Molecular and functional differences in voltage-activated sodium currents between GABA projection neurons and dopamine neurons in the substantia nigra

Abbreviated title: Na channels in nigral GABA projection neurons

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ABSTRACT

GABA projection neurons (GABA neurons) in the substantia nigra pars reticulata (SNr) and dopamine projection neurons (DA neurons) in SN pars compacta (SNc) have strikingly different firing properties. SNc DA neurons fire low frequency long duration spikes, whereas SNr GABA neurons fire high frequency short duration spikes. Since voltage-activated sodium (NaV) channels are critical to spike generation, the different firing properties raise the possibility that compared with DA neurons, NaV channels in SNr GABA neurons have higher density, faster kinetics and less cumulative inactivation. Our quantitative RT-PCR (qRT-PCR) analysis on immunohistochemically identified nigral neurons indicated that mRNAs for pore-forming NaV1.1 and NaV1.6 subunits and regulatory NaVβ1 and NaVβ4 subunits are more abundant in SNr GABA neurons than SNc DA neurons. These α subunits and β subunits are key subunits for forming NaV channels conducting the transient NaV current (INaT), persistent Na current (INaP) and resurgent Na current (INaR). Nucleated patch clamp recordings showed that INaT had a higher density, a steeper voltage-dependent activation, and a faster deactivation in SNr GABA neurons than in SNc DA neurons. INaT also recovered more quickly from inactivation and had less cumulative inactivation in SNr GABA neurons than in SNc DA neurons. Furthermore, compared with nigral DA neurons, SNr GABA neurons had a larger INaR and INaP. Blockade of INaP induced a larger hyperpolarization in SNr GABA neurons than in SNc DA neurons. Taken together, these results indicate that NaV channels expressed in fast-spiking SNr GABA neurons and slow-spiking SNc DA neurons are tailored to support their different spiking capabilities.
INTRODUCTION

As a key component of the basal ganglia motor circuitry, substantia nigra (SN) is populated largely by two types of projection neurons: GABA neurons in SN pars reticulata (SNr) and DA neurons in SN pars compacta (SNc) and also in SNr (Nelson et al. 1996; Bolam et al. 2000; Gonzalez-Hernandez and Rodriguez, 2000; Parent et al. 2000; Deniau et al. 2007). SNr GABA neurons and SNc DA neurons have strikingly different firing properties. SNr GABA neurons fire high frequency, brief action potentials (Atherton et al. 2005; Zhou et al. 2006), whereas SNc DA neurons fire low frequency, long duration spikes (Hyland et al. 2002; Zhou et al. 2006), indicating potential differences in voltage-gated potassium (Kv) and sodium (NaV) channels in these two cell types (Ding et al. 2011; Seutin and Engel 2010). Indeed, our recent study showed that a Kv3-like current is essential to the sustained high frequency firing in SNr GABA neurons (Ding et al. 2011).

NaV channels are composed of one α subunit and two β subunits (Catterall 2000). The α subunit forms the channel pore while the two auxiliary β subunits regulate the kinetics and function of the α subunit. Four of the 9 identified α subunits, NaV1.1, NaV1.2, NaV1.3 and NaV1.6, and all four β subunits, NaVβ1, NaVβ2, NaVβ3, and NaVβ4, are expressed in the central nervous system (Goldin 2001; Yu et al. 2003; Catterall et al. 2005). NaV channels formed by different α and β subunits conduct the classical fast transient Na current (I_{NaT}) with different kinetics (Smith and Goldin 1998; Goldin 2001; Hille 2001). Consequently, different neuron types expressing NaV channels comprised of different subunits may have different action potential waveforms and patterns (Bean 2007). For example, I_{NaT} in hippocampal fast-spiking interneurons recovers rapidly from inactivation and thus promotes fast spiking (Martina & Jonas 1997). NaV channels containing NaV1.6 and/or NaVβ4 may have a resurgent property that generates a resurgent Na current (I_{NaR}) after the inactivation of the I_{NaT} and upon repolarization that may facilitate fast spiking (Raman and Bean 1997; Khaliq et al. 2003; Mercer et al. 2007; Bant and Raman 2010). Additionally, NaV channels also conduct a small but long-lasting or persistent Na current (I_{NaP}) that increases neuronal excitability and promotes pacemaking firing (Crill 1996; Bean 2007).

mRNAs for NaV1.1, NaV1.2, NaV1.3, NaV1.6 α subunits and NaVβ3 and NaVβ4 β subunits, and also NaV1.1 and N1.2 α subunit proteins have been detected in the nigral region (Furuyama et al. 1993; Gong et al. 1999; Chen et al. 2000; Burbidge et al. 2002; Morgan et al. 2000; Yu et al. 2003). Furthermore, mRNA for NaVβ4, a key β subunit for
Na\textsubscript{v} channels with resurgent kinetics is expressed at a high level in the SNr but not in SNc (Yu et al. 2003). Since the major neuron types in SNr and SNc are the GABA projection neurons and DA neurons, respectively, SNr GABA neurons may express more Na\textsubscript{v}\beta4 than SNc DA neuron. It also raises the possibility that gene expression levels for other Na\textsubscript{v} \alpha and \beta subunits are different in SNr GABA and SNc DA neurons, leading to functional differences in Na\textsubscript{v} channels in SNr GABA and SNc DA neurons. More specifically, we reasoned that, compared with SNc DA neuron, Na\textsubscript{v} \alpha and \beta subunits expressed in SNr GABA neurons may form Na\textsubscript{v} channels that conduct a larger transient Na\textsubscript{v} current (I\textsubscript{NaT}) with faster kinetics and less cumulative inactivation, and also a larger persistent Na current (I\textsubscript{NaP}) and a resurgent Na current (I\textsubscript{NaR}).

