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## Nicotine activates and desensitizes midbrain dopamine neurons

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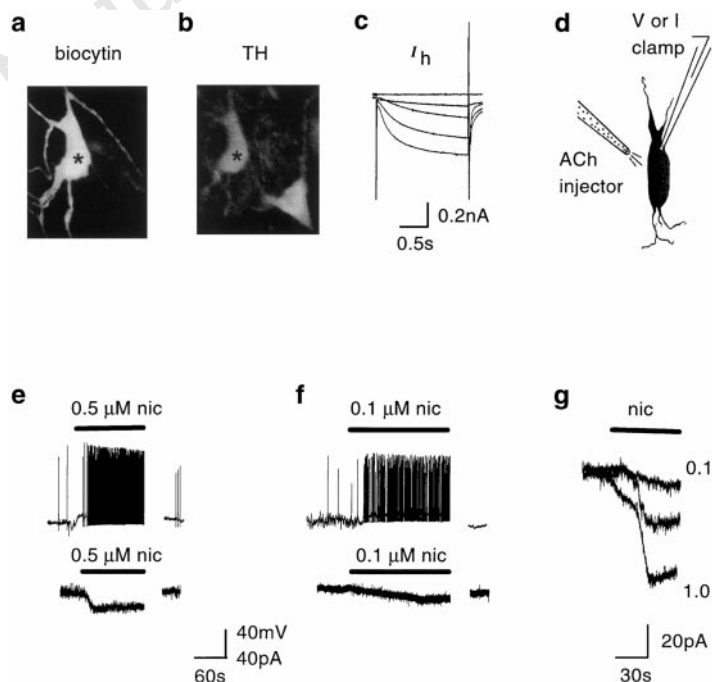
Tobacco use in developed countries is estimated to be the single largest cause of premature death<sup>1</sup>. Nicotine is the primary component of tobacco that drives use, and like other addictive drugs, nicotine reinforces self-administration and place preference in animal studies<sup>2–5</sup>. Midbrain dopamine neurons normally help to shape behaviour by reinforcing biologically rewarding events, but addictive drugs such as cocaine can inappropriately exert a reinforcing influence by acting upon the mesolimbic dopamine system<sup>3–6</sup>. Here we show that the same concentration of nicotine achieved by smokers activates and desensitizes multiple nicotinic receptors thereby regulating the activity of mesolimbic dopamine neurons. Initial application of nicotine can increase the activity of

the dopamine neurons, which could mediate the rewarding aspects of tobacco use. Prolonged exposure to even these low concentrations of nicotine, however, can cause desensitization of the nicotinic receptors, which helps to explain acute tolerance to nicotine's effects. The effects suggest a cellular basis for reports that the first cigarette of the day is the most pleasurable, whereas the effect of subsequent cigarettes may depend on the interplay between activation and desensitization of multiple nicotinic receptors<sup>5</sup>.

A common feature of addictive drugs such as cocaine, amphetamine, morphine and nicotine is that at the doses at which they are self-administered those drugs increase the concentration of dopamine in the nucleus accumbens (NAcc)<sup>3–7</sup>. The main dopaminergic projections to the NAcc arise from ventral tegmental area (VTA) neurons of the mesolimbic dopamine system. *In vivo* studies have shown that nicotine self-administration is reduced by lesions of this mesolimbic pathway or by nicotinic antagonists micro-infused into the VTA<sup>8,9</sup>. Our study explores the cellular basis for these *in vivo* observations.

VTA neurons express nicotinic acetylcholine receptors (nAChRs), as has been shown by autoradiography for ligands and *in situ* hybridization for specific nicotinic subunits<sup>10–12</sup>. Previous work has also shown that high concentrations of nicotine can depolarize and activate dopaminergic neurons<sup>13</sup>. There are no results, however, in the range of nicotine concentrations achieved by smokers. A smoker's level of nicotine reaches about 0.5  $\mu\text{M}$  immediately after smoking a cigarette; 0.1 to 0.5  $\mu\text{M}$  is the general range achieved by smokers, a range that has been shown in animal studies to have addictive power<sup>7–9,14</sup>. We have found that such low levels of nicotine can have multiple effects on VTA dopaminergic neurons.

