Differential effects of nicotine on the activity of substantia nigra and ventral tegmental area dopaminergic neurons *in vitro*

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Abstract. Despite resembling each other in many respects, dopaminergic neurons of the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) exhibit dissimilar responses to nicotine *in vivo*. To investigate this in an *in vitro* model, the acute effects of nicotine on the firing of SNc and VTA neurons were compared in transverse juvenile rat midbrain sections (300-350 μm) using extracellular recording. Levels of nicotine comparable with those encountered in smokers (0.2-1.0 μM, 3 min) not only increased firing rate, but also evoked prolonged irregular firing, as indicated by the increase in the coefficient of variation of discharge frequencies. Pre- and postsynaptic nicotinic cholinergic receptors (nAChRs) were involved, as both effects persisted, although at an attenuated level, in low Ca^{2+}/high Mg^{2+}. Only the nicotine-induced elevation of firing rate was sensitive to the glutamate receptor antagonists APV and CNQX, implying that enhanced glutamate release and glutamate receptor activation are involved in the effects of nicotine on discharge frequency but not pattern. Furthermore, nicotine (1.0 μM) exerted a greater increase in the firing frequency of VTA neurons relative to SNc neurons, suggesting that the differential effects on the two populations previously reported *in vivo* were due to a difference in the postsynaptic nAChR response and/or local synaptic circuits. Low concentrations of nicotine can thus profoundly modulate the activity of dopaminergic mesencephalic neurons through a local action within the ventral midbrain *in vitro*, and, similarly to *in vivo* conditions, evoke stronger effects in the VTA.

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INTRODUCTION

The precise function of nicotinic cholinergic receptors (nAChRs) in the CNS remains unclear, despite their presence in a number of brain regions. High expression levels of these receptors have been detected in the rat substantia nigra pars compacta (SNc), ventral tegmental area (VTA), and their striatal terminal fields *via* autoradiography (Clarke and Pert 1985), immunohistochemistry (Arroyo-Jimenez et al. 1999, Goldner et al. 1997, Sorenson et al. 1998), RT-PCR (Charpantier et al. 1998, Klink et al. 2001) and *in situ* hybridization (Wada et al. 1989). The localization of acetylcholinesterase, choline acetyltransferase (ChAT) (Butcher and Marchand 1978, Henderson and Sherriff 1991), ChAT-immunoreactive fibres (Bolam et al. 1991, Henderson and Sherriff 1991, Martinez-Murillo et al. 1989) and acetylcholine (ACh) (Jacobowitz and Goldberg 1977) in the SNc and VTA, together with the demonstration of high-affinity Na^+-dependent $[^3H]$-choline uptake and Ca^{2+}-dependent release of $[^3H]$-ACh in rat SN tissue (Massey and James 1978), support the role of ACh as an endogenous neurotransmitter in the ventral mesencephalic region. Cholinergic input to the SNc originates predominantly from the ipsilateral pedunculopontine tegmental nucleus (Blaha and Winn 1993, Lavoie and Parent 1994, Oakman et al. 1995), whereas the VTA receives bilateral input primarily from the laterodorsal tegmental nucleus (Blaha et al. 1996, Oakman et al. 1995).

Activation of nAChRs increases the activity of SNc and VTA dopaminergic neurons *in vivo* (Fa et al. 2000, Grenhoff et al. 1986, Mereu et al. 1987) and *in vitro* (Calabresi et al. 1989, Grillner and Svensson 2000, Pidoplichko et al. 1997, Sorenson et al. 1998, Yin and French 2000), and enhances dopamine release from striatal nerve terminals (Blaha and Winn 1993, Blaha et al. 1996, Nisell et al. 1994a,b). Although both are dopaminergic, the SNc and VTA mediate distinct functions (see Discussion) and may exhibit different responses to nicotine, as has been suggested previously (Fa et al. 2000, Grenhoff et al. 1986, Imperato et al. 1986, Mereu et al. 1987). However, whether a true difference exists between SNc and VTA neurons in their response to this drug is unclear. To date, there have only been a few studies directly comparing the effects of nicotine on firing of these neurons *in vivo* (Fa et al. 2000, Grenhoff et al. 1986, Mereu et al. 1987), which have reported a differential response of the two neuronal pools to systemic application of nicotine. This may, however, reflect differences in afferent inputs from regions outside the ventral midbrain, rather than in local pre- and/or postsynaptic responses. Thus, the aim of the present study was to compare the acute response of SNc and VTA dopaminergic neurons in brain slices to nicotine using extracellular recording. This allowed us to study the direct effects of nicotine on local ventral mesencephalic structures within the slice. In addition to discharge frequency, the effect on firing pattern was also examined, as both parameters are important determinants of dopamine release from striatal terminals (Gonon 1988, Gonon and Buda 1985, Suaud-Chagny et al. 1992).

METHODS

Experiments were conducted on juvenile Wistar rats and were approved by the University of Auckland Animal Ethics Committee. All measures were taken to minimize any pain or discomfort experienced by the animals.

Retrograde labeling of SNc and VTA neurons

Rat pups (P12-14) were anaesthetized with 0.042 mg g^{-1} ketamine/0.05 mg g^{-1} xylazine (i.m.) and mounted onto a stereotaxic frame. SNc and VTA neurons were labeled by bilateral injection of 0.5 μl Fluoro-Gold (0.4% in H_2O; Fluorochrome Inc.) into the dorsal and ventral striatum at the following respective coordinates: level of bregma, 3 mm lateral from midline, 3.5 mm and 4.5 mm deep from cortical surface. As Fluoro-Gold has been observed to diffuse great distances from the injection site (>2 mm, even at 2-3 days after injection), only dorsal striatal applications were made in some animals (Fig. 1A,B). After recovery, pups were returned to lactating females.