To test these ideas, we first used single cell RT-PCR on electrophysiologically characterized SNr GABA and nigral DA neurons and quantitative RT-PCR on laser capture-microdissected immunohistochemically identified SNr GABA and SN DA neurons to profile the mRNAs expression of Na\textsubscript{v} channels. Then we used electrophysiological techniques to compare Na\textsubscript{v} currents in SNr GABA neurons and SNc DA neurons. Consistent with our hypothesis, we found that: (1) mRNAs for Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 and Na\textsubscript{v}\beta1 and Na\textsubscript{v}\beta4 are more abundant in SNr GABA neurons than in SNc DA neurons; (2) I\textsubscript{NaT} was larger and activated and deactivated more quickly in SNr GABA neurons than in SNc DA neurons; the recovery of I\textsubscript{NaT} from inactivation was faster and thus the cumulative inactivation was smaller in SNr GABA neurons than in nigral DA neurons; (3) SNr GABA neurons had a larger I\textsubscript{NaP} and also a larger I\textsubscript{NaR} than SNc DA neurons. These differences in Na\textsubscript{v} currents support the different spiking behaviors in fast-spiking SNr GABA neurons and slow-spiking nigral DA neurons. A preliminary analysis of these results has appeared in an abstract (Ding and Zhou 2010).
METHODS

Preparation of brain slices

Sixteen- to 24-day old male and female Sprague-Dawley rats were used. All procedures were carried out in accordance with the NIH guidelines and were approved by the Institutional Animal Care Committee of The University of Tennessee Health Science Center. Midbrain slices were prepared as previously described (Atherton & Bevan. 2005; Zhou et al. 2006, 2008, 2009; Ding et al. 2011). In brief, rats were deeply anesthetized with urethane. After transcardiac perfusion with an oxygenated ice-cold high sucrose cutting solution (see below), their brains were quickly dissected out and immersed in the ice-cold oxygenated cutting solution for 2 min. Coronal midbrain slices (300 μm thickness) containing the midrostral part of the substantia nigra were prepared in an ice-cold, oxygenated high sucrose cutting solution using a Vibratome 1000 Plus (Vibratome Company, St. Louis MO, USA) or Leica Zero Z VT1200S vibratome (Leica Microsystems, Wetzlar, Germany). Slices were transferred to a holding chamber containing the normal extracellular solution (see below) at 30 °C for 45 min and then kept at room temperature.

Electrophysiological cell identification and recording of Na, currents in nucleated membrane patches

Recordings were made under visual guidance of a video-microscope (Olympus BX51WI and Zeiss Axiocam MRm digital camera) equipped with Nomarski optics and a 60X water immersion lens. Patch pipettes with resistances of 1-3 MΩ were pulled from borosilicate (KG-33) glass capillary tubing (1.10 mm id, 1.65 mm od; King Precision Glass, Claremont, CA) using a PC-10 puller (Narishige, Tokyo, Japan). A Multiclamp 700B amplifier, pClamp 9.2 software and Digidata 1322A interface (Molecular Devices, Sunnyvale, CA) were used to acquire and analyze data. After electrophysiologically fingerprinting nigral GABA and DA neurons with conventional whole cell patch clamp within the first 10 s after obtaining access to the cell interior, gentle negative pressure was applied and the patch pipette was withdrawn slowly to isolate nucleated membrane patches (Martina and Jonas 1997; Ding et al. 2011).

Voltage clamp waveforms were generated by pClamp 9.2 software. Signals were digitized at 50 kHz and filtered at 10 kHz using the built-in low-pass Bessel filter in the patch clamp amplifier. This 10 kHz filtering, often necessitated by reducing noise, plus
stray capacitance-induced filtering, was likely to have affected the measurements of the activation and deactivation time courses, since these two kinetic parameters were very fast. Cell membrane capacitive transients were compensated with the auto compensation function of the Multiclamp 700B. The capacitance readings were taken as the capacitance estimates of the nucleated membrane patch to calculate current and conductance density. Series resistance was not corrected since it was estimated to be between 4-8 MΩ and the current amplitude was under 500 pA such that the voltage error caused by series resistance was minimal. Since both electrode capacitance and cell capacitance were compensated, the filtering from residual equivalent electrode-cell resistor-capacitor circuit should also be minimal. However, unknown stray capacitance may still exist in the recording system, leading to underestimation of the rapid activation and deactivation kinetics. Leak currents were subtracted on-line with a P/4 protocol. K⁺ channels were blocked by internal Cs⁺ and/or external tetraethylammonium chloride (TEA, 20 mM) and 4-aminopyridine (4-AP, 5 mM). Ca²⁺ currents were eliminated by replacing extracellular Ca²⁺ with Mg²⁺. GABA_A receptors and ionotropic glutamate receptors were routinely blocked by 100 μM picrotoxin and 20 μM D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV) and 10 μM 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), respectively. Na⁺ currents were isolated by off-line digital subtraction following application of 1 μM tetrodotoxin (TTX). Test pulses were applied every 5 s. Most recordings were made at 30 °C. Additional recordings (Figs. 12 and 13) were obtained at room temperature (25 °C), filtered at 30 kHz and sampled at 200 kHz.

**Composition of solutions**

The high sucrose cutting solution contained (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 20 D-glucose. The normal extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 1.3 MgCl₂, 10 D-glucose, maintaining pH at 7.4 by continuously bubbling with 95% O₂ and 5% CO₂. During recording of Na⁺ currents, 2.5 mM CaCl₂ were substituted by 2.5 mM MgCl₂, and extracellular NaCl was reduced on an equal molar basis when 20 mM TEA and 5 mM 4-AP were used. The Cs-based intracellular solution contained (in mM): 135 CsCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, 4 Na₂-phosphocreatine. pH was adjusted to 7.25 with CsOH. To record normal membrane potential and action potentials, a KCl-based intracellular solution was used, containing (in mM): 135 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 4 Na₂-phosphocreatine. pH was...
adjusted to 7.25 with KOH. The osmolarity for the intracellular solutions was 280-290 mOsm.