Figure 1a shows a biocytin back-filled VTA neuron from a rat brain slice, and Fig. 1b shows the same neuron labelled for tyrosine hydroxylase (TH), an enzyme for catecholamine (dopamine) synthesis. That same neuron (Fig. 1c), and all the neurons we studied, displayed an inward cationic current ( $I_h$ ), which is indicative of dopaminergic neurons<sup>15</sup>. Figure 1d illustrates the general experimental setup for whole-cell voltage or current clamping of a VTA neuron from the slice. In current-clamp mode, 0.5 to 0.1  $\mu\text{M}$  nicotine applied to the whole bath depolarized the neuron and caused spontaneous firing of action potentials (Fig. 1e, f, upper). When bath-applied, 0.1  $\mu\text{M}$  nicotine produced a  $4.3 \pm 0.2$  mV

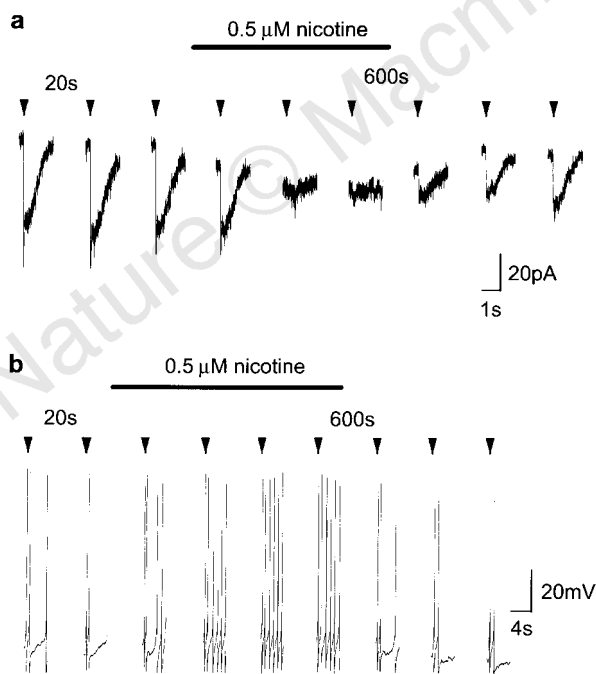


**Figure 1** Dopaminergic neurons respond to nicotine. **a**, A biocytin back-filled neuron from the VTA. The neuron was visualized with fluorescein-conjugated avidin. **b**, The same neuron (\*) and a nearby neuron were labelled with a monoclonal anti-TH antibody that was visualized with a Texas red-conjugated IgG. **c**, The  $I_h$  current is shown for the same neuron as in **a** and **b**. The neuron was voltage-clamped at a holding potential of  $-60$  mV, and hyperpolarizing voltage steps of  $-10$  mV were applied ( $-60$  to  $-100$ ) to activate the inward-going current indicative of dopaminergic neurons. **d**, Schematic drawing of a whole-cell clamped dopaminergic neuron in the VTA slice. **e**, Current-clamp recording (above) from a VTA neuron. Bath-application of  $0.5 \mu\text{M}$  nicotine (solid bar) caused a 12 mV depolarization and a burst of action potentials. The record is shown with a 10 min gap during the washout of nicotine. In voltage-clamp mode (below),  $0.5 \mu\text{M}$  nicotine in the bath caused a 27 pA inward current. There is a 5 min gap during washout. **f**, Current-clamp (above, 5 mV depolarization) and voltage-clamp (below, 16 pA current) recordings in response to  $0.1 \mu\text{M}$  nicotine applied to the bath. The neurons generally responded more slowly to lower agonist concentrations. **g**, Example currents induced by 0.1, 0.5 and  $1.0 \mu\text{M}$  nicotine applied to different neurons.