Preparation of midbrain slices

Following a minimum 3-day post-injection period, P16-P25 rats (33-72 g) were anaesthetized by CO_2 inhalation and decapitated. The brain was rapidly removed and immersed in a semi-frozen solution containing (in mM): sucrose 240, KCl 3, $MgSO_4$ 1.3, NaH_2PO_4 2.5, $NaHCO_3$ 26, $CaCl_2$ 0.1, glucose 10; bubbled with 95% O_2/5% CO_2. Transverse sections (300-350 μm) containing the SNc and VTA were cut with a Vibratome. The sections were incubated in a chamber filled with carbogenated artificial cerebrospinal fluid (ACSF) at
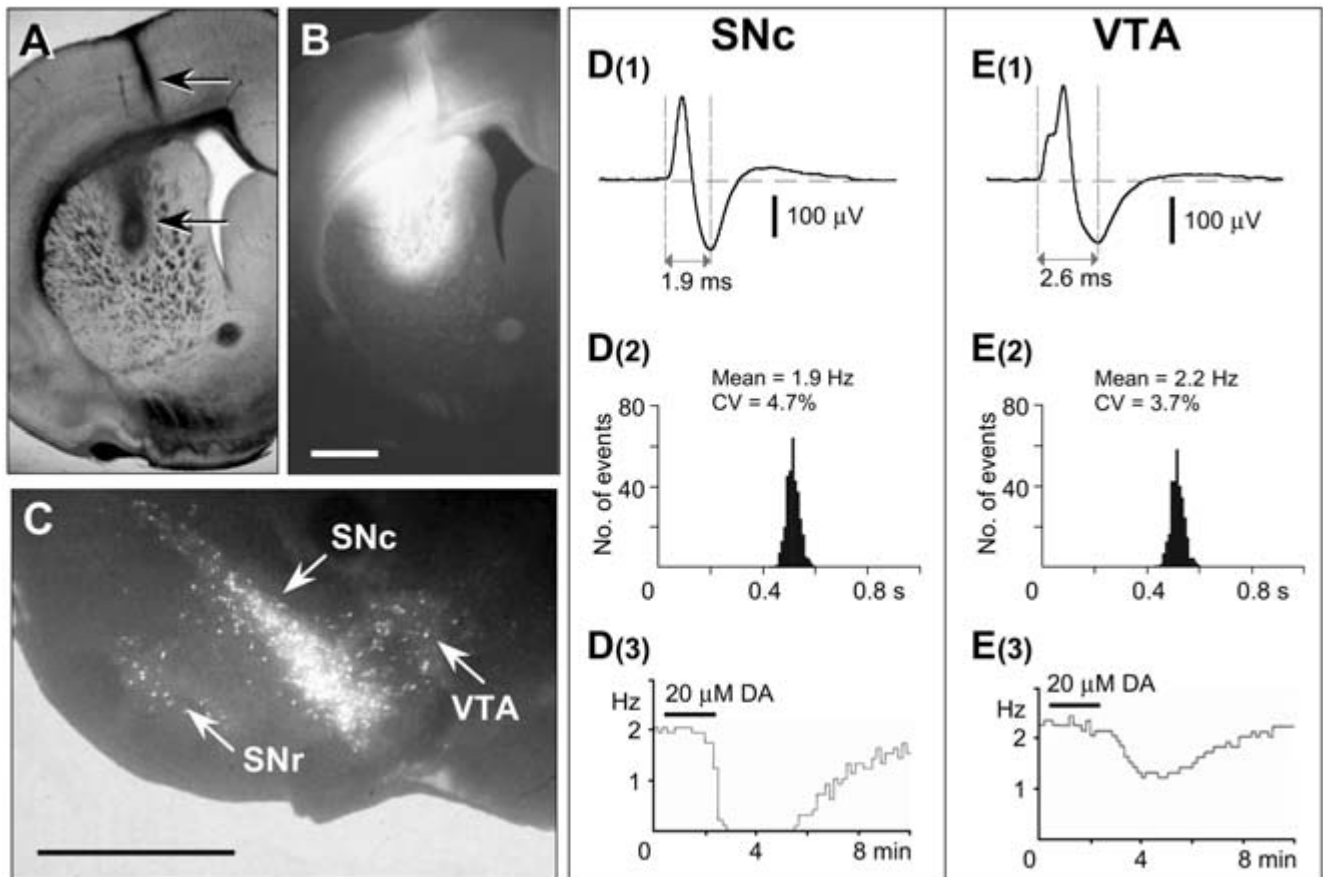
Fig. 1. Identification of SNc and VTA dopaminergic neurons. (A) Transverse section (150 µm) of the rat forebrain viewed under brightfield illumination. The needle track (Fluoro-Gold injection) is marked by arrows. (B) The same section viewed under epifluorescence, showing a deposit of Fluoro-Gold 3 days after striatal injection. Scale bar = 1 mm. (C) Ventral aspect of one half of a 300 µm-thick transverse section of the midbrain viewed under both transillumination and epifluorescence, with SNc and VTA neurons labeled with Fluoro-Gold. Labeling of the substantia nigra pars reticulata (SNr) is also evident (as scatter of labeled cells ventrolateral to the SNc). Scale bar = 1 mm. Firing of an SNc (D) and a VTA (E) neuron, observed during simultaneous extracellular recording from both neurons. (D1), (E1) Action potential waveforms. Spike duration measured as shown. (D2), (E2) Time interval histograms (300 consecutive spikes in each). The high regularity of firing is indicated by the low CV values and normal distribution of interspike intervals. (D3), (E3) Inhibition of firing by 20 µM dopamine (DA).