Data analysis
The liquid junction potential between the 135 mM KCl- or CsCl-based intracellular solution and 100 mM NaCl- and 20 mM TEA-Cl-based extracellular bathing solution, estimated by the liquid junction potential calculator in Clampex, was 4.6 mV and 4.7 mV, respectively, and not corrected in the data presented below. To obtain activation curves, Na$^+$ conductance was calculated with the equation $g_{Na} = I/(V-E_{\text{rev}})$, where $E_{\text{rev}}$ was estimated to be +50 mV under our recording conditions, following the method of Safronov and Vogel (1995) and Raman et al. (2000) (see Fig. 5). Activation and inactivation curves were fitted with Boltzmann equation: $f = 1/[1+\exp\{±(V-V_{1/2})/k\}]$, where $V$ is the membrane potential, $V_{1/2}$ is the potential at which the value of the Boltzmann function is 0.5, and $k$ is the slope factor. Data are reported as mean ± SEM. Two-sample independent t tests were used to make comparisons with $p$ value < 0.05 being considered statistically significant.

Single-cell reverse transcription polymerase chain reaction (scRT-PCR)
scRT-PCR procedures generally followed well established principles and methods (Surmeier et al. 1996; Liss et al. 2001; Zhou et al. 2008, 2009; Ding et al. 2011). Patch pipettes were autoclaved to eliminate RNase. The intracellular solution for scRT-PCR contained (in mM): 135 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, 4 Na$_2$-phosphocreatine, and was prepared using DNase-RNase free water. After electrophysiologically fingerprinting SNr GABA neurons and nigral DA neurons, gentle suction was applied to aspirate the cytoplasm without disrupting the seal. The aspirated cell content was expelled into a 0.2 ml PCR tube and treated with DNase I (5 min at 25 °C) to remove genomic DNA contamination. cDNA was synthesized using SuperScript III reverse transcriptase-based Cells-Direct cDNA Synthesis kit (Invitrogen). The synthesized cDNA was amplified using a hot-start Platinum PCR SuperMix (Invitrogen). RT-minus controls, in which the reverse transcriptase was omitted while all other reaction components were exactly the same, were performed to verify complete removal of genomic DNA. Negative controls were performed by lowering patch pipettes into the tissue and taking them out without seal formation and suction to exclude nonspecific harvesting of surrounding tissue components.
Two-stage PCR amplification was used as previously described (Zhou et al. 2008, 2009; Ding et al. 2011). Briefly, 5 μl of 30-μl cDNA was amplified for 45 cycles in the presence of primers for the first stage (see Table 1 for primer pair sequences). The thermal cycling protocol was 2 min at 94 °C for the initial denaturation, than 45 cycles of 15 s at 94 °C to denature, 30 s at 48 °C to anneal, and 50 s at 72 °C to extend, followed by a 10 min final extension. In the second stage PCR, the product of 1 μl from the first stage PCR amplification was used as template and the same primer pair that used in the first stage was used, and 40 cycles were run.

We first detected GAD1 (Glutamate decarboxylase 1) and TH (tyrosine hydroxylase) mRNA to identify GABA and DA neurons and to confirm the success of cytoplasm aspiration and cDNA synthesis. Then we used 5 μl of the remaining cDNA from the original 30-μl cDNAs to detect the target genes. The products from the second stage amplification were separated by 1.5% agarose gel electrophoresis, visualized by ethidium bromide (0.05 mg/100 ml gel) or Gelgreen under UV light and photographed. The positive bands were then cut out and extracted using a Qiagen extraction kit. The extracted products were sequenced at the Molecular Resource Center of University of Tennessee Health Science Center in Memphis, Tennessee, and positively identified.

The web-based Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm) (MIT, Cambridge, MA) was used to design PCR primers according to sequences published in GenBank. The sequences of these primers are listed in Table 1. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The effectiveness of the primers was positively confirmed by using whole brain total RNA. All primers yielded amplicons of expected sizes with correct sequences.

qRT-PCR assay on laser-captured nigral neurons

Rats were deeply anesthetized with urethane (1.5 g/Kg). Brains were rapidly removed and frozen with Freeze Spray. Cryostat coronal midbrain sections (10 μm) were collected and stored at –80 ºC. Tyrosine hydroxylase (TH) and parvalbumin (PV) were used as markers for DA and GABA neurons, respectively (Gonzalez-Hernandez and Rodriguez 2000; Zhou et al. 2009). To quickly immunostain DA neurons or GABA neurons, slide-mounted cryostat sections of unfixed rat midbrain sections were removed from –80 ºC storage and allowed to thaw before fixation in ice-cold 100% methanol for 3 min. The slide-mounted sections were briefly dipped in cold 0.02 M PBS and incubated for 3 min with 1:25 rabbit anti-TH polyclonal antibody or 1:25 mouse anti-PV monoclonal
antibody diluted in PBS. This was followed by four brief rinses in cold PBS. The tissue sections were incubated for 3 min in red fluorescent secondary donkey anti-rabbit or donkey anti-mouse antibody diluted at 1:50 in PBS. The slide-mounted sections were washed four times in cold PBS and dehydrated (1 min each in 75%, 95% and 100% EtOH), followed by 5 min in xylene twice. After air-drying for 10 min, brain sections were visualized on the ArcturusXT fluorescent Laser Capture Microdissection (LCM) System (Applied Biosystems). TH-positive or parvalbumin-positive cells were picked by LCM and collected on the Arcturus plastic sample caps. Approximately 300-400 TH-positive or parvalbumin-positive neurons from six coronal sections of SNc or SNr were collected and pooled into a single cap. Six caps were used to collect TH-positive or parvalbumin-positive neurons for each animal. RNA was extracted and purified using the PicoPure RNA isolation kit (Applied Biosystems). RNA samples were further treated with DNase to remove any potential genomic DNA contamination and then used as template to generate cDNA using SuperScriptTM III CellsDirect cDNA Synthesis kit (Invitrogen).