( $n = 6$ , s.e.m.) depolarization and  $0.5 \mu\text{M}$  produced  $8 \pm 1 \text{ mV}$  ( $n = 12$ ). In voltage-clamp mode (Fig. 1e, f, lower), an inward current activated by bath application of nicotine was revealed as the underlying cause of the depolarization that initiated action potentials in the VTA neurons. When bath-applied,  $0.1 \mu\text{M}$  nicotine produced a  $13 \pm 1 \text{ pA}$  ( $n = 11$ , s.e.m.) inward current,  $0.5 \mu\text{M}$  produced  $36 \pm 3 \text{ pA}$  ( $n = 16$ ), and  $1 \mu\text{M}$  produced  $57 \pm 9 \text{ pA}$  ( $n = 7$ ). Examples of the nicotine dose-dependence are shown in Fig. 1g.

To investigate the effect of nicotine in greater detail, the muscarinic acetylcholine receptors were inhibited and a pipette containing  $1 \text{ mM}$  ACh was placed  $15$  to  $30 \mu\text{m}$  from the soma to allow brief, local pressure injections of ACh (Fig. 1d). The brief, local pressure pulses of ACh (arrow heads in Fig. 2a) produced inward currents. Upon applying  $0.5 \mu\text{M}$  nicotine to the bath, the brief ACh-induced currents were desensitized and there was a downward shift in the baseline. The best explanation is that the local injection of ACh activated a small number of nicotinic receptors, but bath application of  $0.5 \mu\text{M}$  nicotine reached all the nAChRs on the neuron. The majority of the nAChRs were desensitized, which greatly reduced the current activated locally by the ACh injection. However, a small fraction of the total number of nAChRs stochastically opened in response to the bath application of nicotine, producing a slow inward current seen as a downward shift in the baseline.

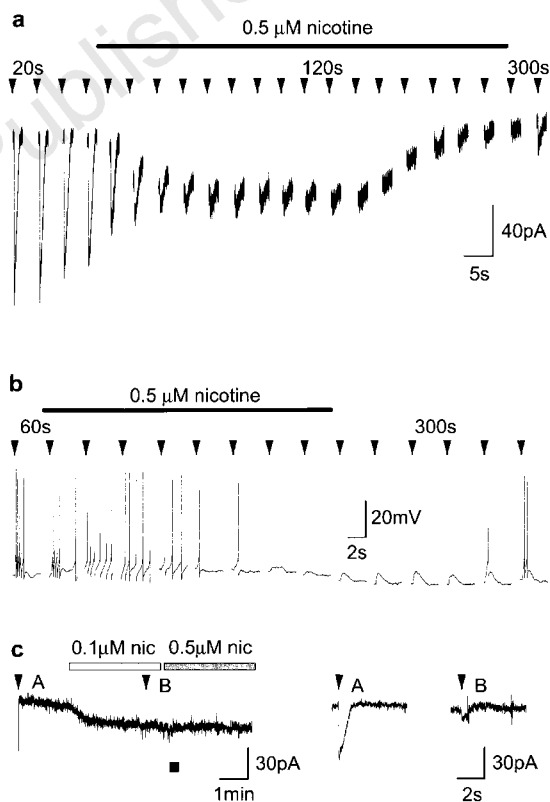
In current-clamp mode, which more closely approximates the biological situation, the pressure injection of ACh caused a burst of action potentials (Fig. 2b). Bath application of  $0.5 \mu\text{M}$  nicotine (solid bar in Fig. 2b) caused a depolarization of the resting membrane potential. After  $1 \text{ min}$  in  $0.5 \mu\text{M}$  nicotine, the neuron fired continuous spontaneous action potentials that were no longer



**Figure 2** Bath-applied nicotine affects ACh-activated responses. **a**, When a voltage-clamped neuron experienced pressure injections of ACh ( $1 \text{ mM}$  for  $100 \text{ ms}$ , arrow heads) separated by  $20 \text{ s}$  inward currents were induced. Bath application of nicotine desensitized the ACh-induced currents and induced a baseline current of  $25 \text{ pA}$ . During the washout and recovery from nicotine, ACh injections were still given every  $20 \text{ s}$ , but for clarity a  $600 \text{ s}$  gap is placed between the displayed currents. Atropine ( $1 \mu\text{M}$ ) was present to inhibit muscarinic ACh receptors. **b**, Under current clamp, injections of ACh ( $1 \text{ mM}$  for  $30 \text{ ms}$ ) separated by  $20 \text{ s}$  activated action potentials. Bath-application of  $0.5 \mu\text{M}$  nicotine depolarized the cell by  $7 \text{ mV}$  and produced action potentials, and the ACh injections became less effective at inducing action potentials.

influenced by the pressure injections of ACh, as would be expected if most of the nAChRs were desensitized. Recovery of the ACh-induced currents following washout of nicotine was slow, requiring nearly  $30 \text{ min}$ .

