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The mechanism of ethanol action on midbrain dopaminergic neuron firing: a dynamic-clamp study of the role of I_h and GABAergic synaptic integration

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Abstract

Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels are expressed in dopaminergic (DA) neurons of the ventral tegmental area (VTA), as well as in DA and GABAergic neurons of the substantia nigra (SN). The excitation of DA neurons induced by ethanol has been proposed to result from its enhancing HCN channel current, $I_h$. Using perforated patch-clamp recordings in rat midbrain slices, we isolated $I_h$ in these neurons by voltage-clamp. We showed that ethanol reversibly increased the amplitude and accelerated the activation kinetics of $I_h$, and caused a depolarizing shift in its voltage-dependence. Using dynamic-clamp conductance injection, artificial $I_h$ and fluctuating GABAergic synaptic type conductance inputs were injected into neurons, following block of intrinsic $I_h$. This demonstrated directly a major role of $I_h$ in promoting rebound spiking following phasic inhibition, which was enhanced as the kinetics and amplitude of $I_h$ were changed in the manner induced by ethanol. Similar effects of ethanol were observed on $I_h$ and firing rate in non-DA, putatively GABAergic interneurons, indicating that in addition to its direct effects on firing, ethanol will produce large changes in the inhibition and disinhibition (via GABAergic interneurons) converging on DA neurons. Thus, the overall effects of ethanol on firing of DA cells of the VTA and SN in vivo, and hence on phasic dopamine release in the striatum, appear to be determined substantially by its action on $I_h$ in both DA cells and GABAergic interneurons.

Keywords
**INTRODUCTION**

Ethanol may exert its effects by directly or indirectly affecting signaling through a variety of receptors, channels, and effector proteins. Recent molecular pharmacology studies, however, have demonstrated that alcohol has several primary targets (Harris 1999; Spanagel 2009). Multiple genetic factors influence an individual’s response to ethanol (Schuckit et al. 2004), and a series of neural networks in the brain are thought to mediate the reinforcing effects of ethanol consumption. Many of these brain regions have been identified in neuropharmacological studies (Gessa et al. 1985; Kohl et al. 1998; Pontieri et al. 1995). In particular, the amygdala and mesolimbic dopaminergic (DA) pathway, including the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex, includes the primary sites in the brain that mediate ethanol reinforcement (Ericson et al. 2008; Lof et al. 2007), together with other brain systems including the mesocortical and nigrostriatal pathways. Ethanol has been shown to increase dopamine levels in regions of the VTA in rats and mice (Di Chiara and Imperato 1988; Zapata et al. 2006), and it directly stimulates VTA DA neurons in rat and mouse brain slices, causing an increase in the spontaneous firing frequency (Brodie and Appel 2000; Brodie et al. 1990). This effect is thought to underlie the behavioral reinforcement of ethanol intake (Brodie 2002). Indeed, administration of DA antagonists to the NAc suppresses behavioral responses associated with the reinforcing effects of ethanol (Kaczmarek and Kiefer 2000; Myers and Robinson 1999; Samson et al. 1993; Weiss et al. 1990).
Consistent with the role of the mesolimbic DA system in the behavioral effects of ethanol, active or passive administration of ethanol elevates extracellular DA levels in the NAc (Di Chiara and Imperato 1988; Weiss et al. 1993), probably via activity at VTA DA cell bodies rather than NAc terminals (Budygin et al. 2001; Yim and Gonzales 2000). Okamoto et al. (2006) demonstrated a clear link between ethanol-induced increases in the firing of mouse pacemaker DA neurons in vitro and the hyperpolarization-activated and cyclic nucleotide-gated (HCN) channel current $I_h$. In addition to its effects on $I_h$, ethanol may also modify firing in DA VTA neurons through reduction of a barium-sensitive potassium current (Appel et al. 2003; McDaid et al. 2008) and M current (Koyama et al. 2007).

In the present study, we attempted to elucidate how ethanol modulates firing through its effect on HCN channels expressed in VTA and substantia nigra (SN) DA neurons and SN GABAergic neurons. Firstly, we characterized ethanol-induced changes in $I_h$ kinetics in DA and GABAergic neurons. Then, we addressed the way in which synaptic integration in DA neurons is influenced by $I_h$ and GABA$_A$ synaptic inputs, using a computational model of $I_h$ combined with dynamic-clamp / conductance injection experiments. The results showed that the level of HCN channel activation regulates phasic DA neuron firing and brief pauses of firing, both of which are thought to be important in reinforcement learning, and that ethanol modulates $I_h$ to shape the integration of GABAergic inhibition differentially in distinct subsets of DA neurons.
MATERIALS AND METHODS

Slice preparation

All procedures involving animals were approved by Osaka University and complied with the NIH Guidelines on Animal Use. Coronal or horizontal slices were prepared from basal ganglia of 15- to 16-day-old Wister rats using standard techniques (Sakmann and Stuart 1995). During slicing, tissue was kept in sodium-free solution containing 254 mM sucrose, 2.5 mM KCl, 26 mM NaHCO₃, 10 mM glucose, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, and 1 mM MgCl₂. Slices were cut on a vibrating slicer (thickness, 300 μm; Microslicer DTK-3000, D.S.K., Kyoto, Japan) and kept in artificial cerebrospinal fluid (ACSF) at room temperature for at least 2 hours before recording. The ACSF contained 125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 25 mM glucose, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, and 1 mM MgCl₂. The slicing and recording solutions were equilibrated with 95% O₂/5% CO₂ to a final pH of 7.4. Slices were viewed using an upright microscope (Olympus BW60WI, Olympus Japan, Tokyo) equipped with infrared differential interference contrast optics.

Electrophysiological recording

Gramicidin-perforated patch-clamp recordings (Akaike 1996) were made from the somas of neurons in the VTA and SN. The patch pipette was tip-filled with internal solution and back-filled with gramicidin-containing internal solution (20–50 μg/ml). For cell filling, we used gentle suction to switch to a standard whole-cell patch clamp configuration after the perforated-patch experiments while monitoring
capacitive transient changes in voltage-clamp mode. Cells were then filled for 2–10 min. During recordings, the slices were perfused continuously with ACSF solution at a flow rate of 1.20 ml/min. Somatic patch-pipette recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in current-clamp and voltage-clamp modes, correcting for the prenulled liquid junction potential. Only voltage-clamp experiments with uncompensated series resistances less than 10 MΩ were included in the study, and series resistances were electronically compensated. Gramicidin-perforated patch recording pipettes (Clark GC150T) of 1.5–3.2 MΩ resistance were filled with standard intracellular solution: 105 mM K-gluconate, 30 mM KCl, 10 mM HEPES, 10 mM phosphocreatine Na₂, 4 mM ATP-Mg, 0.3 mM Na-GTP, and 5 mg/ml biocytin, balanced to pH 7.3 with KOH (Sakmann and Stuart 1995). Signals were filtered at 5 kHz and sampled with 16-bit resolution at 20 kHz. All experiments were performed at 35 ± 1°C.

**Immunocytochemical detection of tyrosine hydroxylase in recorded neurons**

Immunocytochemical detection of tyrosine hydroxylase (TH) was carried out in electrophysiologically characterized neurons to confirm that the classic physiological properties of dopaminergic (DA) neurons in the VTA and SN are consistent predictors of a DA phenotype (Grace and Onn 1989; Richards et al. 1997) under the conditions of this study (Fig. 1Aa,b). Putative DA and nondopaminergic (non-DA) neurons that were recorded using the gramicidin-perforated patch-clamp configuration were then filled with biocytin in the whole-cell patch-clamp configuration. In the substantia nigra pars reticulata (SNr), TH-negative non-DA neurons were classified as putative GABAergic neurons. After recordings, the slices were fixed in 4%
paraformaldehyde solution overnight at room temperature (23–25°C). Slices were then rinsed three times in 0.1 M phosphate buffered saline (PBS; pH 7.4) and placed in cryoprotectant (30% sucrose in 0.05 M phosphate buffer at pH 7.4) for 2 hours. Slices then were subjected to between one and three cycles of rapid freezing in liquid nitrogen followed by thawing in cryoprotectant to maximize penetration of the immunoreagents. They were then rinsed in PBS, soaked in 5% bacto-agar solution, and resectioned at 50 μm using a vibratome (Leica VT1000, Leica Microsystems, Nussloch, Germany). All incubations were performed on free-floating sections at room temperature under gentle agitation in TBS containing 1% bovine serum albumin (Vector Laboratories, Burlingame, CA), 0.5% Triton X-100 (Sigma–Aldrich, St Louis, Missouri, USA), and 1% normal goat serum (Vector Laboratories). Sections were incubated overnight in 0.1% mouse anti-tyrosine hydroxylase monoclonal antibodies (Millipore, Billerica, Massachusetts). Sections were then rinsed in TBS and incubated in 1% Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, San Diego, CA) for 2 hours. Sections were washed in PBS before incubation in 0.2% Alexa Fluor 594-conjugated streptavidin (Invitrogen) for 1 hour. Finally, sections were rinsed and mounted on a glass slide. Fluorescent images of recorded and biocytin-filled neurons and of TH immunoreactivity were obtained using a confocal imaging system (Olympus FV-1000, Olympus).

**Ethanol and drugs**

Human and rodent lethal blood alcohol concentrations are reported to range from 0.22 to 0.50 % wt/vol (50–110 mM), with a usual lethal concentration of 0.36 % (79.2 mM) (Gable, 2007). Since ethanol is a small
amphiphilic molecule which easily crosses the blood–brain barrier (Thomasson et al., 1993), it is usually assumed that blood alcohol concentrations are similar to alcohol concentrations at molecular targets in the brain. In this study, to resolve strong effects on $I_h$ at clinically-relevant levels of ethanol, we usually used 0.25 % wt/vol (55 mM), a concentration corresponding to strong inebriation.

To block Na$^+$ or K$^+$ channel conductance, the slices were perfused continuously with ACSF containing 0.5 μM tetrodotoxin (TTX) or 3 mM tetraethylammonium (TEA; Tocris Cookson, Bristol, UK), respectively. During conductance injection experiments (see below), 100 μM picrotoxin or 10 μM gabazine (Sigma–Aldrich), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Tocris Cookson), and 10 μM AP5 (Tocris Cookson) were included to block most intrinsic synaptic conductances. The $I_h$ blocker ZD 7288 (Tocris Cookson, Ballwin, MO) was prepared as a 30 mM stock solution in ACSF and stored at 4°C. The effects of the drug were assessed using bath applications of ACSF containing dilutions of the stock solution. ZD 7288 in the perfused ACSF was used at concentrations in the range 30–100 μM (usually 60 μM).

**Resting membrane potential and action potential threshold in spontaneously active cells**

To characterize firing of spontaneously active cells (see Fig. 1Ba), the pseudo “resting” membrane potential observed during spontaneous firing was defined as the mean stationary voltage during interspike intervals; the decaying phase of hyperpolarizing afterpotentials and the depolarizing ramps that preceded the discharge of each action potential (AP) were excluded. Defining stationary phases, however, is difficult during periods of...
relatively fast firing (5–20 Hz). To determine the mean “resting” potentials of spontaneously active cells, we used the method described in Ghamari-Langroudi and Bourque (2000). Briefly, histograms (bin width, 0.05–0.1 mV) of membrane voltage excerpts digitized at 4–5 kHz (4–10 s in duration) were constructed. The frequency distribution of the voltage samples showed two clear peaks in the absence of ion channel blockers (Fig. 1Bb). The more negative peak represented the brief stationary phase at the trough of the hyperpolarizing afterpotentials, whereas the larger, slightly more depolarized peak reflects the slowest phase of membrane potential change later in the interspike interval (Fig. 1Bb). The resting membrane potential was defined as the voltage corresponding to the latter peak. The AP threshold of spontaneously active cells was defined as the voltage at which the second derivative of the membrane voltage waveform exceeded three times the value of its standard deviation in the period preceding AP onset (Erisir et al. 1999). An example trajectory of APs in the phase plane of voltage ($V$) vs. $dV/dt$ is shown in Fig. 1Bc.