Levels of mRNA for Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.6, Na\textsubscript{v}\beta1-4 and β-actin (internal control) were measured by using a Roche LightCycler 480 (LC 480) quantitative real-time PCR system and the Universal ProbeLibrary probes and primers (Roche Applied Science, Indianapolis, Indiana). The sequences for these primers are listed in Table 2. Using β-actin mRNA as internal control, Na\textsubscript{v} mRNA quantification was performed employing the comparative crossing point (C\textsubscript{p}) method in the form of \(2^{-[\text{delta}C_p]}\) (Luu-The et al. 2005). The C\textsubscript{p} values of the real-time fluorescence intensity curve were calculated using the second derivative method. The calculation was performed by the built-in software on LC 480. For Na\textsubscript{v} mRNA levels in DA neurons, \([\text{delta}C_p]_{\text{Nav,DA neuron}} = C_{\text{p,Nav,DA neuron}} - C_{\text{p,β-actin,DA neuron}}\). For Na\textsubscript{v} mRNA levels in GABA neurons, \([\text{delta}C_p]_{\text{Nav,GABA neuron}} = C_{\text{p,Nav,GABA neuron}} - C_{\text{p,β-actin,GABA neuron}}\). Finally, \(2^{-[\text{delta}C_p]}\) values for Na\textsubscript{v} mRNAs were normalized to those in SNr GABA neurons.

RESULTS

**SNr GABA neurons fire faster spikes with larger amplitude than nigral DA neurons**

As shown in **Fig. 1 A-C**, putative SNr GABA neurons exhibited high frequency spontaneous spiking with brief action potential duration and weak or no “sag” in response to hyperpolarizing current injections (**Fig. 1A,C**). In contrast, the presumed DA
neurons in SNc and SNr (nigral DA neurons hereafter) spiked spontaneously at low frequency with long action potential duration and displayed a pronounced I_h current-induced sag in response to hyperpolarizing current injection (Fig. 1B,C, Table 3). These characteristics are consistent with published studies for these two neuron types (Tepper et al. 1995; Richards et al. 1997; Atherton and Bevan. 2005; Blythe et al. 2007; Lee and Tepper. 2007; Zhou et al. 2006, 2008). We have previously shown that a Kv3-like channel-mediated potassium current is critical to the fast repolarization, short spike duration, and high frequency firing in SNr GABA neurons (Ding et al. 2011). We also noticed that compared with nigral DA neurons, the action potentials of SNr GABA neurons have more negative threshold (–40.3 ± 0.5 mV vs. –36.2 ± 0.4 mV), a larger amplitude (69.5 ± 1.6 mV vs. 59.3 ± 1.4 mV), shorter duration (1.1 ± 0.1 ms vs. 2.9 ± 0.2 ms), shorter 10-90% rise time (0.27 ± 0.02 ms vs. 0.69 ± 0.05 ms), and faster rise rate (183.8 ± 17.3 mV/ms vs. 71.8 ± 8.8 mV/ms) (Fig. 1, p values < 0.05, see Table 3). Also, SNr GABA neurons showed no or little adaptation in both firing frequency and spike amplitude, whereas nigral DA neurons were adaptive in both firing frequency and spike amplitude (Fig. 1D, E). These results indicate that in addition to differences in Kv channel-mediated repolarization that we have already characterized (Ding et al. 2011), SNr GABA neurons and nigral DA neurons may also have qualitative and/or quantitative differences in Na_v channel expression.

**Na_v channel gene expression in identified SNr GABA and nigral DA neurons**

After electrophysiologically fingerprinting SNr GABA and nigral DA neurons with a 135 mM KCl-based intracellular solution, we performed scRT-PCR to profile mRNAs for Na_vα and Na_vβ subunits. As illustrated in Fig. 2, mRNAs for Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.6, Na_vβ1, Na_vβ2, Na_vβ3, and Na_vβ4 were detected in both SNr GABA neuron and SNc DA neuron, although there were apparent differences in the rate of detection. Since scRT-PCR is a qualitative method, these results indicate that SNr GABA neurons and SNc DA neurons may express identical types of Na_v subunits at different levels. Testing this idea requires quantitative comparison of Na_v subunit mRNA levels in SNr GABA neurons and SN DA neurons. However, the quantity of mRNA obtained in a single neuron was not sufficient for qRT-PCR. To overcome this difficulty, we collected and pooled SNr GABA neurons and SN DA neurons, separately, by using laser capture microdissection (LCM) and performed qRT-PCR analysis on Na_v channel subunit mRNAs isolated from these neurons. SNr GABA neurons and nigral DA neurons were
identified by parvalbumin (PV) and tyrosine hydroxylase (TH) fluorescent immunoreactivity, respectively (Gonzalez-Hernandez and Rodriguez 2000; Zhou 2009). As shown in Fig. 3, we found that the mRNA expression level of Na_v1.1, Na_v1.6, Na_vβ1 and Na_vβ4 in SNr GABA neurons was 2.07, 2.03, 2.15 and 1.96 fold of that in SNc DA neurons, respectively (p < 0.005). In contrast, the level of Na_v1.2, Na_vβ2 and Na_vβ3 mRNAs in SNc DA neurons was 1.65, 1.69 and 3.26 fold of that in SNr GABA neurons (p < 0.005). As in scRT-PCR, Na_v1.3 was detectable in qRT-PCR, but the C_P values (defined in Methods) of SNr GABA neurons and SNc DA neurons were too high (more than 40), indicating low abundance and potentially unreliable quantification. Thus, Na_v1.3 was excluded from quantitative comparison in this study.

Based on these qRT-PCR data on Na_v mRNAs (Fig. 3), the differences in action potential waveforms in SNr GABA neurons and SNc DA neurons (Fig. 1), and the different electrophysiological characteristics of Na_v subunits in heterologous expression systems (Goldin 2001), we made the following prediction: compared with Na_v channels in SNc DA neurons, Na_v channels in SNr GABA neurons (1) are expressed at a higher density, (2) activate at a lower threshold and/or with a steeper voltage dependence, and (3) recover faster from inactivation and thus have less cumulative inactivation, and (4) conduct larger persistent sodium current and resurgent sodium current. These Na_v channel properties would support the sustained fast spiking in SNr GABA neurons.