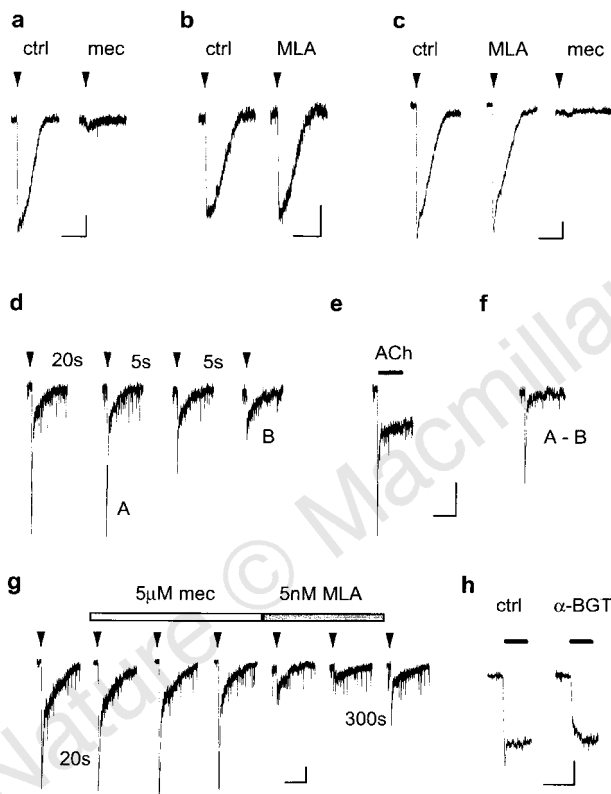
Because the half-life of nicotine in the body is about  $2 \text{ h}$  (ref. 16), smokers experience long exposures to nicotine. Therefore, we treated slices with  $0.5 \mu\text{M}$  nicotine for longer times. When nicotine was applied to the bath for  $19 \text{ min}$  (Fig. 3a), initially the response was as seen previously. There was an inward current that shifted the baseline downward and the nicotinic currents caused by pressure injection of ACh were desensitized. As the nicotine application continued beyond  $5 \text{ min}$ , however, a new process was observed. In the presence of nicotine, the currents activated by pressure injection of ACh were more strongly desensitized, and the baseline current decreased nearly to its original pre-nicotine position. Similar results were found in current-clamp mode (Fig. 3b). Initially, nicotine caused the usual depolarization and consequent action potentials, but the action potentials subsequently stopped as the resting membrane potential moved toward the original pre-nicotine



**Figure 3** Longer bath applications of  $0.5 \mu\text{M}$  nicotine have multiple effects. **a**, Under voltage clamp, injections of ACh ( $1 \text{ mM}$  for  $40 \text{ ms}$ , arrow heads) induced inward currents that were desensitized by bath application of nicotine (solid bar). The injections of ACh were always given every  $20 \text{ s}$ , but the time interval between displayed traces is initially  $20 \text{ s}$  then  $120 \text{ s}$  and then  $300 \text{ s}$ . The current activated by bath-application of nicotine is displayed by a downward displacement of the currents induced by injection of ACh, and at later times in nicotine, the baseline current desensitizes toward its original value. **b**, Under current clamp, injection of ACh ( $1 \text{ mM}$  for  $40 \text{ ms}$ ) initially depolarized the neuron and caused action potentials, but at later times in nicotine the neuron did not respond to ACh. The injections of ACh were given every  $20 \text{ s}$ , but the time interval between displayed traces is altered for clarity from  $60 \text{ s}$  to  $300 \text{ s}$ . **c**, Left, bath application of  $0.1 \mu\text{M}$  nicotine activated a  $17 \text{ pA}$  current. After  $3 \text{ min}$  in  $0.1 \mu\text{M}$ , applying  $0.5 \mu\text{M}$  nicotine activated very little additional current. If there had been no desensitization, the black square marks the average size of the current activated by  $0.5 \mu\text{M}$  nicotine. ACh pressure injections ( $1 \text{ mM}$ ,  $30 \text{ ms}$ , arrow heads) were applied before (A) and near the end (B) of the  $0.1 \mu\text{M}$  nicotine. Those ACh-induced currents on an expanded time scale (right) illustrate the extent of desensitization.