**Parameter estimation of $I_h$ kinetic model**

The voltage dependence of HCN channel conductance can be assessed experimentally by plotting the relative amplitude of current tails evoked at a fixed potential after pulses delivered to different conditioning potentials (McCormick and Pape 1990). In this study, however, we used a more direct method, of subtraction of $I_h$ current using a specific HCN channel blocker ZD 7288 in the external solution from control current evoked at a fixed potential after pulses delivered to different conditioning potentials (Ghamari-Langroudi and Bourque
To fit the kinetics of HCN channels precisely, we constructed a Hodgkin–Huxley-type conductance-based model (Tateno, unpublished observations). $I_h$ was modeled by the following equation:

$$I_h = g_h m(V - E_h),$$

where $V$, $g_h$, and $E_h$ respectively represent the membrane voltage, maximum conductance, and reversal potential for $I_h$ (Amini et al. 1999). The parameters $g_h$ and $E_h$ are assumed to be constant. In Eq. (1), $m$ is an activation rate variable that is governed by a first-order ordinary differential equation,

$$\frac{dm}{dt} = \frac{(m - m_\infty(V))}{\tau_m(V)}.$$  

Moreover, we assume that $m_\infty$ is the following function of $V$:

$$m_\infty(V) = \frac{1}{1 + \exp \left\{ \frac{(V - V_h)}{s_h} \right\}},$$

where $V_h$ and $s_h$ are the half-maximal voltage and slope factor, respectively. The voltage-dependent time constant $\tau_m$ is described by

$$\tau_m(V) = a_0 + a_1 \exp \left\{ -\left( \frac{(V - V_a)}{s_a} \right)^2 \right\},$$

where $a_0$, $a_1$, $V_a$, and $s_a$ are all constant parameters. To determine the parameters $g_h$, $V_h$, $s_h$, and $E_h$ of Eqs. (1) and (2), we minimized the mean square error between the predicted current $I_h = g_h m_\infty(V - E_h)$ and measured current at various potentials $V$ in the range from $-40$ mV to $-140$ mV. Similarly, from the time constants measured over the range of potentials, we estimated the parameters $a_0$, $a_1$, $V_a$, and $s_a$ in Eq. (3).

**Conductance injection method**
For dynamic-clamp or conductance-injection stimulation (Robinson and Kawai 1993; Sharp et al. 1993), an SM-2 conductance-injection system (Cambridge Conductance, Cambridge, UK) was used (Robinson 2008; Tateno and Robinson 2006). This method enabled us to selectively examine the effect of changing particular properties of HCN channel kinetics. The artificial $I_h$ determined by Eq. (1) was injected into DA cells. For each time step $\Delta t$, the activation variable ($m$) was updated from the current time $t$ to $t + \Delta t$, as follows:

$$m(t + \Delta t) = m(t) + \left[ m_\infty - m(t) \right] / \tau_m \times \Delta t,$$

(4)

Thus, $I_h$ was calculated as follows:

$$I(t + \Delta t) = g_h \times m(t + \Delta t) \times [V(t) - E_h]$$

(5)

The conductance-injection system was run at a frequency of 40 kHz (i.e., $\Delta t = 25$ µsec). In this study, all parameter values in Eqs. (1)–(5) that were used as default values were obtained from our experiments. In some conductance-injection experiments, the gating properties of $I_h$ were altered from the original model by changing parameter values. First, the maximum HCN channel conductance $g_h$ was changed in the range from 0 to 15 nS after blocking $I_h$ with ZD 7288. Second, the activation time course was changed by multiplying $\tau_m(V)$ by a constant factor $f_\tau$ (in the range 0.1 – 20) i.e., for the gating variable $m$, the original first-order differential equation was substituted for $dm/dt = (m - m_\infty(V)) / \left[ f_\tau \times \tau_m(V) \right]$. This allowed a selective change in the activation time course without affecting the voltage dependence of $m_\infty(V)$. Third, the voltage dependence of $m_\infty(V)$ was shifted by a constant offset ($-10$ mV to $+10$ mV). Fourth, the reversal potential ($E_h$) of $I_h$ was shifted in the range from $-30$ mV to $-45$ mV.
To mimic synaptic inputs, the opening of a population of synaptic receptor channels was simulated using an excitatory (AMPA) receptor synaptic conductance \( g_E(t) \) and an inhibitory (GABA\(_A\)) receptor synaptic conductance \( g_I(t) \) (Destexhe et al. 2001; Piwkowska et al. 2009). Depending on the changing membrane voltage \( V(t) \), the injected current \( I_s(t) \) was described by

\[
I_s(t) = g_E(t) [V(t) - E_E] + g_I(t) [V(t) - E_I],
\]

(6)

where \( E_E \) and \( E_I \) are the reversal potentials for the AMPA-type and GABA\(_A\)-type conductances, respectively. \( E_I \) was set at \(-63.5 \text{ mV} \) in DA neurons and at \(-72.6 \text{ mV} \) in non-DA GABAergic neurons (Gulacsi et al. 2003), and \( E_E = 0 \text{ mV} \) (Hollmann and Heinemann 1994; Macdermott 1987). The time courses of unitary synaptic events \( u_E(t) \) and \( u_I(t) \) were modeled by differences of two exponentials,

\[
u_j(t) = \begin{cases} 
0 & (t < 0) \\
C_j \left[ \exp(-t / \tau_r) - \exp(-t / \tau_d) \right] & (t \geq 0)
\end{cases},
\]

(7)

for \( j = E \) and \( I \). For the two types of receptor conductances, the waveform and parameters of a single synaptic input were based on our previous reports; the scaling factor values were \( C_E = 1,000 \text{ pS} \) for the AMPA receptors and \( C_I = 300 \text{ pS} \) for the GABA\(_A\) receptors, while the time constant values were \( \tau_r = 2.0 \text{ ms} \) and \( \tau_d = 0.5 \text{ ms} \) for the AMPA receptors, and \( \tau_r = 7.0 \text{ ms} \) and \( \tau_d = 0.5 \text{ ms} \) for the GABA\(_A\) receptors (for details, see Tateno and Robinson, 2006). Two channels of conductance stimulation were constructed by summing the unitary conductance transients such that

\[
g_j(t) = \sum_k u_j(t - T'_k)
\]

(8)
where \( j \) denotes E or I, and \( k = 1, 2, \ldots \), \( \{T_k^j\} \) is a point process for initiating synaptic unitary events. Once the parameters of the unitary synaptic input are fixed, the time series \( \{T_k^j\} \) \((j = E \text{ or } I, k = 1, 2, \ldots \)) completely determines the time course of conductance stimuli. Here, Poisson stimulus trains were constructed by summing unitary events—e.g., AMPA-type or GABA\(_A\)-type—at intervals denoted by a random variable \( t_i \), with the probability density \( p(t_i) = \lambda \exp(-\lambda t_i) \). The intensity of stimulation was varied by changing the Poisson rate \( \lambda \).

**Spike statistics and other data analysis**

Spikes were counted when the membrane potential crossed above the AP threshold. The instantaneous firing rate (reciprocal of the interspike interval) was computed from trains of APs evoked by 0.6–1.0 s pulses. Current or conductance strength was usually progressively increased or decreased in small steps of 10 pA or 20 pA, or 50 or 100 pS, respectively. The instantaneous firing rate (frequency) was plotted as a function of the injected current or conductance strength, resulting in frequency–current (f–I) or frequency–conductance (f–g) relationships. The maximum firing rate of a neuron was computed from the number of spikes per trial at the highest current strength before a depolarization block. Membrane time constants were obtained by fitting a single exponential function to the initial portions of more than 10 time-averaged voltage responses to small –20-pA or –10-pA hyperpolarizing current pulses of 0.6 s or 1.0 s in duration. Input resistance was calculated by dividing the maximal average voltage deflection by the amplitude of the same current pulses. AP threshold was determined as described above, and spike amplitude was measured as the difference between the peak and
the threshold of the AP. The afterhyperpolarization (AHP) was measured as the difference between the spike
threshold and the voltage minimum following the AP peak. Spike width was measured at half the spike
amplitude. To characterize properties of HCN channels, we used several parameters—$\Delta V_{\text{min}}$, $\Delta V_{\text{ss}}$, and sag amplitude $\Delta V_{\text{sag}}$—of membrane voltage changes in response to a long hyperpolarizing current (typically 2 s or
3 s, and $-150$-pA or $-200$-pA current steps; see Fig. 1C). In this study, $\Delta V_{\text{min}}$ and $\Delta V_{\text{ss}} (> 0)$ represented the
differences in membrane voltage from the baseline ($-50$ mV) to the minimum and steady-state voltages,
respectively, during the hyperpolarizing current application. The sag amplitude $\Delta V_{\text{sag}}$ was defined as the
difference between $\Delta V_{\text{min}}$ and $\Delta V_{\text{ss}}$: $\Delta V_{\text{sag}} = \Delta V_{\text{min}} - \Delta V_{\text{ss}}$. The relationship between firing frequency and each
parameter was modeled by fitting a second- or third-order polynomial or a sigmoid function by least-squares.
In addition, the relationship between firing frequency ($f$) and two parameters (e.g., $\alpha$ and $\beta$) was modeled
using the product of the individual functions $f_1(\alpha)$ and $f_2(\beta)$: $f_{12}(\alpha, \beta) = f_1(\alpha) \times f_2(\beta)$; this assumes that the two
parameters independently affect the firing frequency, an assumption that was tested experimentally. Results
are reported as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using Student's
$t$-tests, and $P < 0.05$ was considered significant.
RESULTS

Firing properties of VTA and SN cells

Coronal midbrain slices were used to obtain recordings from 102 DA cells in the VTA, 148 DA cells in the SN pars compacta (SNc), and 98 non-DA cells in the SN pars reticulata (SNr) of 15–16-day-old rats. We studied the electrophysiological properties of these cells by combining gramicidin-perforated patch-clamp techniques with double-labeling confocal immunohistochemistry (Fig. 1Aa, b). The anatomical locations of the recorded cells are plotted in Fig. 1Ac. As shown in Fig. 1Ba, regular spontaneous firing was observed, with an overall average firing rate of $2.21 \pm 0.97$ spike/s in DA cells of the VTA and the SNc (VTA: $2.12 \pm 1.12$ spike/s, $n = 42$; SNc: $2.27 \pm 1.28$ spikes/s, $n = 36$; $P > 0.4$; Table 1). In each of the spontaneously active DA cells, the histogram of the membrane voltage trace had two peaks (Fig. 1Bb), one located at approximately $-55$ mV ($-54.8 \pm 2.2$ mV, $n = 78$) and the other at approximately $-65$ mV ($-65.3 \pm 2.6$ mV). Here, we refer to the level of the more depolarized peak as the “pseudo” resting potential (see Methods). Plotting the membrane voltage ($V$) vs. $dV/dt$ on the phase plane showed that the pseudo-resting potential occurs at a point of relatively slower velocity in the trajectory, and the state variable remains close to this position for a relatively long period (Fig. 1Bc). The AP threshold corresponds to the onset of a rapid rise in $dV/dt$ (Fig. 1Bc, Table 1).
DA cells in the SNC (a) and VTA (b) of 15-day-old rats were tested for spontaneous firing and responses to a negative step current of $-200$ pA (Fig. 1C). Similar to results for 12- to 15-day-old C57BL/6J mice (Neuhoff et al. 2002), the latency of the rebound spike (or postinhibitory delay) after the current step offset was shorter in DA cells from the rat SNC ($292 \pm 25$ ms, $n = 36$) than in DA cells from the rat VTA ($818 \pm 65$ ms, $n = 42$; $P < 0.0001$; Fig. 1Da). However, the sag amplitude of membrane voltage responses to negative step currents was not significantly different between the SNC and VTA ($41.0 \pm 2.4$ mV vs. $45.6 \pm 2.3$ mV, respectively; $P > 0.5$; Fig. 1Da). The rebound firing rate in a short time window (2 sec) immediately after the first rebound spike was also higher in SNC DA cells than in VTA DA cells ($3.15 \pm 0.26$ spike/s vs. $2.07 \pm 0.12$ spike/s; $P < 0.01$; Fig. 1Db), substantially as a result of the much longer postinhibitory delay seen in VTA cells (Fig. 1Da). Input resistances for DA cells in the SNC and VTA were not significantly different ($476 \pm 28$ M$\Omega$ vs. $518 \pm 27$ M$\Omega$, respectively; $P > 0.05$; Fig. 1Db). Thus, our results show a heterogeneous population of DA neurons in the rat midbrain, consistent with results obtained using mice: Sarpal et al. (2004) reported a mediolateral gradient in HCN channel expression for mouse midbrain DA neurons, and Neuhoff et al. (2002) reported that HCN conductance was heterogeneous and significantly different between the mouse SNC and VTA.