33°C for 45 min, then at room temperature until use. The ACSF contained (in mM): NaCl 126, NaHCO$_3$ 26, KCl 3, CaCl$_2$ 2.6, NaH$_2$PO$_4$ 2.5, MgSO$_4$ 1.3, glucose 20.

Extracellular recording

Slices were transferred to a recording chamber (RC-27L/M, Warner Instr.) mounted on an upright microscope (Eclipse E600FN, Nikon), equipped with a fluorescence attachment (filter block UV-2A, Nikon). The slice, maintained in between two meshes of Lycra threads, was continuously perfused from above and below with carbogenated ACSF (approx. 2.5 ml min^{-1}, 30°C). Retrogradely labeled striatum-projecting SNc and VTA neurons were identified under epifluorescence (Fig. 1C).

Extracellular recordings were made with glass microelectrodes (1.5-4 MΩ) filled with 0.02% Lucifer Yellow in a solution containing (in mM): NaCl 145, KCl 3, CaCl$_2$ 2.5, MgCl$_2$ 1, HEPES 10, glucose 15. Lucifer Yellow assisted with the directing of microelectrodes into retrogradely labeled regions under fluorescent illumination. Recordings were made from one of the two regions at a time, or simultaneously from both (Werkman et al. 2001). Extracellular signals were amplified (NL104, Digitimer Ltd.), filtered (70 Hz - 3 kHz), displayed on an oscilloscope, and saved on a VCR recorder (Vetter 3000) for off-line analysis. Action potentials (APs) were dis-

criminated with a custom-designed time-amplitude window discriminator. Interspike interval histograms, mean firing rate and coefficient of variation (CV = standard deviation of firing frequency/mean firing frequency × 100) were calculated from 200-500 successive APs using LabView (National Instr.). A digital frequency meter was used for calculation of the number of spikes within consecutive time intervals (bins). Spike waveforms were displayed on a PC (pCLAMP software, Axon Instr.).

Nicotine was applied through bath perfusion for 3 min, and effects on neuronal firing rate and pattern were monitored over a minimum period of 20 min. Measurements were made from periods in which the firing rate had stabilized, and from equivalent numbers of spikes in pre- and post-nicotine epochs. Regular firing was characterized by CV values <10%, while irregular firing was defined by CV values >10%. For some neurons, the irregular firing observed following nicotine qualitatively resembled a bursting pattern, defined as trains of 2-12 spikes fired in rapid succession that exhibit progressively decreasing amplitudes and increasing interspike intervals, with each burst separated by a period of quiescence (Diana and Tepper 2002). However, specific burst parameters were not quantified in the present study; hence, such neurons were described as "burst-like" rather than exhibiting a true burst pattern. For antagonist studies, slices were pre-treated with mecamylamine or hexamethonium for 3 min, then exposed to a solution containing nicotine and the appropriate antagonist for a further 3 min. To investigate the role of glutamate in nicotine-mediated effects, slices were treated with the NMDA receptor (NMDAR) antagonist APV or the non-NMDAR antagonist CNQX for 3 min after nicotine-mediated effects had developed. To assess the contributions of pre- and postsynaptic nAChRs to nicotinic effects, slices were perfused with low Ca^{2+} (0.2 mM)/high Mg^{2+} (3.7 mM) ACSF for 20-25 min, then exposed to nicotine (1 μM in low Ca^{2+}/high Mg^{2+} ACSF) for 3 min. In general, only one neuron per slice was tested and each slice was exposed to nicotine only once.

Reagents

DL-2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), hexamethonium bromide, Lucifer Yellow, mecamylamine hydrochloride and (-)-nicotine (+)-bitartrate were purchased from Sigma.

Data analysis

All data are presented as mean ± SEM. Effects of nicotine on firing rate and CV are reported as percentage changes from control levels. Statistical comparisons were made using Student's unpaired t-test (Bonferroni-protected where necessary). Modulation of nicotinic effects by APV or CNQX was examined by comparing neuronal firing rate and CV before and after APV/CNQX application with Student's paired t-test. For all tests, a criterion of two tailed $P<0.05$ was considered significant.

RESULTS

Identification of SNc and VTA neurons

Identification of SNc/VTA dopaminergic neurons was based on the following criteria: (i) localization within Fluoro-Gold-labeled regions (Fig. 1C); (ii) triphasic (positive-negative-positive) spike waveform, often with an inflection on the rising phase (Fig. 1D, E); (iii) spike duration ≥1.5 ms (defined as the time between onset of the rising positive phase and peak of the negative phase); (iv) slow and regular firing (≤6 Hz, CV <10.0%); and (v) inhibition of firing rate by dopamine (20 μM, 1-2 min) (Fig. 1D,E) (Diana and Tepper 2002, Wooltorton et al. 2003, Yin and French 2000).