value. Based on previous studies of nAChRs<sup>5,17–22</sup>, a reasonable explanation is that during longer exposure to nicotine the nAChRs can enter deeper levels of desensitization, causing a greater fraction of the nAChRs to become desensitized. Thus, as the time in nicotine increases, the depolarized baseline desensitizes towards its initial resting membrane potential as fewer nAChRs are available to open stochastically to contribute to the depolarizing inward current. The rate at which the baseline depolarization recovered in nicotine toward the original pre-nicotine baseline was highly variable:  $t_{1/2}$  for recovery in 0.5  $\mu\text{M}$  nicotine was  $6 \pm 2$  min ( $n = 6$ ). In three other experiments, the depolarization was constant and did not recover during 15-min exposure to nicotine. Figure 3c shows that even the lower concentration of nicotine (0.1  $\mu\text{M}$ ) that may be maintained during much of a smoker's day can cause significant desensitization of nAChRs.

The variability in  $t_{1/2}$  and in the appearance of the currents



**Figure 4** Pharmacology of the nicotinic currents from dopaminergic VTA neurons. All currents were activated (arrow heads) by 1 mM ACh pressure-injected onto the neurons for 30–50 ms. All scale bars are 25 pA and 1 s. **a**, ACh induced an inward current (control, ctrl) that was inhibited by bath-application of 5  $\mu\text{M}$  mecamylamine (mec). **b**, ACh induced a current (ctrl) similar to **a** that was not inhibited by bath-application of 20 nM MLA. **c**, The ACh-induced current from another neuron was not inhibited by 10 nM MLA but was inhibited by 20  $\mu\text{M}$  mec added to the bath sequentially. **d**, A more rapid ACh-induced current maintained its peak amplitude if 20 s separated ACh pressure injections. Allowing only 5 s between ACh injections caused the rapid peak of the current to desensitize. **e**, A 1 s injection of ACh (solid bar) onto the same neuron shows the 2 components of the current: a rapid peak and a more sustained component. **f**, Subtracting the currents of **d** (A – B) revealed the rapid, desensitizing component of the ACh-induced current. The currents were digitally filtered before the subtraction. **g**, The rapid peak of the ACh-induced current is not significantly inhibited by 5  $\mu\text{M}$  mec, but the peak is inhibited by 5 nM MLA. ACh injections were separated for 20 s, but a 300 s gap separates the last record that is displayed. **h**, ACh-induced (solid bars) current that has 2 components (ctrl). The fast component of the current was inhibited by 100 nM  $\alpha$ -BGT (6 min incubation) but the sustained component was not ( $n = 3$ ). Two currents were averaged under each condition.