All firing statistics for the SNC and VTA DA cells are shown in Table 1.

Voltage dependence of $I_h$ and a kinetic model
As reported previously (Neuhoff et al. 2002), DA neurons in both the VTA and SNc expressed a hyperpolarization-activated current $I_h$, which typically activates slowly in midbrain DA cells, and the time constant of activation decreases for hyperpolarizations below $-60$ mV. The different subunits that form functional $I_h$ channels (HCN1-4) show different activation kinetics (Baruscotti et al. 2010; Biel et al. 2009; Wahl-Schott and Biel 2009). To model $I_h$, we analyzed its voltage dependence using voltage-clamp recordings.

To assess the effects of $I_h$ blockade on spontaneous firing in DA cells, we perfused the slices with ACSF containing $60 \mu$M ZD 7288, a specific blocker of $I_h$ (Harris and Constanti 1995).

The amplitude of the ZD 7288-sensitive current evoked by hyperpolarizing voltage steps applied from a holding potential of $-40$ mV or $-45$ mV was measured. Current–voltage ($I–V$) relationships were obtained by delivering a series of prolonged (2–3 s) voltage steps to values between $-120$ mV and $-45$ mV at a frequency slow enough to allow complete deactivation of $I_h$ between pulses ($< 0.02$ Hz; Fig. 2A). These trials were repeated at regular intervals before and during application of ZD 7288. $I_h$ was resolved by subtraction of averaged current trances obtained in control recordings (Fig. 2Aa) from those recorded in the presence of ZD 7288 (Fig. 2Ab). $I–V$ relationships of steady-state $I_h$ were plotted as shown in Fig. 2Ac and 2Ba, in which $I_h$ is shown normalized to the maximum value in each cell (at $V = -120$ mV). The mean values of $g_h$ ($\pm$ SEM) normalized by the maximum conductance obtained from fitting distributions to the steady-state activation described by Eq. (2) (Methods) are shown for SNc and VTA DA cells in Fig. 2Bb. Using a Hodgkin–Huxley-type formulation, the maximum conductance $g_h$ and the reversal potential $E_h$ in Eq. (1), and
the half-maximal voltage $V_k$ and the slope factor $s_k$ in Eq. (2) were estimated for each $I-V$ relationship obtained for individual DA cells (see Methods for details). Thirty-two SNc DA neurons gave the following parameter values: $g_h = 7.57 \pm 0.54$ nS; $E_h = -39.1 \pm 0.6$ mV; $V_h = -69.0 \pm 0.3$ mV; and $s_h = 10.5 \pm 0.3$ ms. Twenty-six VTA DA neurons gave the following parameter values: $g_h = 5.46 \pm 0.28$ nS; $E_h = -41.0 \pm 0.9$ mV; $V_h = -68.3 \pm 0.4$ mV; and $s_h = 9.7 \pm 0.4$ ms. These parameter values, therefore, were used as the default values in the model. Notably, however, values for the maximum conductance $g_h$ were distributed over a wide range, from 1.0 nS to 11.0 nS in the SNc DA cells and from 1.5 nS to 8.0 nS in the VTA DA cells, as shown in Fig. 2Bc. The mean $g_h$ values in the SNc and VTA were significantly different ($P < 0.05$).

The time course of $I_h$ activation was obtained by analysing the rising phase of the ZD 7288-sensitive current evoked by hyperpolarizing steps to various voltages. As shown in Fig. 2Ac, ZD 7288-sensitive current traces were well fitted by a series of single exponential functions of the form $A(t) = A_\infty (1 - e^{-t/\tau_a})$, where $A(t)$ is the amplitude of $I_h$ at time $t$ after voltage stimulus onset, $A_\infty$ is the amplitude of $I_h$ at a steady state, and $\tau_a$ is the activation time constant. The mean value of the activation time constant $\tau_a$ at each of the voltages is shown in Fig. 2Bd. The four parameters in Eq. (3) were estimated for 31 SNc DA neurons: $a_0 = 0.32 \pm 0.05$ s; $a_1 = 1.85 \pm 0.04$ s; $V_a = -81.0 \pm 0.2$ mV; and $s_a = 18.1 \pm 0.2$ mV, and in 22 VTA DA neurons: $a_0 = 0.55 \pm 0.07$ s; $a_1 = 1.86 \pm 0.05$ s; $V_a = -83.6 \pm 0.3$ mV; and $s_a = 19.4 \pm 0.2$ mV. Curves plotted from Eq. (3) with the mean estimated parameter values are shown superimposed on the data in Fig. 2Bd.
Next, we confirmed the estimated values of the $I_h$ reversal potential ($E_{h}$) using voltage-clamp experiments, since this is an important parameter in determining the characteristics of rebound firing and rebound delays in response to hyperpolarizing current steps. $E_h$ was determined using a voltage protocol in which hyperpolarization to $-120$ mV was followed by different step depolarizations, allowing measurement of the tail current amplitude of fully-activated $I_h$, immediately following each step, at different potentials. Extrapolating a linear fit between $-120$ and $-65$ mV reveals the reversal potential of $I_h$. The mean reversal potential of $I_h$ for 13 DA neurons in the SNc was $-39.0 \pm 1.3$ mV, which was not significantly different from the mean reversal potential estimated from the $I-V$ relationship ($P > 0.05$). Similarly, in 11 DA neurons from the VTA, the mean reversal potential of $I_h$ was $-41.5 \pm 3.7$ mV, which was not significantly different from that in SNc neurons ($P > 0.05$). These results indicate that the voltage-dependent characteristics of $I_h$ in DA neurons are similar in the SNc and VTA.

### Effects of ethanol on $I_h$ in SNc and VTA DA cells

Ethanol reportedly enhances the intrinsic pacemaker activity of midbrain DA neurons, leading to increased DA transmission in the mesolimbic system (Brodie and Sampson 1990; Brodie and Appel 1998). This effect is thought to underlie the behavioral reinforcement of alcohol intake (Brodie 2002). Recently, Okamoto et al. (2006) described an increase in pacemaker activity frequency in mouse DA neurons in vitro after acute ethanol application and a role for $I_h$ in the effects of ethanol. These authors also reported that ethanol reversibly augmented the $I_h$ amplitude and accelerated the activation kinetics of this current. The effect of
ethanol was accompanied by a shift in the voltage dependence of $I_h$ activation to more depolarized potentials and an increase in the maximum $I_h$ conductance. Therefore, we next examined the effects of ethanol in DA cells from rat midbrain slices. Previous studies have examined the effects of ethanol on rat VTA DA cells using extracellular recordings and ethanol-induced inhibition of firing (for example, McDaid et al. 2008). However, for a quantitative understanding and modeling of the effect of ethanol, more detailed biophysical characterization is required, and hence we have used patch-clamp recordings in rat brain slices.

Monitoring spontaneous firing of SNc and VTA DA neurons showed that superfusion of 55 mM ethanol reversibly increased the firing frequency by 29.0 ± 0.4% (2.10 ± 0.02 Hz to 2.71 ± 0.02 Hz; $n = 41$; $P < 0.001$). Figure 3A shows an example of firing properties in a VTA DA cell under control, ethanol-treated, and washout conditions. In addition, 23 mM ethanol superfusion increased the firing frequency by 10.7 ± 2.3% (2.15 ± 0.05 Hz to 2.38 ± 0.04 Hz; $n = 10$; $P < 0.05$). After 55 mM ethanol application, the spike AHP decreased ($-72.3 ± 0.4$ mV vs. $-63.1 ± 0.4$ mV; $n = 41$; $P < 0.01$). Although the firing rate increased after ethanol superfusion, the derivative $dV/dt$ of the membrane voltage ($V$) in the downward phase of the AHP became more negative (data not shown).

Ethanol (55 mM) produced nearly identical increases in firing frequency in the rat SNc and VTA (27.1 ± 0.5% ($n = 22$) vs. 27.5 ± 0.6% ($n = 19$), respectively; $P = 0.92$). ZD 7288 treatment dramatically reduced the stimulating effect of ethanol on firing, giving only a 0.55 ± 0.40% increase in 7 DA cells in the VTA and 5
DA cells in the SNc. Ethanol slightly inhibited firing after ZD 7288 treatment in 3 of the 12 cells, suggesting that ethanol may be inhibitory when \( I_h \) is blocked (data not shown).

To examine the role of \( I_h \) in ethanol-induced firing, \( I_h \) was separated as described above, using voltage-clamp recording and the \( I_h \) blocker ZD 7288 (Fig. 3Ba). Ethanol increased the amplitude of the \( I_h \) component (17.8 ± 0.8% in 47 cells from the VTA and SNc; Figs. 3Bb and Ca). Washing out ethanol decreased \( I_h \) to the control level, demonstrating that the change was reversible (data not shown). Ethanol produced nearly identical increases in the \( I_h \) amplitude in the SNc and VTA (14.9 ± 0.9% (\( n = 27 \)) vs. 20.4 ± 1.0% (\( n = 20 \)), respectively; \( P = 0.32 \); Fig. 3Bb). In four SNc DA cells, however, the \( I_h \) amplitude decreased (−5.6 ± 1.6%), which was never observed in the VTA DA cells.

We next examined the effect of ethanol on the voltage dependence of \( I_h \). As shown in Fig. 3C, \( I_h \) was evoked once per minute by a 2- or 3-s hyperpolarizing voltage step from −55 mV to −125 mV. Superfusion of 55 mM ethanol reversibly increased the \( I_h \) amplitude by 26.2 ± 1.3% (501 ± 19 pA to 621 ± 21 pA; \( n = 11 \); \( P < 0.001 \)). \( I_h \) was also enhanced by 11.7 ± 0.8% in the presence of 23 mM ethanol (436 ± 30 pA to 484 ± 32 pA; \( n = 5 \); \( P < 0.01 \)).

We then investigated the effect of ethanol on the voltage dependence of \( I_h \) activation, using the same subtraction method described above. \( I_h \) activation curves were examined under three conditions: control, 55 mM ethanol superfusion, and 60 μM ZD 7288 treatment after washing out ethanol (Fig. 3Ca). For 17 DA cells in both the VTA and SNc, 55 mM ethanol increased the maximal current and shifted the \( I_h \) activation curve to
more depolarized potentials (Fig. 3Cb). The half-activation potential \((V_h)\) was shifted from \(-69.2 \pm 0.3\) mV to \(-65.3 \pm 0.3\) mV \((n = 17; P < 0.01)\). For SNc and VTA DA cells, ethanol produced nearly identical voltage shifts in the half-activation potentials: \(4.2 \pm 0.3\) mV \((n = 8)\) and \(4.0 \pm 0.3\) mV \((n = 9)\), respectively \((P > 0.4)\).