SNc neurons ($n = 66$) exhibited a mean firing rate of 2.4 ± 0.1 Hz, mean CV of 4.7 ± 0.2% and mean AP duration of 1.8 ± 0.1 ms. The corresponding values for VTA dopaminergic neurons ($n = 58$) were 2.2 ± 0.1 Hz, 5.1 ± 0.3% and 2.0 ± 0.1 ms. While there were no significant differences between SNc and VTA neurons in their basal discharge frequency or CV, VTA neurons displayed significantly longer spike durations ($P<0.05$). An example of simultaneous recording from a SNc neuron and a VTA neuron is shown in Fig. 1D (SNc) and E (VTA).

Effects of nicotine

Both SNc and VTA neurons responded to nicotine with increased firing rate (Fig. 2A, D). On average, 0.2 μM and 1.0 μM nicotine raised the firing frequency of SNc neurons by 25.0 ± 11.8% ($n = 14$) and 20.1 ± 6.1% ($n = 19$), respectively, with no significant difference between the responses evoked with the two concentrations (Fig. 2D). The effect lasted for 7-10 min with a return to pre-nicotine levels in most neurons (88%), but persisted
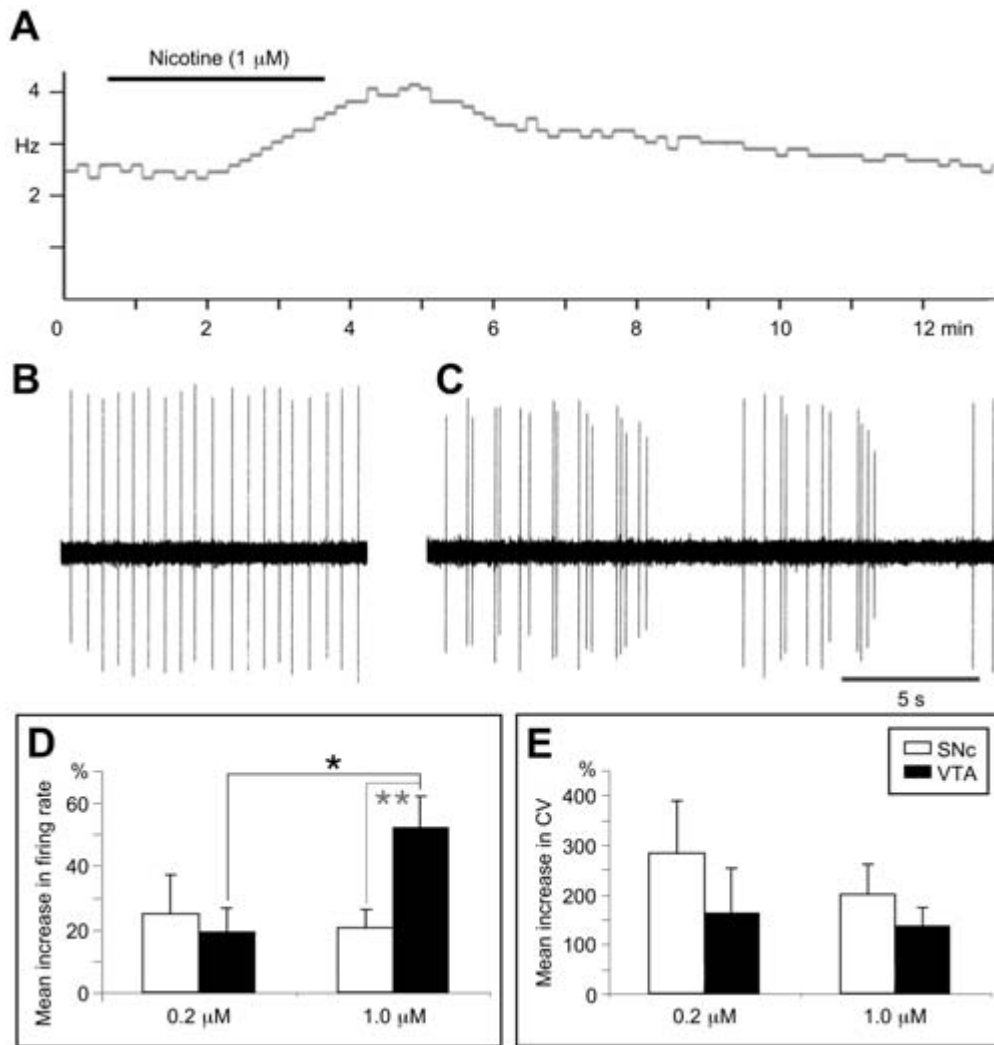
Fig. 2. Acute responses of SNc and VTA neurons to nicotine. (A) Increase in firing frequency observed in an SNc neuron (1 μM nicotine); control firing prior to nicotine application (B) and development of irregular burst-like firing in a VTA neuron following 0.2 μM nicotine (C); (D) mean effects of nicotine on firing rate; (E) mean effects of nicotine on firing regularity. Data presented as mean ± SEM (significant difference: (*) $P<0.05$, (**) $P<0.01$; Bonferroni-protected unpaired student's t-test).

throughout the duration of recording (20 min after nicotine application) in the remaining cells. For VTA neurons, 1.0 μM nicotine increased discharge frequency by an average of $52.1 \pm 9.9\%$ ($n = 21$), which was significantly greater ($P<0.05$) than the $18.9 \pm 7.7\%$ ($n = 13$) increase observed after 0.2 μM (Fig. 2D). The effect was more prolonged, with recovery to pre-nicotine levels observed in only 50% of neurons (within 7-13 min) but persisting throughout the duration of recording for the remaining cells. Relative to the SNc, 1.0 μM nicotine elicited a significantly larger increase in discharge frequency of VTA neurons ($P<0.01$), although both populations exhibited comparable responses to the 0.2 μM concentration (Fig. 2D).