Higher I_NaT density in SNr GABA neurons than in DA neuron

To study Na_v currents in SNr GABA neurons and nigral DA neurons, we performed nucleated patch clamp recordings in rat brain slices (Martina and Jonas 1997; Ding et al. 2011). The key advantage of nucleated patch clamp is the substantially reduced spatial and voltage clamp problems because of the elimination of axonal and dendritic processes. To help isolate Na_v currents, a 135 mM CsCl-based intracellular solution was used to block potassium channels. Cs^+ also blocks the I_h current that is prominent in nigral DA neurons but lacking or small in SNr GABA neurons (Fig. 1). Thus, we performed electrophysiological identification of SNr GABA and nigral DA neurons within the first 10 s after obtaining access to the cell interior. A similar method was used by Martina and Jonas (1997). As shown in Fig. 4A,B, during this short time window, SNr GABA neurons and nigral DA still displayed distinct electrophysiological properties. SNr GABA neurons exhibited spontaneous firing at frequencies ≥ 10 Hz and little or no “sag” in response to hyperpolarizing current injection. In contrast, nigral DA neurons exhibited
spontaneous firing at frequencies $\leq 5$ Hz and prominent “sag” in response to hyperpolarizing current injection. After electrophysiological identification, nucleated membrane patches were isolated from these neurons (Fig. 4. C-E).

We first compared $I_{\text{NaT}}$ density in nucleated membrane patches from SNr GABA neurons and nigral DA neurons. $I_{\text{NaT}}$ was evoked from a holding potential of $-100$ mV to test potentials ranging $-80$ mV to $+30$ mV in $5$ mV steps. As shown in Fig. 5, $I_{\text{NaT}}$ reached its peak amplitude around $-10$ mV in both SNr GABA neurons and nigral DA neurons. The peak $I_{\text{NaT}}$ and thus conductance density were clearly higher in SNr GABA neurons than in nigral DA neurons: $148.9 \pm 17.8$ pA/pF and $2481.8 \pm 296.2$ pS/pF for SNr GABA neurons ($n = 12$) and $86.0 \pm 14.1$ pA/pF and $1433.5 \pm 234.2$ pS/pF for nigral DA neurons ($n = 9$, $P < 0.05$, Table 3). If we assume the specific membrane capacitance to be $1$ µF/cm$^2$ (Hille 2001), then $I_{\text{NaT}}$ conductance density was $24.8 \pm 3.0$ pS/µm$^2$ for SNr GABA neurons and $14.3 \pm 2.3$ pS/µm$^2$ for nigral DA neurons. The reversal potential used for calculating the conductance was obtained by linear-fitting the data points between $0$ mV and $30$ mV (Fig. 5), following the method of Safronov and Vogel (1995) and Raman et al. (2000). The estimated reversal potential was $49.8$ mV for GABA neurons and $49.9$ mV for DA neurons. The higher $I_{\text{NaT}}$ density may contribute to the faster rise and larger amplitude of the action potentials in SNr GABA neurons.

**Steeper voltage-dependent $I_{\text{NaT}}$ activation in SNr GABA neurons than in nigral DA neurons**

We next compared the voltage-dependence and kinetics of $I_{\text{NaT}}$ in SNr GABA neurons and nigral DA neurons. The voltage-dependent activation of $I_{\text{NaT}}$ was studied by holding the nucleated membrane patch at $-100$ mV and then stepping to test membrane potentials between $-80$ mV to $30$ mV (Fig. 6A1). Representative $I_{\text{NaT}}$ traces at these test membrane potentials and averaged activation curves were shown in Fig. 6A2-A4. $I_{\text{NaT}}$ started to activate around $-50$ mV in both cell types. However, with increasing depolarization, the increase of $I_{\text{NaT}}$ conductance was faster and reached the maximum earlier in SNr GABA neurons than in DA neurons, indicating that $I_{\text{NaT}}$ had a steeper voltage-dependent activation in SNr GABA neurons than in SN DA neurons (Fig. 6A4). Boltzmann equation fitting revealed that the activation slope factor K was $6.2 \pm 0.2$ for SNr GABA neurons ($n = 12$) and $7.3 \pm 0.3$ for nigral DA neurons ($n = 9$, $p < 0.05$). The activation midpoint potential $V_{1/2}$ was also different for the two cell types: $V_{1/2}$ was $-30.2 \pm 0.6$ mV for SNr GABA neurons ($n = 12$) and $-26.7 \pm 0.5$ mV for nigral DA neurons,
respectively \( n = 9, \ p < 0.05, \) Table 3). The steeper activation curve with a more negative midpoint indicates that \( I_{NaT} \) in SNr GABA neurons can be activated more readily than those in nigral DA neurons, contributing to the more negative spike threshold and faster spike rise rate in SNr GABA neurons.

As expected for \( I_{NaT} \), both the activation (measured by 10-90% rise time) and inactivation (measured by 10-90% decay time) of \( I_{NaT} \) were fast (Fig. 6A2-3, B2-2, Table 3). However, we did not detect any significant difference in \( I_{NaT} \) rise time and decay time in these two cell types. For example, when stepped from –100 mV to 0 mV, the 10-90% rise time was 85 ± 5 µs for SNr GABA neurons \( n = 12 \) and 84 ± 5 µs for nigral DA neurons \( n = 9 \); the 10-90% decay time was 191 ± 12 µs for SNr GABA neurons and 195 ± 15 µs for nigral DA neurons.

**Similar steady-state inactivation of \( I_{NaT} \) in SNr GABA neurons and nigral DA neurons**

Next, we studied the steady-state inactivation of \( I_{NaT} \) in SNr GABA and nigral DA neurons. Representative traces evoked by a steady-state inactivation protocol were shown in Fig. 6B. \( I_{NaT} \) in SNr GABA neurons and nigral DA neurons showed a similar steady-state inactivation. Boltzmann equation fitting revealed that the slope factor of steady-state inactivation was 8.1 ± 0.5 for SNr GABA neurons \( n = 12 \) and 10.1 ± 1.6 for nigral DA neurons \( n = 9 \), respectively \( p > 0.05 \). The midpoint potential of steady-state inactivation was –63.3 ± 1.3 mV for SNr GABA neurons \( n = 12 \) and –61.3 ± 2.5 mV for nigral DA neurons \( n = 9 \), respectively \( p > 0.05 \). These results show that steady-state inactivation of \( I_{NaT} \) did not differ between the two types of neurons.