Ethanol enhancement of \(I_h\) was also accompanied by acceleration of the activation kinetics. Thus, at \(-80\) mV from a holding potential of \(-45\) mV, 55 mM ethanol reduced the activation time constant of \(I_h\) from \(2.43 \pm 0.12\) s to \(1.89 \pm 0.16\) s in SNc DA cells \((n = 11; P < 0.01)\) and from \(2.29 \pm 0.14\) s to \(1.72 \pm 0.20\) s in VTA DA cells \((n = 9; P < 0.01)\).

Some neurotransmitters are thought to cause an apparent reduction in \(I_h\) in DA neurons by reducing the membrane input resistance, which would worsen the space clamp during voltage-clamp recordings (Watts and Neve 1996; Williams and Mitchell 2008). Thus, it is possible that ethanol-induced augmentation of \(I_h\) was a result of an increase in the membrane input resistance, and hence improved space clamp. Yet 55 mM ethanol actually reduced the membrane input resistance from \(506 \pm 12\) MΩ to \(295 \pm 11\) ΩM \((n = 20; P < 0.001)\). This result is consistent with a similar finding in Okamoto et al. (2006).

**Injecting synthetic \(I_h\) via dynamic-clamp**

To examine the functional roles of HCN channels in SNc and VTA DA cells and characterize the relevant gating properties quantitatively, we developed a strategy which complements pharmacological blockade or genetic disruption of the channels. Using the conductance-based Hodgkin–Huxley-type model described
above, which accurately describes HCN channel gating in DA neurons, we implemented a dynamic-clamp conductance injection system allowing us to add this model conductance electrically to real neurons (Robinson and Kawai, 1993; Sharp et al., 1993; Robinson, 2008; Tateno, 2010). This technique allowed us to determine quantitatively the functional impact of HCN channels and the effects of ethanol in DA cells. The advantage of this approach is that it combines electrophysiological recordings from real cells in which all other conductances are the natural ones of the biological membrane, with the flexibility of a computational model approach for studying the role of $I_h$, allowing a systematic modification of defined parameters of $I_h$ kinetics.

Addition of synthetic HCN conductance following pharmacological block of intrinsic $I_h$

Subtraction of endogenous HCN conductance in DA neurons led to elimination of the membrane voltage sag evoked by long hyperpolarizing current pulses (2 s, −200 pA), leaving a near-exponential decay. Thus, subtraction of the HCN conductance using dynamic clamping mimics the effects of ZD 7288 (data not shown). This subtraction method, however, is demanding, because it requires precise adjustment of the various parameters of the model for individual cells (i.e., simultaneous adjustment of at least eight parameters used in Eqs. (1)–(3)). Next, therefore, we performed a “gain of function” experiment in which an artificial HCN conductance was injected after pharmacological blockade by ZD 7288 (Fig. 4A).
We first examined whether the addition of artificial HCN conductance could rescue the hyperpolarizing voltage response patterns elicited by long negative current pulses (Fig. 4Aa, Ba) using a conductance $g_h$ that restored the voltage sag evoked by a brief pulse train in the same cells (Fig. 4Ab, Bc). Adding 60 μM ZD 7288 reduced the sag amplitude from 34.6 ± 1.4 mV to 4.82 ± 0.68 mV (2 s, −200 pA current pulse; $n = 16$).

Subsequent addition of artificial HCN conductance restored the intrinsic DA-cell membrane potential phenotype (Fig. 4Bc); the restored sag amplitude was 33.9 ± 1.3 mV ($g_h = 5.3 ± 0.4$ nS; $n = 16$). In conclusion, these results indicate that the sag amplitude in response to hyperpolarizing current pulses and the normal DA-cell response phenotype can be rescued by adding the artificial HCN conductance.

Together with previous data from mice (Okamoto et al., 2006), our results show that ethanol increases the maximal conductance ($g_h$) of HCN channels in DA cells in the SNc and VTA, as described above. Using the dynamic-clamp technique, we next varied the value of $g_h$ to characterize ethanol-induced changes in both subthreshold properties (sag amplitude and input resistance) and suprathreshold properties such as rebound delay and the spike rate in a 2-s window immediately after the long hyperpolarizing currents were turned off.

Figure 4Ca shows typical repetitive membrane voltage traces for a SNc DA cell. As $g_h$ increased, the sag amplitude (Fig. 4Cb) increased monotonically up to around 5 nS and decreased slightly at 7.5 nS ($n = 5$). In addition, increasing $g_h$ was accompanied by monotonically decreasing input resistance (Fig. 4Cc) and delay (Fig. 4Cd) of the rebound spikes ($n = 5$), whereas the rebound spike rate, measured immediately after switching off the long hyperpolarizing currents increased ($n = 5$). In conclusion, the value of $g_h$ influenced not
only the subthreshold properties and time course of membrane voltage, but also the latency and rate of spikes and the input resistance for the hyperpolarizing current inputs. These results imply that the posthyperpolarization firing rate evoked by inhibitory synaptic inputs can be modulated by changes in the HCN conductance.

Similarly, to examine quantitatively the impact of activation kinetics on subthreshold and firing properties of DA cells, we added artificial HCN channel conductance with an altered activation rate \((f_t = 0.2-10\); Fig. 4Da), and applied 2 s hyperpolarizing current steps. If the activation rate was accelerated \((f_t = 0.2)\), the sag amplitude decreased. The sag amplitude was maximal at approximately \(f_t = 2.0-3.0\), and decreased again with further slowing of \(I_h\) activation (Fig. 4Db). In contrast, as the activation rate slowed \((f_t > 1)\), the input resistance increased monotonically (Fig. 4Dc). The minimum delay of rebound spikes was observed at approximately \(f_t = 2.0\) (Fig. 4Dd), and the maximum spike rate immediately after a hyperpolarizing current was at approximately \(f_t = 1.0\) (Fig. 4Dd). As can be seen for the case \(f_t = 5.0\), activation is too slow for the current to reach steady-state during the 2 second period of hyperpolarization, doubtless accounting in part for the effects on sag amplitude, delay and rebound firing rate at such slow activation rates. The results show that the intrinsic level of \(I_h\) in DA cells is close to optimal for a minimum delay and maximal firing rate of rebound spikes, following 2 s hyperpolarizations, a duration which is well-matched to its activation kinetics.
In agreement with previous studies (Mercuri et al. 1995; Neuhoff et al. 2002), we observed that ZD 7288 not only inhibited the large sag component (Fig. 4Cb), but also significantly prolonged the rebound delay (Figs. 4Bb and 5Ab) and weakened or eliminated transient posthyperpolarization excitation. Our evidence also strongly suggests that these effects of ZD-7288 are solely mediated by ZD 7288-sensitive $I_h$ channels, because restoring the HCN conductance by dynamic-clamp reversed these effects. Contrary to the results reported by Neuhoff et al. (2002), however, we observed that the timing of the first few APs after a hyperpolarizing current was reliable and independent of the preceding membrane potential at the moment of hyperpolarization under control conditions (Figs. 4B and 5A; $n = 10$), whereas in the presence of ZD 7288, timing depended on the preceding membrane potential, leading to a scattering of spike times in the ensemble of trials. The involvement of $I_h$ in this phenomenon was confirmed in a rescue experiment, in which artificial HCN conductance was added to DA cells (Fig. 5Ac). Although it was difficult to recover precisely the same spike timing and firing rates by exactly matching the amount of intrinsic $I_h$, the fidelity of the timing of the first and second spikes after negative step currents increased greatly in the rescue experiments (Fig. 5Ad).

As stated before, the intrinsic reversal potential of DA cells in the SNC and VTA was approximately $-40$ mV. The reversal potential, however, varied in individual DA cells of the SNC and VTA. Next, we tested if the reversal potential ($E_h$) of $I_h$ affects the rebound delay and posthyperpolarization excitation. Using the dynamic-clamp technique, we shifted $E_h$ in the range $-29$ mV to $-44$ mV while injecting artificial $I_h$ based on Eq. (1) after ZD 7288 application (Fig. 5Ba–d). The results showed that the rebound delay was shortest at $-34$
mV, slightly more depolarized than natural $E_h$. In addition, the extent of posthyperpolarization excitation was also largest at $E_h = -34$ mV. In conclusion, the dynamic-clamp experiments revealed that the reversal potential of $I_h$ in DA cells strongly influences both rebound delay and posthyperpolarization excitation.

To systematically investigate the relationships between mean AP frequency and the conductance $g_h$ or the activation time constant factor $f_\tau$, we varied both $g_h$ and injected current intensity ($I$), or $f_\tau$ and $I$, while measuring the mean AP frequency. Figure 6Aa shows the mean AP frequency during a short current pulse, plotted against $g_h$ for 7 levels of $I$. At low and high values of stimulus current, the relationship increased monotonically with $g_h$, while for intermediate currents, the relationship showed a shallow maximum at $g_h = 5.0$ nS to 12.5 nS (i.e., 80 pA $\leq I \leq 120$ pA in Fig. 6Aa). The mean AP frequency during the short current pulses is plotted against $f_\tau$ for 7 levels of $I$ in Fig. 6Ab. Similarly, for a given stimulus current, the relationship did not increase monotonically with $f_\tau$, but instead showed a shallow local maximum at around $f_\tau = 0.5$ or 1.0.

In summary, these results indicate that the activation rate is approximately optimized for SNc and VTA DA neurons to produce near-maximal AP frequency, while the real $g_h$ is only slightly lower than the optimal value.

Next, to examine how activation curves of HCN channels affect the mean AP frequency and time course of hyperpolarized membrane voltage in response to positive and negative current injection, we added artificial $I_h$ conductance with an altered half-maximal voltage $V_h$ in Eq. (2) (Fig. 6B) after blocking endogenous $I_h$. If $V_h$ was shifted by +10 mV, the threshold for initiation of a repetitive AP train decreased
from $-42.3 \pm 0.5$ mV to $-46.3 \pm 0.7$ mV ($n = 6; P < 0.05$; Fig. 6Bb). In addition, the firing rate increased from 10.3 ± 1.4 Hz to 15.7 ± 0.9 Hz in response to injection of 2 s, 100-pA positive current pulses ($n = 6; P < 0.05$; Fig. 6Bc), although strong adaptation to repetitive firing was observed (Fig. 6Bb). Moreover, the amplitude of the APs was reduced to those observed under control conditions (57.4 ± 1.1 mV to 53.8 ± 1.3 mV; $n = 6$; $P < 0.05$; Fig. 6Bb). Under the same conditions, the sag amplitude was reduced from 55.6 ± 1.3 mV to 51.5 ± 1.1 mV ($n = 6; P < 0.05$; Fig. 6Bb). In contrast, if $V_h$ was shifted by $-10$ mV, the threshold for the initiation of multiple APs increased ($-38.8 \pm 0.7$ mV; $n = 6; P < 0.05$) and the firing rate decreased (7.7 ± 0.8 Hz at 100-pA current intensity; $n = 6; P < 0.05$; Fig. 6Bc). In addition, the peak amplitude of the APs increased (61.2 ± 0.9 mV; $n = 6; P < 0.05$) and the sag amplitude increased (39.4 ± 0.5 mV; $n = 6; P < 0.05$; Fig. 6Bb). Figure 6Bc shows the mean AP frequency during the 2 s pulse plotted against a wide range of injected currents (0–0.18 nA) for control conditions and three different shifts ($-10$, 0, and $+10$ mV) of the midpoint voltage ($V_h$) all in the presence of $I_h$ blockade. In conclusion, these results show that a depolarized activation threshold of HCN channels facilitates DA neuronal activity. In contrast, a more hyperpolarized activation threshold confers AP frequency adaptation during long stimuli and increased sag amplitude in response to hyperpolarizing inputs.