Nicotine also caused dopaminergic neurons to fire less regularly, as evident in the large increases of CV. At 0.2 μM and 1.0 μM, nicotine raised the CV of SNc neuron firing by an average of $281.5 \pm 108.5\%$ ($n = 14$) and $199.1 \pm 60.6\%$ ($n = 19$), respectively, while the corresponding values for VTA neurons were $162.0 \pm 92.1\%$ ($n = 13$) and $136.4 \pm 36.8\%$ ($n = 21$). No significant concentration-dependence was evident in either population, although responses evoked by the lower nicotine concentration appeared to be greater (Fig. 2E). The effect on firing regularity was prolonged, persisting throughout the duration of recording in >90% of SNc and VTA neurons at both concentrations used. In some neurons, a burst-like discharge pattern developed (25%

of SNc neurons and 22% of VTA neurons after 0.2 μM nicotine; 8% of SNc neurons after 1.0 μM nicotine) (Fig. 2B,C). Furthermore, many neurons (33% of SNc neurons and 33% of VTA neurons after 0.2 μM nicotine; 46% of SNc neurons and 6% of VTA neurons after 1.0 μM nicotine) displayed a progressive exacerbation of irregularity followed by complete arrest of firing. There were no significant differences between SNc and VTA neurons with respect to the effect of nicotine on firing regularity, although the increase in CV appeared to be greater in SNc neurons (Fig. 2E).

The effects of nicotine were strongly reduced by the non-competitive nAChR antagonists mecamylamine and hexamethonium (Fig. 3). In the presence of 5 μM mecamylamine, 1 μM nicotine increased the firing rate of SNc and VTA neurons by $1.1 \pm 2.7\%$ ($n = 10$) and $5.4 \pm 3.1\%$ ($n = 13$) respectively, and elicited $6.5 \pm 8.0\%$ ($n = 10$) and $20.8 \pm 12.6\%$ ($n = 13$) increases in CV of SNc and VTA neurons, respectively. These responses were

significantly smaller than those induced by nicotine alone ($P<0.05$). An attenuation of nicotine effects by 50 μM hexamethonium was also observed. Mecamylamine or hexamethonium alone caused a slight transient decrease in firing rate, with no effect on firing pattern (data not shown).

Effects of nicotine in low Ca^{2+}/high Mg^{2+} conditions

To assess and compare the role of pre- vs. postsynaptic nAChRs in nicotine-induced effects in SNc and VTA neurons, recordings were performed in low Ca^{2+}/high Mg^{2+}. Nicotine (1.0 μM) was still efficacious under this condition, but the responses were attenuated. SNc and VTA neurons exhibited mean increases of discharge frequency of $11.5 \pm 6.8\%$ ($n = 10$) and $10.7 \pm 7.2\%$ ($n = 7$), respectively, which was significantly reduced relative to responses observed in ACSF ($P<0.05$)
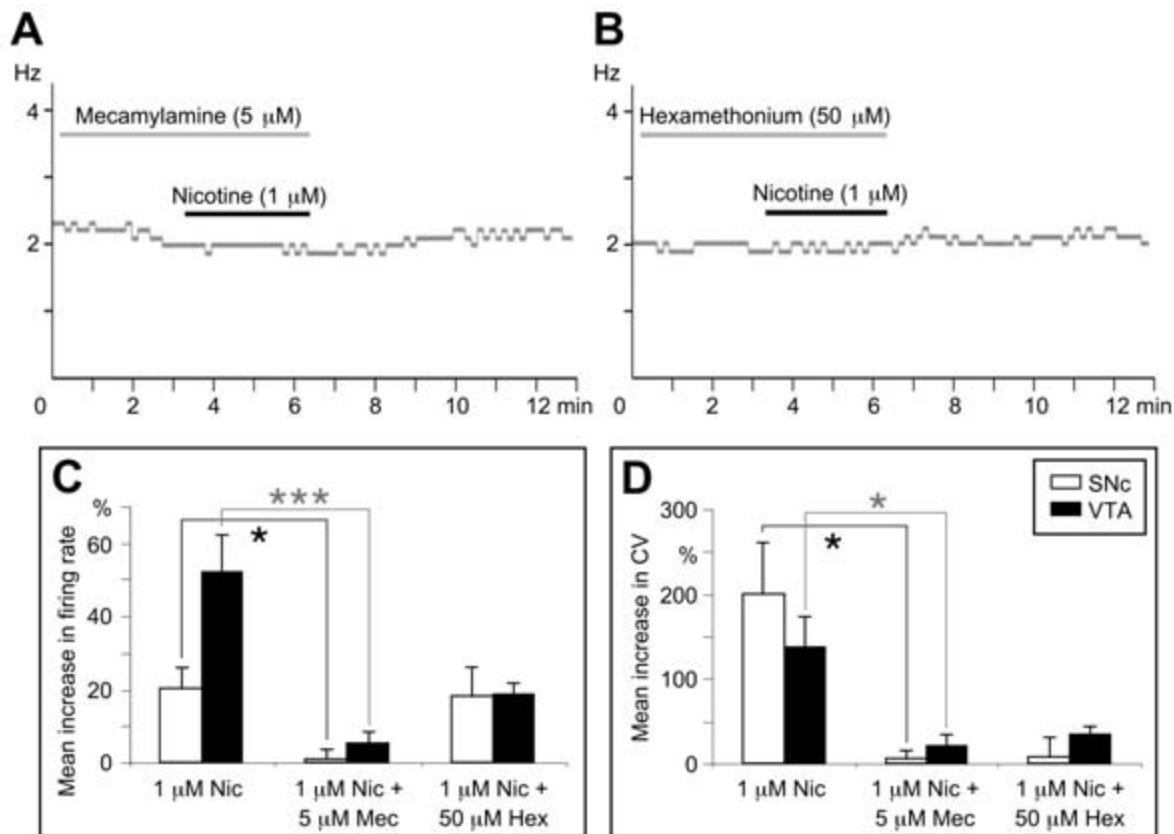