**\( I_{NaT} \) recovery from inactivation is faster in SNr GABA neurons than in nigral DA neurons**

Fast recovery of \( I_{NaT} \) from inactivation is essential for high frequency repetitive firing. Based on the fact that the spike amplitudes decline less in SNr GABA neurons than in nigral DA neuron (Fig. 1D, E), we hypothesized that \( I_{NaT} \) may recover more rapidly from inactivation such that more functional Na\(_V\) channels are available in SNr GABA neurons than in nigral DA neurons. We examined the recovery time course from inactivation with a double-pulse protocol (Fig. 7A): the holding potential was –90 mV, then a 50 ms prepulse at –120 mV, then a 300 ms pulse at 0 mV to completely inactivate \( I_{Na} \), then stepping to –120 mV for increasing duration \( (\Delta t) \) to allow \( I_{NaT} \) recover, and finally
a 20 ms test pulse at 0 mV (Fig 7A). We found that the time course for \( I_{\text{NaT}} \) to recover from inactivation was bi-exponential in both SNr GABA neurons and SNc DA neurons (Fig. 7B-D). The fast recovery component contributed 52.6 ± 5.7% and 35.8 ± 3.4% (recovered within 5 ms) to the total current in SNr GABA neurons and nigral DA neurons, respectively. Mostly importantly, \( I_{\text{NaT}} \) recovered faster in SNr GABA neurons than in SNc DA neurons. The fast recovery \( \tau_f \) was 0.59 ± 0.07 ms for SNr GABA neurons (n = 5) and 1.15 ± 0.1 ms for SNc DA neurons (n = 5, p < 0.05). The slow recovery \( \tau_s \) was 35.1 ± 6.4 ms for SNr GABA neurons (n = 5) and 79.7 ± 13.1 ms for SNc DA neurons (n = 5, p < 0.05, Table 3). These results indicate that \( I_{\text{NaT}} \) in the fast spiking SNr GABA neurons recovered more rapidly from inactivation than that in the slow spiking SNc DA neurons.

Because of the different recoveries from inactivation, we reasoned that compared with nigral DA neurons, SNr GABA neurons may incur less cumulative \( I_{\text{NaT}} \) inactivation during repetitive depolarization. To test this idea, we performed the experiment shown in Fig. 8. The membrane patch was held at −70 mV, then stepped to a train of 5 pulses (3 ms at 0 mV with an inter-pulse interval of 100 ms at −70 mV, Fig. 8A). The ratio of the \( I_{\text{NaT}} \) evoked by a pulse over that evoked by the first pulse, \( I_r/I_1 \), was used as a measure of cumulative inactivation. The ratio becomes small when cumulative inactivation is large. As illustrated in Fig. 8B-D, by the third pulse, the ratio was already significantly larger in SNr GABA neurons (n = 6) than in SNc DA neurons (n = 5), though without reaching statistical significance. By the fourth pulse, the difference was statistically significant (p < 0.05). These results indicate that during repetitive depolarization such as spike firing, \( \text{Na}_V \) channels undergo less cumulative activation in SNr GABA neurons than in nigral DA neurons. This difference may contribute to the non-adaptive spiking in SNr GABA neurons and the adaptive spiking in nigral DA neurons, respectively (Fig. 1D, E).

\( I_{\text{NaT}} \) deactivation is faster in SNr GABA neurons than in nigral DA neurons

\( \text{Na}_V \) channels with slower deactivation kinetics can conduct longer-lasting inward current upon membrane repolarization and prolong action potential duration. We compared the deactivation properties of \( I_{\text{NaT}} \) in SNr GABA neurons and nigral DA neurons. As illustrated in Fig. 9D, \( I_{\text{NaT}} \) was evoked by a 200 μs pulse to 0 mV. Based on our data on \( I_{\text{NaT}} \) activation (Fig. 6A2,A3), at the end of a 200 μs pulse at 0 mV, \( I_{\text{NaT}} \) and thus \( \text{Na}_V \) channel opening were at or near their peak. To deactivate \( \text{Na}_V \) channels, the membrane was stepped to −100 mV through −20 mV in a 10 mV step. As shown in Fig. 9A,B, \( I_{\text{NaT}} \) deactivated very quickly as reflected by the rapid decay of the tail currents in
both neuron types. These tail currents were well fitted with a single exponential function. At very negative membrane potentials (~100 mV and ~90 mV), the deactivation was only slightly faster in SNr GABA neurons than in nigral DA neurons. For example, the deactivation time constant at ~100 mV was 48.1 ± 2.6 µs for SNr GABA neurons (n = 7) and 54.9 ± 5.3 µs for SNc DA neurons (n = 6), but the difference was not statistically significant (p > 0.05). When the deactivation pulse became more positive, particularly at ~50 mV to ~20 mV that is in the range of action potential repolarization, the deactivation time course became significantly faster in SNr GABA neurons than in SNc DA neurons (Fig. 9A-C). For example, at ~40 mV, the deactivation time constant was 99 ± 8.9 µs for SNr GABA neurons (n = 7) and 127.8 ± 12.2 µs for nigral DA neurons (n = 6, p < 0.01). The faster deactivation of I_{NaT} together with a robust Kv3-like current (Ding et al. 2011) can thus repolarize the membrane more quickly in the fast-spiking SNr GABA neurons than in slow-spiking SNc DA neurons (see Fig. 1).