**Effects of ethanol on spontaneous activity and $I_h$ in non-DA SNr cells**

The GABAergic neurons of the SNr convey signals from the basal ganglia to the thalamus and superior colliculus (Bentivoglio et al. 1979; Deniau et al. 1978). SNr neurons also innervate the SNC, where they
modulate the activity of DA neurons (Grace and Bunney 1979; Mailly et al. 2003; Tepper et al. 1995). SNr neurons are tonically active \textit{in vivo} and \textit{in vitro} (Bevan and Wilson 1999; Yung et al. 1991). Brief changes in firing, therefore, result in disinhibition or further inhibition of neurons in target nuclei of the basal ganglia (DeLong 1990). To assess the effects of ethanol on neural activity in the local SN circuit, we recorded from non-DA cells in the SNr. To the best of our knowledge, no previously published reports have examined how ethanol modulates the intrinsic pacemaker activity and HCN channel kinetics of non-DA neurons in the rat SNr.

As reported in Yung et al. (1991), TH-negative non-DA cells in the SNr were spontaneously active \textit{in vitro} (15.2 ± 5.5 Hz; \(n = 68\)), and fired at over 40 Hz (53.8 ± 17.8 Hz; \(n = 68\)) in response to 100-pA current injections (Figs. 7Aa1 and b1). ZD 7288-sensitive HCN currents were also observed in SNr neurons. Non-DA neurons in the SNr, however, were heterogeneous and the HCN channel conductance varied. The difference between two typical types of non-DA cells in the SNr are shown in Figs. 7A. Figures 7Aa2 and b2 show a series of membrane voltage traces in response to negative current step injections (−20-pA steps from −20 pA to −200 pA). The population of cells showed some variation in sag amplitude of the membrane voltages, which indicates that HCN channel expression levels vary considerably from cell to cell in the SNr. There was little correlation in the SNr neurons between the sag amplitude and spontaneous firing rate (Fig. 7Ba, correlation coefficient \(r = 0.077; n = 68\)) or the maximal evoked firing rate in response to current step injections just before depolarization block (Fig. 7Bb, \(r = 0.024; n=68\)).
Next, we injected hyperpolarizing current (−200 pA) into SNr non-DA neurons, and monitored membrane voltage. Slices were exposed to control conditions, superfusion with 55 mM ethanol, or 60 μM ZD 7288 after washout of ethanol (Fig. 7Ca). After 55 mM ethanol superfusion, the sag amplitude increased from the control level in all of the tested cells (Fig. 7Cb; 16.7 ± 2.0 mV to 24.8 ± 1.8 mV; n = 16; P < 0.05), whereas ZD 7288 decreased the sag amplitude (Fig. 7Ca). The size of the changes in sag amplitude after ethanol superfusion varied from cell to cell (Fig. 7Cb), and consequently, we further analyzed only cells in which ethanol induced at least 20% change in the sag amplitude. Ethanol reversibly increased spontaneous firing frequency from 11.0 ± 3.5 Hz to 27.7 ± 4.1 Hz (n = 16; P < 0.001).

Figure 8A shows example membrane voltage waveforms obtained under these three experimental conditions. The average minimum voltage immediately after individual spikes depolarized in response to ethanol superfusion (from −58.3 ± 0.4 mV to −54.1 ± 0.5 mV; n = 16; P < 0.01). The firing rate returned to control levels after ethanol washout (10.5 ± 1.0 Hz; n = 16; P > 0.05). ZD 7288 further reduced the firing rate to 4.7 ± 1.1 Hz (n = 7; P < 0.01). The average minimum voltage after spikes also decreased slightly in the presence of ZD 7288, although the change was not significant (−59.8 ± 0.8 mV; n = 7; P > 0.05).

The time course of changes in the average firing rate in these ethanol superfusion experiments are shown in Fig. 8B. Ethanol superfusion gradually and reversibly increased the firing rate in SNr non-DA cells (n = 7). In the presence of ZD 7288, ethanol did not increase the firing rate. This indicated that $I_h$ is a major contributor to increased spontaneous firing rates in these cells. In addition, ethanol slightly inhibited firing
after ZD 7288 treatment in 3 of 7 cells, suggesting that ethanol may be inhibitory when $I_h$ is blocked. In the 7 cells treated with both ethanol and ZD 7288, the magnitude of the ethanol-induced enhancement of firing was positively correlated with that of ZD 7288-induced suppression (correlation coefficient $r < 0.76$; data not shown). Together, these data suggest that $I_h$ contributes to ethanol-induced stimulation of pacemaker activity in non-DA cells. We next directly examined the effect of ethanol on $I_h$ using voltage-clamp recording. $I_h$ was evoked once per minute by 2-s or 3-s hyperpolarizing voltage steps from $-50$ to $-120$ mV. Superfusion of 55 mM ethanol reversibly increased steady state $I_h$ amplitude by $43.2 \pm 5.2\%$ (298 ± 16 pA to 427 ± 19 pA; $n = 9$; $P < 0.01$; Fig. 8Cb). ZD 7288 irreversibly reduced the current amplitude, and ethanol did not increase the amplitude after ZD 7288 was applied.

To gain further mechanistic insights into the effects of ethanol on non-DA cells in the SNr, we examined the voltage dependence of $I_h$ activation with the same ZD 7288 subtraction voltage-clamp protocol used to examine $I_h$ kinetics in DA neurons. As before, three conditions were tested: control, 55 mM ethanol superfusion, and 60 μM ZD 7288 treatment after ethanol was washed out. Current amplitudes during a series of hyperpolarizing voltage steps ($-60$ to $-120$ mV) were measured (Fig. 8D) to construct an $I_h$ activation curve. Ethanol shifted the $I_h$ activation curve to more depolarized potentials (Fig. 8Ea) and increased the maximal current (Fig. 8D). The half-activation potential ($V_{h}$) in Eq. (2) shifted from $-76.6 \pm 0.6$ mV to $-72.4 \pm 0.7$ mV in 8 non-DA cells ($P < 0.01$; Fig. 8Ea), and the maximum conductance ($g_h$) changed from $0.642 \pm 0.047$ nS to $0.855 \pm 0.112$ nS ($P < 0.05$). In addition, the slope factor $s_h$ did not change significantly
(11.5 ± 0.7 to 9.7 ± 1.1 mV, $P > 0.05$). Ethanol shifted the plot of the voltage-dependent time constant $\tau_m$ to the right, accelerating $I_h$ below $-75$ mV, and delaying it at more positive potentials above its peak. Finally, the reversal potential $E_h$ was not significantly changed ($-41.3 ± 0.5$ mV to $-41.0 ± 0.6$ mV; $n = 8$; $P > 0.05$; Fig. 8Eb). In summary, ethanol enhanced spontaneous firing and increased $I_h$ in non-DA cells from the SNr, and shifted voltage-dependence of activation and kinetics to more depolarized voltages.

Effects of ethanol on local circuits in the striatum and the SN

In many slice preparations, it is known that ethanol enhances GABAergic synaptic inhibition, primarily via a direct allosteric facilitation of the activity of GABA$_A$ receptors (for review, Weiner and Valenzuela 2006). The striatum is a major source of basal ganglia inputs to the DA system (for review, see Joel and Weiner 2000). Striatal GABAergic innervation of the VTA mainly originates in the limbic striatum, particularly the nucleus accumbens (NAc) shell (Berendse et al. 1992; Heimer et al. 1991). The dorsal SNC is only innervated by the limbic striatum, whereas the ventral SNC is innervated by the three striatal subregions: caudate, putamen, and NAc (Gerfen 1985; Nauta et al. 1978; Walaas and Fonnum 1980; Zahm and Heimer 1993). Striatal GABAergic projections to the SN are also directed to the SNr, where they synapse on the ventrally extending dendrites of DA cells located in the ventral SNC (Somogyi et al. 1981; Wassef et al. 1981). Here, we focused on the effects of ethanol on these local circuits of the DA systems, i.e., the DA neurons and striatal GABAergic projections to DA neurons in the SN and the VTA.
Figure 9A shows an overview of the neural circuits containing the VTA and SN. Despite the simplicity of the network, little is known about how ethanol affects DA neuronal activity, although the anatomical details of the striatoni gral projections are well-described (Tepper et al. 2007). Some evidence suggests that ethanol enhances GABA<sub>A</sub> receptor activity in DA cells (Lobo and Harris 2008; Michaeli and Yaka 2010; Weiner and Valenzuela 2006). Inputs from inhibitory synapses regulate DA neuronal excitability. Although a number of recent studies have identified both pre- and postsynaptic mechanisms that contribute to the acute and long-term effects of ethanol on GABAergic synaptic transmission, we focused on postsynaptic GABA<sub>A</sub> receptors, which mediate the majority of fast synaptic inhibition.

The VTA, in particular, has been implicated in the effects of ethanol (Di Chiara and Imperato 1988; Gessa et al. 1985; Pontieri et al. 1995). Alcohol has also been shown to increase extracellular dopamine levels in the striatum, especially in the NAc. Increased levels of dopamine alter GABAergic feedback into VTA DA neurons (Spanagel and Weiss 1999). There are two possible scenarios, which we illustrate by injection of GABA<sub>A</sub> type shunting conductance in a VTA DA cell. Firstly, a burst of activity in a direct inhibitory projection within the VTA or from the ventral striatum may directly silence ongoing activity in a DA cell (Figs. 9Aa and B). Secondly, increased activity in a striatal inhibitory neuron could depress activity in an inhibitory neuron target in SNr (GABAergic neuron 3 in Fig. 9Ab) which in turn projects to a DA cell, thereby causing a disinhibition of DA cell activity (Fig. 9C). Since ethanol will therefore, in addition to its
direct effect on $I_h$, also produce changes in the firing of the inhibitory input to DA cells, we sought to elucidate the way in which the levels of inhibition and $I_h$ interact in determining firing of DA cells.

In Fig. 10Aa, the average firing rate of the VTA DA cells with three levels of inhibitory GABA$_A$ conductance is plotted against the amplitude of excitatory AMPA conductance injected in 2-s steps. The relationship between the firing rate and AMPA conductance ($f-g$) monotonically increased for all 8 cells (4 VTA and 4 SNc DA cells). As the inhibitory conductance increased, the $f-g$ relationship shifted linearly to the right. To complement this, in Fig. 10Ab, the average firing rate of VTA DA cells for three frequency levels of excitatory AMPA inputs is plotted against the frequency of inhibitory synaptic inputs. Increasing inhibitory inputs monotonically reduced the average firing rate, and the relationships could be fitted approximately using sigmoid functions (Fig. 10Ab). Increasing the frequency of excitatory AMPA inputs shifted the curve upward.

To understand postinhibitory firing of VTA DA cells (see Fig. 10Ac), the rebound firing rate was examined during a short time window (1-2 s) immediately after a burst of inhibitory GABA$_A$ synaptic inputs were injected (Fig. 10Ac). In Fig. 10Ad, the average rebound firing rate is plotted against the frequency of inhibitory synaptic inputs. The average rebound firing rate at each frequency level was higher than the spontaneous firing rate under control conditions ($2.12 \pm 0.33$ Hz; $n=3$; $P < 0.01$), and the rate increased linearly as the inhibitory input frequency increased (Fig. 10Ad).

Figure 10B shows similar measurements for TH-negative SNr cells (putatively inhibitory relay cells).

The $f-g$ relationship rose monotonically with increasing AMPA input frequency in each of 8 of these cells
(Fig. 10Ba), although the dynamic range of the $f$-$g$ curves was nearly three times larger than that obtained using DA cells with the same range of AMPA conductance. As the inhibitory conductance increased, the $f$-$g$ relationship of the SNr cells was linearly shifted to the right. In Fig. 10Bb, the average firing rate of SNr cells for three frequency levels of excitatory AMPA inputs is plotted against the frequency of inhibitory synaptic inputs. Increasing inhibitory input monotonically reduced the average firing rate (Fig. 10Bb). In addition, the higher levels of excitatory AMPA input frequency shifted the curve upward. As shown in Fig. 10Bc, the rebound firing rate was measured in a 1-2 s time window after injection of inhibitory GABA$_A$ synaptic inputs.