Fig. 3. Antagonism of nicotine effects by mecamylamine and hexamethonium. (A) Recording from an SNc neuron, demonstrating the lack of effect of 1 μM nicotine in the presence of 5 μM mecamylamine; (B) similar effect observed in another SNc neuron in the presence of 50 μM hexamethonium; (C) mean effect of nicotine on firing rate in the presence of antagonists; (D) mean effect of nicotine on firing regularity in the presence of antagonists. Data presented as mean ± SEM (significant difference: (*) $P<0.05$, (***) $P<0.001$; Bonferroni-protected unpaired student's t-test).

Table I

Modulation of the effects of nicotine on SNc and VTA neurons by APV and CNQX

	SNc		VTA	
	Mean firing rate (Hz)	Mean CV (%)	Mean firing rate (Hz)	Mean CV (%)
1 μM nicotine	3.0 ± 0.3 (*n* = 4)	6.6 ± 1.0 (*n* = 4)	3.6 ± 0.8 (*n* = 4)	6.5 ± 2.0 (*n* = 4)
50 μM APV	2.5 ± 0.2 (*n* = 4)**	5.1 ± 0.7 (*n* = 4)	2.7 ± 0.6 (*n* = 4)*	7.7 ± 3.0 (*n* = 4)
1 μM nicotine	3.6 ± 0.7 (*n* = 4)	5.4 ± 1.8 (*n* = 4)	3.2 ± 0.3 (*n* = 4)	5.6 ± 2.1 (*n* = 4)
10 μM CNQX	2.9 ± 0.6 (*n* = 4)*	5.5 ± 1.8 (*n* = 4)	2.2 ± 0.3 (*n* = 4)*	5.8 ± 2.2 (*n* = 4)

APV or CNQX was applied after the response to nicotine had developed. All data presented as mean ± SEM. Significantly different from the response to nicotine alone, (*) $P<0.05$ or (**) $P<0.01$, student's paired t-test.

for VTA, but not for SNc neurons. Effects on firing regularity were smaller in low Ca^{2+}/high Mg^{2+}, with SNc and VTA neurons demonstrating a 43.9 ± 26.7% (*n* = 10) and 60.2 ± 48.1% (*n* = 7) mean increase in CV, respectively. No significant differences existed between SNc and VTA neurons with respect to the nicotine-mediated elevation of firing rate and CV in low Ca^{2+}/high Mg^{2+}. Interestingly, low Ca^{2+}/high Mg^{2+} alone induced faster and more irregular firing, causing mean increases in firing rate and CV of 62.1 ± 23.6% and 108.0 ± 25.7% for SNc neurons (*n* = 10), and 38.6 ± 12.2% and 148.7 ± 47.7% for VTA neurons (*n* = 7), respectively.

Effects of APV and CNQX on nicotine effects

The ability of APV (50 μM) and CNQX (10 μM) to reverse the elevation of firing frequency and irregularity following nicotine (1 μM) was investigated (Table I). Both APV and CNQX caused a significant decrease in firing rate ($P<0.05$), but had no significant effect on CV of SNc (*n* = 4) and VTA (*n* = 4) neurons.

DISCUSSION

In this study, extracellular recordings were made to compare the effects of nicotine on SNc and VTA neurons. This is of interest not only because these two regions - which resemble each other in many respects (see Diana and Tepper 2002) - mediate distinct functions, but also because of the way these functions may be modulated by nicotine. While the SNc constitutes the nigrostriatal system that is primarily involved in voluntary motor control, the VTA forms the mesocorticolimbic system that mediates the reinforcing effects of natural rewards and most addictive drugs (Dani and Heinemann 1996, Diana and Tepper 2002, Nestler 1992). Although comparisons between the actions of nicotine on the two midbrain regions have been made previously in rats *in vivo* (Fa et al. 2000, Grenhoff et al. 1986, Mereu et al. 1987), this is the first study to compare the effects of the drug on the firing of the two neuronal pools in brain slices. The employment of multiple criteria for neuron identification ensured that only dopaminergic neurons were examined. In addition, our experiments were conducted on juvenile, rather than adult, brain tissue. The significance of this will be discussed later.