**Resurgent sodium current is larger in SNr GABA neurons than in nigral DA neurons**

Resurgent Na current (I_{NaR}) is generated during membrane repolarization after depolarization and inactivation of I_{NaT} (Raman and Bean 1997; Grieco et al. 2005; Bant and Raman 2010). I_{NaR} is much smaller but longer-lasting than I_{NaT}. Na_{V}1.6 (α6) and Na_{V}β4 (β4) are critical for I_{NaR} generation (Do and Bean 2004; Grieco and Raman 2004; Grieco et al. 2005; Bant and Raman 2010). In cerebellar Purkinje neurons and granule neurons and the GABA neurons in the globus pallidus, the Na_{v} channels containing Na_{V}1.6 and/or Na_{v}β4 have a resurgent property that generates a resurgent Na current (I_{NaR}) after the inactivation of the I_{NaT} and upon repolarization (Raman and Bean 1997; Khaliq et al. 2003; Mercer et al. 2007; Bant and Raman 2010). I_{NaR} has been suggested to facilitate fast spiking. Our qRT-PCR data (Fig. 3) indicated that Na_{V}β4 mRNA is expressed at a higher level in SNR GABA neurons than in nigral DA neurons. Using a voltage protocol illustrated in Fig. 10D, we observed I_{NaR} in both SNr GABA neurons and SNc DA neurons (Fig. 10A,B). Consistent with our qRT-PCR data that compared with nigral DA neurons, SNr GABA neurons had more Na_{v}1.6 and Na_{v}β4 subunits expression, our nucleated patch clamp recordings showed that I_{NaR} was larger in SNr GABA neurons than in nigral DA neurons (Fig. 10, p < 0.01, Table 3). Na_{v}1.6 and Na_{v}β4 subunits expression has been reported to be the main subunits responsible for large resurgent
sodium current in other types of neurons (Grieco et al. 2004; Rush et al. 2005; Aman and Raman 2007; Mercer et al. 2007; Bant and Raman 2010).

**Persistent Na current ($I_{NaP}$) is larger in SNr GABA neurons than in nigral DA neurons**

Separate studies have examined $I_{NaP}$ in SNr GABA neurons (Atherton & Bevan. 2005) and SNc DA neurons (Puopolo et al. 2007). But a direct, side-by-side comparison of $I_{NaP}$ between the two types of neurons was lacking. So we set out to compare the amplitude of $I_{NaP}$ and their possible roles in firing properties and membrane potentials between SNr GABA neurons and SN DA neurons. Since $I_{NaP}$ was slow, conventional whole cell patch clamp recording was suitable. We used the CsCl-based intracellular solution (see Methods section) to block K+ channels. We also used a slow 4 s linear voltage ramp from –80 mV to 0 mV to inactivate the $I_{NaT}$ (Fig. 11A) (Gorelova and Yang 2000; Koizumi and Smith 2008). Since $I_{NaP}$ is known to be blocked by 1 μM TTX (French et al. 1990; Crill 1996; Gorelova and Yang 2000; Koizumi and Smith 2008), the slow TTX-sensitive current was taken as the $I_{NaP}$. After obtaining a stable baseline recording, 1 μM TTX was bath-applied. As shown in Fig. 11, a quite large TTX-sensitive, slow or “persistent” sodium current ($I_{NaP}$) was recorded in both SNr GABA neurons and nigral DA neurons (Fig. 11A2,A3). $I_{NaP}$ started to activate around –60 mV and peaked around –40 mV. The peak amplitude of $I_{NaP}$ was 185.4 ± 17.5 pA in 7 SNr GABA neurons and 121.3 ± 12.7 pA in 6 SNc SN DA neurons (p < 0.05, Fig. 11A4, Table 3). To explore the functional roles of $I_{NaP}$, we recorded the membrane potential and action potential firing in conventional whole cell mode with the 135 mM KCl-based intracellular solution and the normal extracellular solution (see Methods). After obtaining a stable baseline, 10 μM riluzole, an established $I_{NaP}$ blocker (Urbani and Belluzzi 2000; Koizumi and Smith 2008), was bath-applied. As shown in Fig. 11B, bath application of 10 μM riluzole induced a 11.9 ± 1.1 mV hyperpolarization from its baseline potential around –50 mV in SNr GABA neurons (n = 6) and a 7.3 ± 0.6 mV hyperpolarization from its baseline potential around –50 mV in nigral DA neurons (n = 5), leading to a cessation of spontaneous firing in both neuron types. Since the slow hyperpolarization is not likely due to a potential riluzole inhibition of $I_{NaT}$ (Urbani and Belluzzi 2000), these results indicate that $I_{NaP}$ was active at the baseline membrane potential around –50 mV in SNr GABA neurons and nigral DA neurons. The larger $I_{NaP}$ in SNr GABA neurons may contribute to the faster pacemaking activity in SNr GABA neurons than in SN DA neurons.
To verify that our conclusions based on recordings made at 30 °C and filtered at 10 kHz were correct, additional experiments were performed at room temperature (25 °C), low-pass filtered at 30 kHz and sampled at 200 kHz. \( I_{\text{NaT}} \) activation, decay and deactivation were re-examined because these parameters were fast and thus sensitive to recording temperature and signal filtering. As shown in Figs. 12 and 13 and Table 4, although slower than at 30 °C, the kinetic differences seen at 30 °C between these two cell types remained at room temperature, while the kinetic parameters that were similar at 30 °C were still similar at room temperature. The only change in \( I_{\text{NaT}} \) kinetics that needs to be mentioned in particular is that the 10-90% decay time was prolonged more substantially than the 10-90% rise time. Though the mechanisms are not clear, differential temperature effects on the rise and the decay of \( I_{\text{NaT}} \) have been observed (Sah et al. 1988; Magatomo et al. 1998). The \( I_{\text{NaT}} \) density is similarly reduced in both cell types at room temperature compared with that at 30 °C, consistent with literature data (Sah et al. 1988). These data indicate that the kinetics differences in \( I_{\text{NaT}} \) currents in nigral DA neurons and SNr GABA neurons observed at 30 °C may be extrapolated to 37 °C, the normal body temperature, and thus are physiologically important.
DISCUSSION

The main findings of this study are that compared with slow-spiking nigral DA neurons, fast-spiking SNr GABA neurons had higher gene expression levels for \( \text{NaV}_\alpha 1.1, \text{NaV}_\alpha 1.6 \text{NaV}_\beta 1 \) and \( \text{NaV}_\beta 4 \); and larger amplitudes of \( I_{\text{NaT}}, I_{\text{NaP}}, \) and \( I_{\text{NaR}}. \) \( I_{\text{NaT}} \) also had a steeper voltage-dependent activation, a faster deactivation and a faster recovery from inactivation in SNr GABA neurons than in nigral DA neurons. These differences in \( \text{NaV} \) currents may contribute to the striking differences in spiking properties in these two cell types.