induced by pressure injection of ACh suggested that there could be multiple types of nAChRs expressed on the VTA neurons<sup>20–22</sup>. The various nAChR types could have different activation and desensitization characteristics. We investigated this possibility by examining the pharmacology of the currents induced by pressure injection of 1 mM ACh onto voltage-clamped VTA neurons. The majority of the neurons (15 out of 21) expressed predominantly a nicotinic current that peaked in about 50 ms, and that current was inhibited by mecamylamine but not by methyllycaconitine (MLA), a specific inhibitor of  $\alpha$ -bungarotoxin ( $\alpha$ -BGT)-sensitive nAChRs that contain the  $\alpha 7$  subunit (Fig. 4a, b, c)<sup>23–25</sup>. Other VTA neurons, however, also expressed a faster current that peaked in about 30 ms, and that current was more easily desensitized (Fig. 4d). When injections of ACh were separated by 20 s, the fast peak was maintained; but when the injections were separated by only 5 s, the fast peak desensitized but the slower component of the current remained. The two different components of the current from this neuron are seen more clearly in Fig. 4e, where a longer injection of ACh was applied. The fast current rapidly desensitized, but the slower current did not. Figure 4f shows the appearance of the fast current obtained by subtracting the slower component. MLA, the specific inhibitor of  $\alpha 7$ -containing nAChRs, inhibits the fast component of the current but mecamylamine does not (Fig. 4g). Finally, the fast component is inhibited by  $\alpha$ -BGT (Fig. 4h). The inhibition by MLA and  $\alpha$ -BGT, the fast kinetics, and the high sensitivity for desensitization all indicate that this current is carried by  $\alpha 7$ -containing nAChRs. Earlier investigations have not focused on this question, and therefore,  $\alpha 7$ -containing nAChRs have not been previously detected in the VTA.

Behaviour studies indicate that nicotine is an addictive drug that reinforces self-administration, increases locomotion and reinforces place preference<sup>3–5,26</sup>. Our results indicate that the concentrations of nicotine obtained by smoking can acutely excite dopaminergic VTA neurons to fire action potentials. This activity would initiate dopamine release in the NAcc and, in that way, is likely to be an important cellular mechanism for nicotine's rewarding effects<sup>3–7,10</sup>. A smoker, however, maintains a rather steady low-level background of nicotine throughout the day<sup>5,16</sup>, and our results indicate that longer exposures to nicotine can cause severe desensitization of VTA nAChRs. The rate of desensitization and recovery from desensitization can vary depending on which types of nAChRs are expressed on a particular neuron or in different areas of the brain. Thus, multiple phases of nAChR desensitization and recovery could underlie aspects of tolerance such that a second dose of nicotine following immediately after an initial dose does not elicit the same effects. The long half-time of nicotine and slow recovery from deep levels of desensitization may explain why many smokers report that the first cigarette is the most pleasurable of the day<sup>5</sup>. The nAChRs may more completely recover from long-term desensitization only after a night-time of abstinence from nicotine. Thus, the cellular mechanisms underlying some rewarding effects of nicotine and aspects of tolerance may be explained by nicotine activating and subsequently desensitizing nAChRs on VTA neurons. These results further support the hypothesis that the mesolimbic dopamine system contributes in different ways to the cellular and behavioral effects of many addictive drugs<sup>7,26–28</sup>. □

**Methods**

Midbrain horizontal slices containing the ventral tegmental area were prepared from Sprague–Dawley rats (12–25 days) using standard techniques<sup>13</sup>. Animal care was in accordance with institutional guidelines. Slices (200–250  $\mu\text{m}$ ) were cut in cold solution (in mM, 124 NaCl, 2.5 KCl, 2.5 CaCl<sub>2</sub>, MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 30 NaHCO<sub>3</sub>, 2 sodium pyruvate, 10 dextrose) bubbled with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. The slices were submerged in a continuously flowing (about 8 ml min<sup>-1</sup>) bath solution at 32 to 34 °C of (in mM) 135 NaCl, 2.5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 21 NaHCO<sub>3</sub>, 10 dextrose, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and unless stated otherwise 0.25–1  $\mu\text{M}$  atropine was present to inhibit

muscarinic acetylcholine receptors. Occasionally 0.5  $\mu\text{M}$  tetrodotoxin (TTX) was present to inhibit action potentials. The solution in the pipette used for pressure injections contained 1 mM ACh in a bath-like solution except the buffering was with 10 mM HEPES (pH 7.3).