Effect of nicotine on neuronal firing rate

The excitatory effects of nicotine on dopaminergic neuron firing rate have been described, both *in vivo* (Fa et al. 2000, Grenhoff et al. 1986, Mereu et al. 1987) and *in vitro* (Calabresi et al. 1989, Sorenson et al. 1998, Yin and French 2000). However, the underlying mechanisms remain unclear. Nicotine has been postulated to increase firing frequency by depolarizing neurons in two ways: (i) by a direct postsynaptic action on nAChRs in the somatodendritic region of dopaminergic neurons, as suggested by the *in vitro* detection of nAChR-mediated inward currents (Klink et al. 2001, Picciotto et al. 1998, Pidoplichko et al. 1997) and neuronal depolarization in the presence of TTX and Co^{2+} (Calabresi et al. 1989); or (ii) by activation of presynaptic nAChRs on glutamatergic afferents and glutamate release, as supported by the attenuation or reversal of nicotinic effects by glutamate receptor antagonists (Erhardt et al. 2001). Our data implicate both direct and indirect mechanisms, as demonstrated by the persistence – although at an at-

tenuated level – of nicotinic effects when presynaptic transmitter release was eliminated in low Ca^{2+}/high Mg^{2+} conditions. Furthermore, the partial inhibition by APV and CNQX corroborates a role of glutamate in the nicotine-induced elevation of firing rate, although the inability of either drug to completely reverse these effects suggests that other mechanisms are also operative.

Our *in vitro* results demonstrated a larger increase of VTA neuronal firing rate relative to SNc neurons. Previous *in vivo* studies have reported a greater increase in firing rate and burst discharge of VTA neurons (Fa et al. 2000, Grenhoff et al. 1986, Mereu et al. 1987), as well as dopamine release and metabolism in the nucleus accumbens (Imperato et al. 1986) following systemic administration of nicotine. However, in these experiments, the site of action of the drug is unclear. The use of midbrain slices restricts the source of difference between SNc/VTA neurons to postsynaptic or presynaptic nAChRs on local synaptic inputs within the slice. The former is implicated by Klink et al. (2001) and Wooltorton et al. (2003), who have recorded larger nAChR-mediated inward whole-cell currents in VTA neurons relative to SNc neurons in midbrain sections following local application of ACh or nicotine. It should be noted that both these studies used higher concentrations of nicotine or ACh (20 μM or 1 μm, respectively) than those employed here, which makes a comparison difficult. In addition, these previous studies recorded only whole-cell currents, rather than cell firing, in response to a rapid, close-cell application of the drug. This method of application results in little receptor desensitization, unlike the more physiological situation where the drug acts over a longer period of time (in smokers, or during bath application of the drug – an approach chosen in our study; see also Pidoplichko et al. 1997). Therefore, our bath perfusion applications of lower drug concentrations correspond more closely to the physiological situation. Nevertheless, these previous results (Klink et al. 2001, Wooltorton et al. 2003) correspond with our data, as larger nicotine-induced currents would lead to greater depolarization and discharge frequency. Moreover, our findings do not necessarily preclude a role of differential innervation of the SNc and VTA. Local afferents may still be intact within sections and may contribute to the nicotine response, as suggested by the lack of difference between the nicotine responses of SNc and VTA neurons in low Ca^{2+}/high Mg^{2+}.

Effect of nicotine on firing pattern

Low doses of nicotine were also observed to cause SNc and VTA neurons to fire more irregularly. This effect was generally prolonged and tended to be inversely related to the concentration of nicotine. Some neurons developed a burst-like firing pattern. Others exhibited an exacerbation of firing irregularity followed by cessation of activity, reminiscent of "depolarization block" of dopaminergic neuronal activity by excitatory agents (Diana and Tepper 2002). However, while the two processes share some common features (e.g., delayed development, associations with burst firing and spike waveform alterations), post-nicotine inhibition was preceded by a gradual decline of firing rate, whereas depolarization block usually follows a progressive elevation of discharge frequency. Further experiments are required to confirm the identity of and mechanism underlying this novel phenomenon.

Nicotine-induced changes in firing pattern have not been previously reported *in vitro*. A possible reason is that firing irregularity is evoked only by low concentrations of the drug, and therefore would have been missed by studies that employed higher doses. Another factor may be the young age of the animals we used. Mereu et al. (1987) have reported that dopaminergic neurons in brain slices from younger (P15-P21) rats exhibited all discharge patterns (pacemaker, irregular, burst firing), whereas those from adult rats (P40-P70) exhibited pacemaker firing only. Although all dopaminergic neurons examined in this study exhibited regular firing (CV ≤10%), our criteria for neuronal identification may have led to the exclusion of irregular or bursting dopaminergic neurons. Furthermore, the same study showed that NMDA-mediated burst firing could be more readily induced in pacemaker neurons from immature rat brain sections than those from adult tissue. Nicotinic effects on dopaminergic neuronal firing pattern *in vivo* are controversial, with various groups reporting no effect (Lichtensteiger et al. 1976), regularization (Lichtensteiger et al. 1982) or burst induction following nicotine (Carlson and Foote 1992, Erhardt et al. 2001, Fa et al. 2000, Grenhoff et al. 1986).

