotine, ACh is not

permeable to the membrane[34]. We postulate that intra-terminal nicotine affects the synchrony of GABA release, leading to larger amplitude of sIPSCs.

Our pharmacological examinations in mechanically dissociated SNc DA neurons showed that DHβE and MEC, but not α-bungarotoxin, reduce the frequency of sIPSCs in these neurons and that RJR-2403, but not choline, increases the frequency of sIPSCs. This suggests, first, that there is tonic ACh released from cholinergic terminals attached to the isolated SNc DA neurons; second, that the ACh concentration achieved by this release is sufficient to activate the presynaptic nAChRs; and third, that α4β2* nAChRs may be the principal type of nAChR located on the GABAergic terminals. The GABAergic innervation to SNc DA neurons originates from SNr, dorsal striatum, and globus pallidus; however, functional α4β2* nAChRs were detected only in SNr GABAergic neurons[4, 10], while high-affinity nicotine binding and α4 subunit expression are detectable only in the neuronal processes of the dorsal striatum[10, 21]. Therefore, in the substantia nigra, nicotinic modulation of GABAergic tone to SNc DA neurons may be primarily mediated by α4β2* nAChRs in SNr GABAergic neurons.

Author contribution

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