Neurons were visualized for patch-clamp recordings under infrared light using Nomarski optics<sup>25</sup>, and to improve the optics the great majority of the slices were cut 200  $\mu\text{m}$  thick. The pipette solution for voltage-clamp recordings was (in mM) 120  $\text{CsCH}_3\text{SO}_3$ , 10  $\text{CsCl}$ , 10 EGTA, 5  $\text{Mg-ATP}$ , 0.3  $\text{Na}_3\text{GTP}$ , 10 HEPES, pH 7.3 and for current-clamp recordings half of the  $\text{CsCH}_3\text{SO}_3$  was replaced by  $\text{KCH}_3\text{SO}_3$ . In current-clamp mode when the internal cation was almost completely  $\text{Cs}^+$ , the resting membrane potential underwent slow waves that led to bursts of activity<sup>29</sup>. Standard patch-clamp techniques were used<sup>25</sup>. The holding potential for voltage-clamp recordings was  $-60$  or  $-70$  mV. Currents or voltage measurements were amplified and filtered (0.2 to 1 kHz) using an Axopatch 1B with a 4-pole Bessel filter and were usually digitally sampled (1 to 5 kHz). Additional off-line filtering was used for displays. All the programs were written in Axobasic (Axon Inst.).

After recording, slices containing neurons back-filled with biocytin were fixed in 4% paraformaldehyde at 4 °C for 5 h. The slices were washed, and anti-TH antibodies (1:2,000) were added in 0.2% Triton X-100 for 24–48 h. Fluorescein-conjugated avidin (8 mg  $\text{ml}^{-1}$ ) was added, and the primary TH antibody was visualized with goat anti-mouse IgG conjugated to Texas red (probes from Molecular Probes). The images' levels, brightness and contrast were adjusted with Adobe Photoshop.

The pharmacology experiments (Fig. 4) relied on pressure injection of 1 mM ACh in the presence or absence of bath-applied antagonists. Although we attempted to maximize the rate of ACh application and minimize desensitization of the  $\alpha 7$ -type component, the situation in brain slices is not optimal for extremely rapid and reproducible drug or agonist applications<sup>25</sup>. Thus, we expect that the rapid  $\alpha 7$ -type currents may have been underestimated or not detected in some cases. Special effort is required to detect these currents. In addition, 0.5  $\mu\text{M}$  mecamylamine blocked the slower component of current well, but after several minutes it would also start to inhibit the  $\alpha 7$ -type currents, which is consistent with previous reports<sup>30</sup>.

During longer exposures to 0.5  $\mu\text{M}$  nicotine, the membrane potential first depolarized than began to recover toward the initial pre-nicotine baseline potential. A more constant level of desensitization without recovery was usually seen with 0.1  $\mu\text{M}$  nicotine. To determine the  $t_{1/2}$  for recovery to the initial potential, we measured the time when the maximum depolarization was first reached until the time when the potential had recovered to half of its pre-nicotine value. In 2 of the 6 cases,  $t_{1/2}$  was linearly estimated from the slowly recovering membrane potential that had not yet recovered half way.

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## Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance

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Acquired drug resistance is a major problem in the treatment of cancer. Of the more than 500,000 annual deaths from cancer in the United States<sup>1</sup>, many follow the development of resistance to chemotherapy. The emergence of resistance depends in part on the genetic instability, heterogeneity and high mutational rate of tumour cells<sup>2</sup>. In contrast, endothelial cells are genetically stable, homogenous and have a low mutational rate. Therefore, antiangiogenic therapy directed against a tumour's endothelial cells should, in principle, induce little or no drug resistance. Endostatin<sup>3</sup>, a potent angiogenesis inhibitor, was administered to mice bearing Lewis lung carcinoma, T241 fibrosarcoma or B16F10 melanoma. Treatment was stopped when tumours had regressed. Tumours were then allowed to re-grow and endostatin therapy was resumed. After 6, 4 or 2 treatment cycles, respectively, no tumours recurred after discontinuation of therapy. These experiments show that drug resistance does not develop in three tumour types treated with a potent angiogenesis inhibitor. An unexpected finding is that repeated cycles of antiangiogenic therapy are followed by prolonged tumour dormancy without further therapy.

In preliminary studies, drug resistance was not inducible in mice bearing Lewis lung carcinomas treated with TNP-470, a selective angiogenesis inhibitor which inhibits tumour growth<sup>4</sup>. Cancer patients treated with TNP-470 in clinical trials have, to date, not