The mechanisms governing the post-nicotine firing irregularity are unknown. It does not appear to be directly related to the increase in firing rate, as suggested by the lack of correlation between the two effects. Kitai et al. (1999) have proposed that the firing patterns of midbrain dopaminergic neurons are part of a continuous spectrum that ranges from single spiking to burst firing,

in which irregular discharge may be a precursor to bursting. Speculating from burst-generating mechanisms, the post-nicotine firing irregularity may be due to NMDAR activation and elevation of intracellular Ca^{2+} levels (Kitai et al. 1999, Overton and Clark 1997). A role of calcium is supported by the demonstration of increased cytosolic Ca^{2+} levels in mouse SNc neurons following nicotine (Tsuneki et al. 2000). Our results indicate that both pre- and postsynaptic mechanisms may be involved, because nicotine-induced irregularity was attenuated in low Ca^{2+}/high Mg^{2+}. However, glutamate release or NMDAR/non-NMDAR activation does not appear to be involved, as suggested by the lack of effect of APV or CNQX on the CV of neuronal firing. On the other hand, if irregular firing results from some event downstream of glutamate receptor activation, post-nicotine application of APV or CNQX could be ineffective. So, at present, the contribution of glutamate to nicotine-induced irregular firing remains unclear.

Time course of nicotine-induced effects

An apparent anomaly is the prolonged time course of nicotinic effects, despite the susceptibility of nAChRs to desensitization. Indeed, responses of dopaminergic neurons to a wide range of nicotine concentrations have been shown to desensitize rapidly (Calabresi et al. 1989, Pidoplichko et al. 1997, Sorenson et al. 1998). A possible explanation is that nAChR activation initiates, but is not required to sustain a long-term process that is therefore insensitive to receptor desensitization. In this regard, Mansvelder et al. (2002) have demonstrated the ability of brief nicotine exposures to induce NMDAR-dependent long-term potentiation of synaptic efficacy in VTA neurons *in vitro*, which may account for the prolonged elevations of firing rate. Dopaminergic neuronal excitation following nicotine may also be sustained by concomitant GABAergic disinhibition, resulting from nicotine-induced desensitization of nAChRs on local GABAergic neurons and attenuation of their response to excitatory cholinergic inputs (Mansvelder et al. 2002).

Functional significance

Dopamine release from nerve terminals is exponentially related to neuronal firing rate (Chergui et al. 1994, Gonon 1988, Gonon and Buda 1985, Suaud-Chagny et al. 1992). In addition, burst firing has been found to be more effective at inducing dopamine release than regular discharge at the same mean frequency (Chergui et al. 1994, Gonon 1988, Gonon and Buda 1985, Suaud-Chagny et al. 1992), while alternation between regular and irregular or burst firing appears to be even more potent (Gonon 1988). The excitatory effects of nicotine on neuronal firing demonstrated in this study would presumably lead to increased dopamine release in terminal regions and dopaminergic neurotransmission. Indeed, administration of nicotine into the SNc and VTA has been shown to enhance dopamine release in the neostriatum and nucleus accumbens, respectively (Blaha and Winn 1993, Blaha et al. 1996, Nisell et al. 1994a,b).

The subsequent activation of the mesocorticolimbic and nigrostriatal dopaminergic systems has multiple functional implications. The enhancement of dopamine release is thought to mediate the reinforcing and addictive properties of nicotine. Our observations of increased firing irregularity in addition to increased firing rate, as well as the prolonged time course of effects after relatively short exposures to nicotine, suggest that the influence of nicotine on dopamine release may have been previously underestimated. Such potent effects of low nicotine concentrations may explain the strong addictive power of the drug, and the persistence of low nicotine levels in smokers may contribute to the long-term rewarding effects resulting from brief exposures. That our data have been obtained from juvenile rat tissue may have particular significance in understanding the high susceptibility of adolescents to nicotine use, addiction and dependence (Woolf 1997). The aetiology underlying the initiation and maintenance of tobacco use among adolescents is complex, and is thought to involve a multitude of social, cultural, behavioural, psychological and economic factors (Kendler et al. 1999, Kaufman et al. 2002, Simon et al. 1995, Tickle et al. 2001). Moreover, diverse genetic/biological predispositions have been reported to affect vulnerability to smoking initiation and smoking behavior, including gender (Zeman et al. 2002), nicotine metabolism/clearance (Pianezza et al. 1998, Zeman et al. 2002), taste sensitivity (Enoch et al. 2001), psychiatric comorbidities (Keuthen et al. 2000), and D2 dopamine receptor gene polymorphism (Spitz et al. 1998). Our observations of a greater nicotinic stimulation of VTA dopaminergic neuron firing rate in juvenile rat brain sections may represent an additional physiological susceptibility factor among adolescents, which may contribute to the epidemic of teenage smoking and the transition to long-term tobacco use.

CONCLUSIONS

Low levels of nicotine, comparable with those encountered in smokers, not only increased the firing rate of SNc and VTA dopaminergic neurons *in vitro*, but also caused cells to fire more irregularly. Although the underlying mechanisms have yet to be elucidated, our data suggest that these effects are mediated by activation of nAChRs located both presynaptically on local afferents innervating SNc/VTA neurons, as well as postsynaptically on the somatodendritic regions of these neurons. Only the nicotine-induced elevation of neuronal firing rate could be reduced by the glutamate receptor antagonists APV and CNQX, indicating a role of glutamate in this component of the response. VTA neurons exhibited a significantly greater elevation of firing rate in response to 1.0 μM nicotine than SNc neurons. Our findings imply that the preferential activation of VTA neurons previously observed *in vivo* may be due, at least in part, to differences in the postsynaptic nAChR response and/or local innervation. Furthermore, the modulation of neuronal firing pattern in addition to discharge frequency implies that nicotine may have an even greater effect on terminal dopamine release than had been previously suspected. The potent action of low concentrations of nicotine on neuronal elements within the ventral midbrain may contribute to the addictive properties of the drug and its prolonged rewarding effects following brief exposures in smokers.

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