In Fig. 10Bd, the average rebound firing rate at all inhibitory input frequencies exceeded the spontaneous firing rate under control conditions ($15.2 \pm 1.9$ Hz; $n = 3$; $P < 0.05$), and the rate increased linearly as the inhibitory input frequency increased.

Dopaminergic neurons in the SNc and VTA have been found to exhibit phasic responses related to reward-prediction errors (Mirenowicz and Schultz 1994; Tremblay et al. 1998). DA cells are thought to be part of an adaptive system that uses learned expectations to filter reward-related signals. This filtering creates dopamine bursts and pauses that respectively signal positive and negative violations of reward-related predictions. Based on the above results, ethanol should be expected to strongly influence this filtering process.

Thus, to investigate how $I_h$ kinetics is important in shaping the phasic firing responses of DA neurons in the reward system, we examined the interaction between the level of $I_h$ and inhibitory inputs, using conductance injection. After blocking endogenous $I_h$ using ZD 7288, the artificial HCN channel, excitatory AMPA, and
inhibitory GABA_A conductances were simultaneously injected into VTA DA cells, and the average firing rate during a short time window of several seconds was determined (Fig. 11Aa). As seen in Fig. 8, EtOH as well as increasing g_h, shifts the voltage-dependence of I_h in the depolarizing direction, and increases the time constant of decay, both of which also result in increased I_h. For simplicity, we model all of these effects simply as variations in g_h. If the effects of HCN channel conductance g_h and frequency α of the inhibitory inputs on firing rate are combined linearly, the firing rate f_h(g_h, α) can be represented as the product of the individual functions of the two respective variables (i.e., f_h(g_h, α) = f_h(g_h) × f_α(α)). Under this assumption, using the individual functions f_h(g_h) (Fig. 6Aa) and f_α(α) (Fig. 10Ab) obtained at a moderate level of current injection, we estimated the average firing rate of the DA cells, and predicted a three-dimensional firing rate surface, predicting its dependence on g_h and α (Fig. 11Ab). Corresponding results for the conductance injection experiments are shown in Fig. 11Ac. Although the two surfaces are broadly quite similar, there is evidence of some nonlinear interaction between the firing rate and g_h at higher inhibitory input frequencies (> 0.5 kHz), leading to a flattening of the relationship (Fig. 11Ac).

Because HCN channels are activated at voltages more hyperpolarized than the firing threshold of DA cells, we next examined postinhibitory rebound firing (Fig. 11Ba). After blocking I_h with ZD 7288, inhibitory conductance and g_h were simultaneously injected into VTA DA cells, and we determined the firing rate during a short window immediately after the inhibitory input was turned off. Similarly to above, assuming a linear interaction of g_h and frequency (β) of the inhibitory inputs in determining firing rate, the firing rate f_h(g_h, β)
may be represented as the product of the respective firing rate functions $f_0(g_h)$ and $f_\beta(\beta)$ (Fig. 11Bb). The results of corresponding conductance injection experiments are shown in Fig. 11Bc. In this case, there is a greater discrepancy between the simple linear model and experiment: the measurements show a much more pronounced optimum for firing rate, at each level of inhibitory input, at intermediate values of $g_h$ (5.0–10.0 nS), leading to a prominent ridge in the surface (Fig. 11Bc).
DISCUSSION

In this paper, we have carried out voltage-clamp measurements of $I_h$ in DA neurons of the VTA, and in DA and GABAergic neurons of the SN, and current-clamp combined with dynamic-clamp conductance injection to assess the effects of ethanol on spike generation in DA neurons. We found that $I_h$ is expressed in all of these cell types, to a higher degree in SNc DA neurons than in VTA DA neurons, and that ethanol reversibly increased its amplitude and accelerated its activation kinetics, and caused a depolarizing shift in its voltage-dependence. Mimicking these effects by artificial injection of $I_h$ following pharmacological block of intrinsic $I_h$ reproduced ethanol enhancement of postinhibitory rebound firing, implying that this results from its action on $I_h$.

Recent studies have shown that ethanol modulates the excitability of neurons by acting on a multitude of ion channels and receptors, including NMDA, GABA$_A$, glycine, 5-HT3, and nACh receptors (Vengeliene et al. 2008), as well as L-type Ca$^{2+}$ channels and G-protein–activated inwardly rectifying K$^+$ channels (for review, see Harris 1999; Spanagel 2009). Ethanol-induced excitation of VTA DA neurons is thought to contribute to the rewarding and reinforcing properties of ethanol and, in some individuals, alcohol addiction (Koob et al. 1998; Wise 1996). In addition, chronic alcohol consumption alters the balance between inhibitory and excitatory neurotransmission, leading to neuronal degeneration (Vengeliene et al. 2008). Significant adaptive changes and degeneration result in dysfunction in several brain areas, which may contribute to the transition from controlled to compulsive alcohol use.
DA midbrain neurons express variants of the slow-gating HCN2, HCN3, and HCN4 channels (Franz et al. 2000; Wahl-Schott and Biel 2009), and HCN2 appears to be the major type expressed in the VTA (Notomi and Shigemoto 2004). Ethanol may increase the single-channel conductance and/or maximal open probability of individual HCN channels via direct binding, as has been shown for other channel types (Harris 1999). In the present study, ZD7288 (30–100 μM) suppressed the firing of rat DA neurons in both the VTA and SNc, consistent with results in C57BL/6J mice (Okamoto et al. 2006). These observations contrasted, however, with a study by Neuhoff et al. (2002), who reported that $I_h$ contributes substantially to pacemaker frequency control only in a calbindin-negative subpopulation of SNc DA neurons, which may express higher levels of $I_h$-conducting channels compared with other subpopulations of DA neurons. We did not assay for calbindin and thus cannot directly address this issue. However, we did not see clear evidence for a bimodality in the density of $g_h$ (Fig. 3Bc) in the population of SNc DA neurons assessed.

Nevertheless, in the present study, the overall $I_h$ conductance levels in SNc DA cells were larger than in VTA DA cells, in agreement with Neuhoff et al. (2004) but contrasting with Okamoto et al. (2006), possibly owing to differences between animal preparations and ages used. We also found that $I_h$ was measurable in rat DA cells of both the SNc and VTA at P3 (data not shown). In addition, the functional $I_h$ current may undergo developmental changes, as observed in hippocampal pyramidal neurons (Vasilyev and Barish 2002).

Previous studies showed that the firing frequency of VTA DA neurons increases in response to ethanol in vitro and in vivo (Brodie et al. 1990; Gessa et al. 1985). Okamoto et al. (2006) suggested that ZD 7288
antagonizes ethanol-induced excitation of DA VTA neurons in mice. McDaid et al. (2008), however, did not observe a significant effect of ZD 7288 on the peak excitatory response of C57 mouse DA neurons in the VTA, whereas inhibition of firing in response to ethanol was noted in both C57 and DBA mice. The latter study suggested that, because ethanol both excites and inhibits neural activity in C57 mice treated with ZD 7288, the effects may cancel each other out (Appel et al. 2003). Although our results from rat slices support the observations of Okamoto et al. (2006), the electrical properties of VTA DA cells were heterogeneous in the present study, and responses to ethanol varied (Fig. 3Bb). In fact, ethanol reduced firing frequency in some DA neurons after superfusion of ZD 7288 (Fig. 3Bb). The non-$I_h$ effects of ethanol may be mediated by barium-sensitive potassium currents (McDaid et al. 2008) and/or the M current (Koyama et al. 2007).

Different subpopulations of GABAergic neurons, including parvalbumin- and/or calretinin-expressing neurons, have been identified in the rat SN (Lee and Tepper 2007a), with some GABAergic neurons exhibiting plateau potentials and larger sag amplitudes (Lee and Tepper 2007b). We also found that $I_h$ current varied among SNr GABAergic neurons, and that ethanol reversibly increased $I_h$ in SNr GABAergic neurons with larger $I_h$ currents, as well as in DA neurons. A previous in vivo study, however, showed enhanced or suppressed firing of GABAergic neurons in the VTA depending on the ethanol dose (Stobbs et al. 2004).

We directly tested the contribution of $I_h$ to integration using a computational model of HCN channel voltage-dependent kinetics and a dynamic-clamp approach to inject artificial HCN channel conductance. The somatic location of our recording pipette means that we may not adequately measure under voltage-clamp or mimic
the effects of remote dendritic $I_h$. However, on the reasonable assumption that the electrical attenuation and filtering of the cell’s dendritic tree is similar in voltage-clamp and current-clamp, then somatic injection of $I_h$ as measured at the soma should reproduce the effect of somatic and proximal dendritic $I_h$. Several integrative properties of DA neurons were markedly dependent on $I_h$ (Fig. 4). The observed rebound delay in the rescue experiments, however, was shorter than that in actual VTA DA neurons. To understand this difference, we varied the reversal potential $E_h$: shifting $E_h$ to a either more hyperpolarized or depolarized values from $-40$ mV lengthened the rebound delay. Furthermore, we systematically examined the effects of changing the time constant and activation curves associated with $I_h$ on the electrical properties of DA neurons, since ethanol produces strong shifts in the voltage-dependence and speeds the kinetics of $I_h$. Sag amplitude, input resistance, and rebound delay were all highly sensitive to the kinetics of $I_h$ (Fig. 4), and the firing rate was strongly affected by 10 mV shifts in the voltage-dependence of activation (Fig. 6B). Thus, the changes that 50-55 mM ethanol, which are similar to the concentrations expected for strong inebriation (Vengeliene et al. 2008), induce in the level and kinetics of $I_h$ are sufficient to cause a strong increase in firing in DA neurons by up to a factor of two or more, at least when all synaptic input is blocked as in these in vitro experiments.

Ethanol-induced changes in $I_h$ conductance and kinetics in various subpopulations of DA neurons also affect two distinct modes of phasic postsynaptic integration. Because ethanol increased the maximum conductance $g_h$, postinhibitory excitation was enhanced in a manner that was dependent on the inhibitory input frequency (Fig. 11Bc). Thus, a subpopulation of DA neurons characterized by higher $I_h$ conductance, including calbindin-negative SN DA cells (Neuhoff et al. 2002), show $I_h$ conductance-dependent transient, postinhibitory excitation; ethanol
appears to have larger effects on this subpopulation than on others. In contrast, DA neurons that show a lower $I_h$ conductance, including calbindin-positive SN DA neurons and other VTA DA neurons (Neuhoff et al. 2002) display pronounced postinhibitory inhibition (Fig. 1C); ethanol shifts this postinhibitory inhibition to postinhibitory excitation. In DA neurons with low levels of $I_h$ conductance, ethanol may not exert its main effect directly through $I_h$, but by stimulating firing of presynaptic GABAergic neurons, through effects on $I_h$ in these neurons, as we have shown here.

Synaptic inputs to DA neurons are primarily GABAergic (>70% of inputs) from the basal ganglia including the striatum and the globus pallidus (Bolam et al. 2000; Grace and Bunney 1985). The effects of increased striatal GABAergic neuron firing, though, are a combination of direct inhibition (Fig. 9Aa), and disynaptic inhibition (Fig. 9Ab), where increased activity in some GABAergic neurons (Fig. 9Ab, neuron 2) will actually produce a release from inhibition - both phenomena have been observed in DA neurons in vivo (Kiyatkin and Rebec 1998; Paladini et al. 1999). Thus, our results indicate not only that differences in the density of $I_h$ among DA neurons may mediate differential integration of GABAergic signaling, but also that the effects of ethanol on $I_h$ in heterogeneous GABAergic neurons (Fig. 7) will cause a shift in the balance of GABAergic input to DA neurons.

It has been postulated that SN DA neurons which show high $I_h$ conductance operate in a closed striatonigrostriatal loop that phasically releases dopamine in response to coordinated disinhibition by nigral and pallidal GABAergic inputs. Ethanol-induced changes in the firing of SNr GABAergic neurons are likely to contribute to this disinhibition. In contrast, SN and VTA DA neurons with lower $I_h$ conductance are directly inhibited by striatal input in an open-loop configuration with less temporal precision (Joel and Weiner 2000;
Maurin et al. 1999). The differences in \( I_h \) channel density appear to contribute to the polarity and temporal properties of GABAergic integration in DA neurons. Our results suggest, therefore, that ethanol affects the open-loop and closed-loop configurations differently, and will interfere strongly in the balance between them. It is impossible, with our current state of knowledge, to predict the overall effect of ethanol on these networks, but we may conclude that its powerful action on \( I_h \) is very likely to play a key role in changes in the dopaminergic reward system induced by ethanol consumption.
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**Figure legends**

**Figure 1.** A, Anatomical distribution of DA neurons in the SN and VTA. (a), Double immunolabeling for TH (green) and biocytin (red) was used to identify recorded cells as DA neurons in coronal midbrain slices from 15- to 16-day-old rats. Higher magnification views of the area denoted by the white square in (a) are shown in (b): TH (*left*), biocytin (*middle*), and TH and biocytin together (*right*). (c), The anatomical positions of electrophysiologically characterized and immunohistochemically identified DA neurons (open circles) were plotted on a coronal midbrain map, which also contains the TH-negative non-DA neurons analyzed in this study (filled squares). The section is about 6.2 mm anterior to the vertical coronal plane passing through bregma. The origin is approximately at mediolateral/dorsoventral coordinates of (x=-2.8 mm, y=1.2 mm) in flat-skull stereotaxic coordinates. The dashed curve indicates the boundary of the section. B, Spontaneous regular firing of a VTA DA neuron. A voltage histogram (b) was constructed from the membrane voltage signal in (a). Two peaks can be seen in the histogram; the voltage at the higher voltage peak was defined as a “pseudo” resting potential. (c), Trajectories of APs in (a) are plotted on a phase plane of membrane voltage $V$ vs. the derivative $dV/dt$ with respect to time $t$. The AP threshold, which corresponds to the voltage at which $dV/dt$ begins to rapidly increase, is indicated. C, Electrical properties of a SNc DA cell (a) and a VTA DA cell (b) from a 15-day-old rat are shown. Spontaneous firing (*top*) and evoked membrane voltage (*middle*) in response to a negative current pulse (*bottom*) are shown. The sag amplitude ($\Delta V_{sag}$) was calculated as the minimum membrane voltage ($\Delta V_{min}$) relative to baseline ($-50$ mV) minus the steady-state membrane voltage ($\Delta V_{ss}$) relative to baseline. D, Sag amplitudes of SNc and VTA DA cells plotted against their corresponding...
rebound delays in (a), and rebound firing rates during a 2-s time window immediately after a negative current
step offset were plotted against input resistance of the corresponding cells.

**Figure 2.** Current–voltage (I–V) analysis of $I_h$ in DA cells. A, Current responses of a VTA DA neuron to consecutive 2- or 3-s hyperpolarizing voltage steps to potentials between $-120$ mV and $-45$ mV from a holding potential at $-40$ mV. In (a) and (b), averaged ($n = 4$ trials per set) current responses from the same cell (bottom) to voltage steps (top) recorded before (control) and after application of ZD 7288 are shown. (c), Traces represent digitally subtracted ZD 7288-sensitive currents evoked by steps to each indicated voltage. Additionally, each current trace is superimposed on a thin solid line that shows the best monoexponential fit of the data. The time constants ($\tau$) used in the fits are indicated beside each trace. B, Voltage dependence of $I_h$ and associated kinetics. (a), Plots of the mean steady-state amplitude ($\pm$ SEM), normalized to the maximum amplitude at a membrane voltage of $-120$ mV. Membrane voltage values were increased in 5-mV steps between $-50$ mV and $-120$ mV. The half-maximal voltages in the SNc and the VTA were $-86.4$ mV and $-87.7$ mV, respectively. (b), The data shown in (a) were converted to conductance values ($g_h$) according to $g_h = I_h/(V - E_h)$, where $V$ is the test voltage and $E_h$ the estimated reversal potential in Eq. (2): $E_h = -39.1$ mV and $-41.0$ mV in SNc and VTA DA cells, respectively. The superimposed curves show best fits using Eq. (2). (c), A histogram of estimated maximum conductance ($g_h$) values is shown for 32 SNc DA cells and 26 VTA DA cells. (d), Plots of the mean $I_h$ activation time constant ($\pm$ SEM) measured in 32 SNc DA cells and 26 VTA DA cells and estimated relationships between the voltage and time constant (dotted curves) are shown.
The dotted curves are best fit curves from Eq. (3) that minimize mean square errors between data points and the estimated functions at those points.

**Figure 3.** Effects of ethanol in DA neurons. A, Superfusion of 55 mM ethanol enhanced spontaneous firing (b) compared with firing observed in control samples (a) and samples in which ethanol was washed out (c). B, Ethanol augments \( I_h \) in DA neurons. (a), Representative voltage-clamp recording traces of the effects of ethanol on \( I_h \). Traces before and during superfusion of 55 mM ethanol and a trace representing application of 60 \( \mu \)M ZD 7288 after ethanol was washed out. (b), Comparison of the effects of ZD 7288 on \( I_h \) in the VTA and SNc. C, The effects of ethanol on voltage-dependent activation curves and time constants of \( I_h \). (a), Representative traces of \( I_h \) evoked by the indicated series of voltage steps. Traces for control conditions (left), superfusion of 55 mM ethanol (middle), and application of 60 \( \mu \)M ZD 7288 (right) after ethanol was washed out are shown. (b), The subtraction method shown in Fig. 2A was used to plot the normalized HCN channel conductance \( g_h \) against command voltage in control and ethanol-treated conditions. The dashed lines represent fits to a Boltzmann function. The form of the Boltzmann function is given by Eq. (2).

**Figure 4.** Addition of HCN conductance by dynamic clamp conductance injection in SNc DA cells. A, Rescue of the sag by injecting HCN conductance in the presence of 60 \( \mu \)M ZD 7288. (a), Traces of membrane potential responses to −200-pA current injections in the presence and absence of ZD 7288 are superimposed.
(b), Comparison of control conditions and those in which $I_h$ was restored. B, Current injections at five

different intensities (−160 pA to −80 pA in 20-pA steps) were used in control conditions (a), ZD 7288 superfusion (b), and with restoration of $I_h$ (c). For conductance injection, $g_h = 5.1 \text{nS}$ was used. C, Dependence of the maximum conductance $g_h$ on membrane excitability and firing properties in a SNC DA cell after blocking endogenous $I_h$ with 60 μM ZD 7288. (a), Membrane voltage traces (top) in response to HCN conductance injection (bottom) using five different $g_h$ values (0 nS, 1.0 nS, 2.5 nS, 5.0 nS, and 7.5 nS). C, Relationships between HCN conductance $g_h$ and sag amplitude (b), input resistance (c), rebound delay (d), or rebound spike rate immediate after the conduction injection (d). (d), The rebound spike rate was modeled, fitted by a second-order polynomial function ($f_h(g_h) = -0.02 \times g_h^2 + 0.442 \times g_h + 3.82$), and used in Fig. 11. D, Dependence of membrane excitability and firing properties on the $I_h$ time constant, for the same conditions described in D. (a), Five membrane potential traces (top) representing responses to HCN conductance injection (bottom) using five different scale factor values $f_\tau$ (0.2, 0.5, 1.0, 2.0, and 5.0) for the voltage-dependent time constant. Similarly, relationships between HCN conductance $g_h$ and the sag amplitude (b), input resistance (c), rebound delay (d), and rebound spike rate immediate after the conduction injection (d) are shown. Note that all waveforms of injected current are plotted with depolarizing current upwards, following the normal convention for displaying stimulus current, rather than that for plotting voltage-clamp currents (depolarizing current downwards).
Figure 5. Dependence of $I_h$ on AP properties. A, Current- and dynamic-clamp recordings of membrane responses (top) to injections of six different hyperpolarizing currents ($-200 \text{ pA}$ to $-100 \text{ pA}$ using 20-pA steps, bottom). Recordings were obtained from SNc and VTA DA neurons under control conditions (a), after complete inhibition of the $I_h$ current using 30 $\mu$M ZD 7288 (b), and after restoration of $I_h$ using conductance injection (c). The timing of the first and second spikes in the postinhibitory activity were less affected by the hyperpolarizing current intensities under control and $I_h$ restoration conditions than when $I_h$ was blocked. (d), Summaries of rebound delays and spike timing reliability in the postinhibitory activity. B, In SNc and VTA DA cells, synthetic $I_h$ reversal potential $E_h$ affected the rebound delays and spike timing reliability in the postinhibitory activity. Four different $E_h$ values were used: $-29 \text{ mV}$, $-34 \text{ mV}$, $-39 \text{ mV}$, and $-44 \text{ mV}$ in (a), (b), (c), and (d), respectively. Membrane voltage responses (top) to the corresponding $I_h$ injections (middle) are shown with six different hyperpolarizing command currents ($-200 \text{ pA}$ to $-100 \text{ pA}$ in 20-pA steps; bottom). (e), Summaries of the rebound delays and spike rates in the postinhibitory activity.

Figure 6. Dependence of $I_h$ conductance and the activation curve on firing frequency. A, The dependence of the firing rate on parameters from the voltage-dependent activation curves and time constants. (a), A plot of the average firing rate during a 2-s current pulse versus the HCN maximum conductance ($g_h$) for 7 levels (60 to 180 pA in 20-pA steps) of the amplitude ($I$) of the injected current pulse. Each data point represents a mean frequency value in two VTA cells and three SNc DA cells. Dotted lines in the plot show curves at $I = 100 \text{ pA}$
and 180 pA approximated using the least-squares fit third-order polynomial functions \( f_{bh}(g_h) = 0.0026 \times g_h^3 - 0.116 \times g_h^2 + 1.50 \times g_h + 2.50 \) and \( f_{bh}(g_h) = 0.0043 \times g_h^3 - 0.0912 \times g_h^2 + 1.12 \times g_h + 7.40 \). (b), A plot of the average firing rate during a 2-s current pulse as a function of the scaling factor \( (f_i) \) of the activation time constant for 7 levels of the amplitude \( (I) \) of the injected current pulse. B, Effects of shifts in the activation kinetics on DA neurons. (a), A schematic representation of change \((-10 \text{ mV}, 0 \text{ mV}, \text{ and } +10 \text{ mV})\) in the half-point potential \( V_h \) of the activation curves. (b), Trains of APs (top) and hyperpolarized membrane voltage responses (bottom) evoked by 2-s command pulses (middle) while injecting synthetic \( I_h \) after blocking intrinsic \( I_h \) with ZD 7288. (c), The average firing rate as a function of the amplitude of the injected command current under control conditions, and during \( I_h \) conductance injection with different \( V_h \) values for the activation curve. The original value was \(-69 \text{ mV}, \text{ and } -10\text{-mV or } +10\text{-mV shifts from that value are shown.}

**Figure 7.** Electrophysiological properties of non-DA SNr neurons with different HCN conductance values. A, Perforated patch-clamp recording of two TH-negative SNr neurons (a and b), including membrane voltage responses (bottom panels in a1, a2, b1, and b2) to 1-s injection of depolarizing current (100 pA, top panels in a1 and b1) or hyperpolarizing current \((-200 \text{ pA to } -20 \text{ pA using } 20\text{-pA steps, top panels in a2 and b2})\). The neuron shown in (a) produced a larger \( I_h \) sag than that in (b). B, Relationships between firing rate and sag amplitude in SNr neurons. (a), The spontaneous firing rate from 68 TH-negative SNr neurons was plotted against the corresponding sag amplitude obtained with a \(-200\text{-pA hyperpolarizing current. (b), The maximum} \)
firing rate just before the action potentials were blocked in response to depolarizing current injection was plotted against the corresponding sag amplitude obtained with a –200-pA hyperpolarizing current. C, Effects of ethanol on membrane voltage responses in SNr neurons. (a), Hyperpolarizing currents were injected (top), and membrane voltage responses were plotted for control conditions, 55 mM ethanol perfusion, and application of ZD 7288 after ethanol was washed out. (b), Relationship between sag amplitudes in control conditions and in response to 55 mM ethanol perfusion. A 20% increase in the sag amplitude after 55 mM ethanol perfusion is indicated by a dotted line. (c), Relationship between firing rates under the two conditions shown in (b). A 20% increase in the firing rate after 55 mM ethanol perfusion is again represented by a dotted line.

**Figure 8.** Effects of ethanol on TH-negative SNr neurons. A, Superfusion of 55 mM ethanol increases spontaneous firing (b) compared to control (a) or after the ethanol was washed out (c). (d), ZD 7288 superfusion decreased the spontaneous firing rate. B, Graph illustrating the effects of 55 mM ethanol on the average firing rate normalized to the corresponding firing rate in control (n = 7 SNr cells). Application of 60 μM ZD 7288 irreversibly decreased the firing frequency and blocked the effect of 55 mM ethanol. C, Ethanol augments $I_h$ in SNr neurons. (a), Representative traces showing effects of ethanol on $I_h$ during voltage-clamp recordings. Overlaid traces before and during superfusion of 55 mM ethanol, and during application of 60 μM ZD 7288 after ethanol was washed out. (b), Graph of the effects of ZD 7288 on $I_h$ in a SNr cell. D,
Representative traces of $I_h$ (bottom) evoked by a series of voltage steps (top). Traces representing control conditions (left), superfusion of 55 mM ethanol (middle), and application of 60 μM ZD 7288 after ethanol was washed out (right) are shown. E, The effects of ethanol on voltage-dependent activation curves and time constants of $I_h$ in SNr neurons. (a), Using the subtraction method described for Fig. 3Ca, we plotted normalized HCN channel conductance $g_h$ against the command voltage obtained using control conditions and in the presence of 55 mM ethanol ($n = 8$ SNr neurons). Dashed lines represent fits to a Boltzmann function. (b), Comparison of $I_h$ activation time constants before and after ethanol treatment of SNr cells using test voltages from $-120$ to $-50$ mV in 5-mV steps.

Figure 9. Firing rate modulation in VTA and SN neurons by mimicking excitatory and inhibitory synaptic inputs by conductance injection. A, Local circuits that include the VTA, SN, and striatum. A simplified schematic representation of the local circuits. Ethanol stimulates DA release preferentially in the nucleus accumbens shell region, a process that may be involved in the reinforcing effects of alcohol consumption. Although multiple neurotransmitter and neuropeptide systems are involved in the initiation of this neurochemical event, disinhibition of GABAergic neurons appears to be a major underlying mechanism (b). B, Inhibition of firing in a VTA DA cell by inhibitory inputs from GABAergic neurons (Aa, Network 1). C, Disinhibition of firing in a VTA DA cell through two inhibitory synaptic pathways (Ab, Network 2).
Figure 10. A, Firing rate modulation in VTA DA cells by excitatory and inhibitory synaptic inputs. (a), The average firing rate is plotted against excitatory AMPA-type conductance for simultaneous injection of three different inhibitory GABA$_A$-type conductance values (0 nS, 1.5 nS, and 3.0 nS). (b), The average firing rate is plotted against the frequency of inhibitory GABA$_A$-type synaptic inputs for three different excitatory AMPA-type synaptic input frequencies (2.0 kHz, 4.0 kHz, and 6.0 kHz). The relationship between the firing rate and frequency of the inhibitory synaptic inputs was modeled using a sigmoid function $a/(1 + \exp(b(\alpha - c)))$, where $\alpha$ is the frequency of inhibitory inputs in kHz, and $a$, $b$, and $c$ are constant parameters; the values of $a$, $b$, and $c$ were 4.33, 4.49, and 5.52 for 2.0 kHz of excitatory synaptic inputs; 5.09, 2.98, and 5.87 for 4.0 kHz; and 6.05, 2.11, and 6.01 for 6.0 kHz, respectively. (c), The rate of rebound firing (top) in a 1-s time window after the inhibitory inputs (bottom). (d), The rebound firing rate of VTA cells is plotted against the frequency of inhibitory GABA$_A$-type synaptic inputs with excitatory AMPA-type synaptic inputs at a frequency of 4 kHz. The firing rate was linearly fit using $0.713 \times \log(\beta) + 1.408$, where $\beta$ is the frequency of inhibitory inputs in kHz. B, Firing rate modulation in TH-negative SNr cells by excitatory and inhibitory synaptic inputs. (a), The average firing rate is plotted against excitatory AMPA-type conductance with simultaneous injection of three different inhibitory GABA$_A$-type conductance values (0 nS, 1.5 nS, and 3.0 nS). (b), The average firing rate is plotted against the frequency of inhibitory GABA$_A$-type synaptic inputs for three different excitatory AMPA-type synaptic input frequencies (2.0 kHz, 4.0 kHz, and 6.0 kHz). (c), The rate of rebound firing (top) in a 1-s time window after the inhibitory inputs (bottom). (d), The rebound firing rate of TH-negative SNr cells is plotted against the frequency of inhibitory GABA$_A$-type synaptic inputs with
excitatory AMPA-type synaptic inputs at a frequency of 4 kHz. The firing rate was linearly fitted using $1.62 \times \log(\beta) + 9.98$, where $\beta$ is the frequency of inhibitory inputs in kHz.

**Figure 11.** Combined effects of HCN channels and inhibitory synaptic conductance injections on the firing rate of VTA DA cells. A, Firing rate modulation by simultaneous injection of three artificial conductance types (HCN channel, excitatory AMPA-type, and inhibitory GABA_A-type) after blocking the endogenous $I_h$ current with 60 $\mu$M ZD 7288. (a), Burst activity (bottom) of a VTA DA cell was evoked by simultaneous injection of HCN channel (top), excitatory AMPA-type (upper middle), and inhibitory GABA_A-type (lower middle) conductances. The average firing rate was calculated during the period indicated by the bar under the membrane voltage trace. (b), A three-dimensional representation of the average firing rate of VTA DA cells based on $f_{hu}(g_h, \alpha) = f_h(g_h) \times f_{a}(\alpha)$, where $g_h$ and $\alpha$ are the HCN channel conductance and frequency of inhibitory synaptic input, respectively. Here, $f_h(g_h) = 0.0026 \times g_h^3 - 0.116 \times g_h^2 + 1.50 \times g_h + 2.50$ and $f_{a}(\alpha) = 5.09/(1 + \exp(2.98 \times (\alpha - 5.87)))$. (c), A three-dimensional representation of the average firing rate of VTA DA cells based on data obtained from recordings. B, Rebound firing rate modulation by simultaneous injection of two artificial conductance types (HCN channel and inhibitory GABA_A-type) after blocking endogenous $I_h$ current with 60 $\mu$M ZD 7288. (a), Postinhibitory burst firing (bottom) of a VTA DA cell was evoked by simultaneous injection of HCN channel (top) and inhibitory GABA_A-type conductances (bottom). The average firing rate was calculated during the period indicated by the bar under the membrane voltage.
trace. (b), Three-dimensional representation of the average rebound firing rate of VTA DA cells is illustrated by \( f_{\beta}(g_h, \beta) = f_h(g_h) \times f_\beta(\beta) \), where \( g_h \) and \( \beta \) are the HCN channel conductance and frequency of inhibitory synaptic input, respectively. Here, \( f_h(g_h) = -0.02 \times g_h^2 + 0.442 \times g_h + 3.82 \) and \( f_\beta(\beta) = 0.713 \times \log(\beta) + 1.408 \).

(c), Three-dimensional representation of the corresponding average firing rates of VTA DA cells obtained from recordings.
Figure 3, Tateno & Robinson, 01 June 2011
Figure 6, Tateno & Robinson, 01 June 2011
Figure 7, Tateno & Robinson, 01 June 2011

A (a1) Injected current
0 nA
Membrane voltage
-63 mV
0.1 s
5 mV
0.1 s
0.1 nA
Injected current
Membrane voltage

(b1) Injected current
0 nA
Membrane voltage
-60 mV
0.1 s
5 mV
0.1 s
0.1 nA
Injected current
Membrane voltage

B (a)

\[ n = 68, r = 0.077 \]

Spontaneous firing rate (Hz)

Sag amplitude (mV)

B (b)

\[ n = 68, r = 0.024 \]

Maximum firing rate (Hz)

Sag amplitude (mV)

C (a) Injected current
0 pA
-200 pA
Membrane voltage
-65 mV
EtOH 100 mM
ZD 7288 30 µM
Control
0.3 s
50 mV

(b) 20% increase

Sag amplitude (mV)

Maximum firing rate (Hz)

n = 16

(c) 20% increase

Sag amplitude (mV)

Control firing rate (mV)

n = 16
Figure 9, Tateno & Robinson, 01 June 2011
A

DA cells

Firing rate (Hz)

AMP A conductance (nS)

0 nS GABA A

1.5 nS GABA A

3.0 nS GABA A

Inhibitory input voltage

Voltage

10 mV

1 s

1 s

Inhibitory input frequency (kHz)

Firing rate (Hz)

6.0 kHz AMP A

4.0 kHz AMP A

2.0 kHz AMP A

B

Cells in SNr

Firing rate (Hz)

AMP A conductance (nS)

GABA A 0 nS

GABA A 1.5 nS

GABA A 3.0 nS

Inhibitory input frequency (kHz)

Firing rate (Hz)

6.0 kHz AMP A

4.0 kHz AMP A

2.0 kHz AMP A

Spontaneous firing rate in control
Figure 11, Tateno & Robinson, 01 June 2011

A
(a) $g_h$

AMPA

GABA_A

Membrane voltage

(b) Model

(c) Experiment

Firing rate (Hz)

Inhibitory input frequency (kHz)

Conductance $g_h$ (nS)

B
(a) $g_h$

GABA_A

Membrane voltage

(b) Model

(c) Experiment

Firing rate (Hz)

Inhibitory input frequency (kHz)

Conductance $g_h$ (nS)
Table 1. Summary of properties of immunochemically identified SNc and VTA DA neurons and SNr non-DA neurons

<table>
<thead>
<tr>
<th>Cells</th>
<th>Input resistance (MΩ)</th>
<th>Spontaneous FF (Hz)</th>
<th>Rebound delay (ms)</th>
<th>(I_h) sag (mV)</th>
<th>AP threshold (mV)</th>
<th>AP width (ms)</th>
<th>AP amplitude (mV)</th>
<th>Pseudo RP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNc DA (n=36)</td>
<td>476±28</td>
<td>2.27±1.28</td>
<td>−292±25</td>
<td>41.0±2.4</td>
<td>−32.5±1.7</td>
<td>1.93±0.66</td>
<td>55.6±5.1</td>
<td>−54.6±1.7</td>
</tr>
<tr>
<td>VTA DA (n=42)</td>
<td>518±27</td>
<td>2.12±1.12</td>
<td>−818±65(^*)</td>
<td>45.6±2.3</td>
<td>−34.1±2.1</td>
<td>2.01±0.71</td>
<td>56.3±4.9</td>
<td>−55.0±2.1</td>
</tr>
<tr>
<td>SNr non-DA (n=68)</td>
<td>549±17(^*)</td>
<td>15.2±0.7(^*)</td>
<td>−84.4±3.9(^*)</td>
<td>15.1±1.0(^*)</td>
<td>−50.7±2.9(^*)</td>
<td>1.20±0.22(^*)</td>
<td>78.6±3.7(^*)</td>
<td>−59.6±4.1</td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences compared with values obtained from SNc DA neurons (\(P < 0.05\); ANOVA).

SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; DA, dopaminergic; VTA, ventral tegmental area; FF, firing frequency; AP, action potential; RP, resting potential.