Different \( \text{NaV} \) mRNA expression in SNr GABA neurons and nigral DA neurons

The strikingly different spike waveform and spiking pattern in SNr GABA neurons and nigral DA neurons suggest possible molecular and functional differences in \( \text{NaV} \) channels between the two neuron types. Our qualitative scRT-PCR analysis revealed similar types of \( \text{NaV} \) mRNAs in SNr GABA neurons and nigral DA neurons, indicating the two cell types probably express qualitatively similar but quantitatively different \( \text{NaV} \) subunits, leading to the formation of \( \text{NaV} \) channels with different subunit composition, particularly with different regulatory \( \beta \) subunits. Indeed, using qRT-PCR on immunochemically identified SNr GABA and SNc DA neurons, we found that SNr GABA neurons had higher expression levels of \( \text{NaV}_1.1, \text{NaV}_1.6, \text{NaV}_\beta 1 \) and \( \text{NaV}_\beta 4 \) and lower expression levels of \( \text{NaV}_1.2, \text{NaV}_\beta 2 \) and \( \text{NaV}_\beta 3 \) than SN DA neuron (Fig. 3). The high \( \text{NaV}_\beta 4 \) level in SNr GABA neurons compared with SNc DA neurons is also consistent with a histochemical study that detected high \( \text{NaV}_\beta 4 \) mRNA in SNr but not SNc (Yu et al. 2003). These differences in \( \text{NaV} \) channel expression levels may affect \( \text{NaV} \) channel expression density and also subunit composition and hence \( \text{NaV} \) channel properties and function. Specifically, the higher \( \text{NaV}_1.1 \) and \( \text{NaV}_1.6 \) mRNA expression together with \( \text{NaV}_\beta 1 \) and \( \text{NaV}_\beta 4 \) mRNA expression likely contributes to the larger amplitude or higher density of \( I_{\text{NaT}}, I_{\text{NaP}}, \) and \( I_{\text{NaR}} \) in SNr GABA neurons than in nigral DA neurons. \( \text{NaV}_\beta 1 \) may enhance \( \text{NaV} \) channel cell surface expression and increase \( I_{\text{NaT}} \) (Isom et al. 1992, 1995; McEwen et al. 2004). \( \text{NaV}_\beta 1 \) may also promote fast gating for \( \text{NaV}_\alpha \) subunits (Goldin 2001). Recent studies indicate that \( \text{NaV}_\beta 4 \) may interact with \( \text{NaV}_\alpha \) subunit and enhance \( I_{\text{NaP}} \) and \( I_{\text{NaR}} \) (Grieco et al. 2005; Bant and Raman 2010), and will be further discussed below. The significance of higher \( \text{NaV}_1.2, \text{NaV}_\beta 2 \) and \( \text{NaV}_\beta 3 \) mRNA expression in DA neurons than in GABA neurons is not clear. One possibility is that our semi-quantitative qRT-PCR only compared the relative abundance of \( \text{NaV}_1.2 \) mRNA
between the two cell types and did not compare the relative abundance of Na\textsubscript{v}1.1 mRNA, Na\textsubscript{v}1.2 mRNA, and Na\textsubscript{v}1.6 mRNA within each cell type. Thus, it is possible that Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 are the dominant Na\textsubscript{v} subunits such that the difference in Na\textsubscript{v}1.2 is not important. Alternatively, we speculate that while Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 are the major \( \alpha \) subunits with Na\textsubscript{v}β1 and Na\textsubscript{v}β4 as the regulatory subunits in SNr GABA neurons, Na\textsubscript{v}1.2 and Na\textsubscript{v}1.6 may be the major \( \alpha \) subunits with Na\textsubscript{v}β2 and Na\textsubscript{v}β3 as the regulatory subunits in nigral DA neurons. Future molecular studies are needed to determine the absolute mRNA expression levels for these different Na\textsubscript{v} subunits and Na\textsubscript{v} channel protein subunit composition.

Different \( I_{NaT} \) density and kinetics in SNr GABA neuron and nigral DA neuron

In the present study we found that \( I_{NaT} \) and conductance density were considerably higher in fast spiking SNr GABA neurons than in slow spiking nigral DA neurons: 2481.8 \( \pm \) 296.2 pS/pF vs. 1433.5 \( \pm \) 234.2 pS/pF (Fig. 5, Table 3). This may be due to higher expression of Na\textsubscript{v}1.1 and Na\textsubscript{v}1.6 and also auxiliary Na\textsubscript{v}β1 and Na\textsubscript{v}β4. The higher \( I_{NaT} \) density may contribute to the faster rise and larger amplitude of the action potentials in SNr GABA neurons.

In addition to the difference in \( I_{NaT} \) density, we also detected differences in \( I_{NaT} \) kinetics between SNr GABA neurons and SNc DA neurons. To compare the very fast kinetics of \( I_{NaT} \), we used the nucleated patch clamp techniques that provide outstanding space and voltage clamp (Martina and Jonas 1997; Ding et al. 2011). We found that the activation and inactivation time courses of \( I_{NaT} \) were similar in SNr GABA neurons and nigral DA neurons (Fig. 6, Table 3). However, \( I_{NaT} \) had a steeper voltage-dependent activation and a faster deactivation in SNr GABA neurons than those in nigral DA neurons (Figs. 6 and 9, Table 3), potentially contributing to the fast rise and short duration of spikes in the fast spiking SNr GABA neurons. \( I_{NaT} \) also recovered more quickly from inactivation in SNr GABA neurons than in SN DA neurons, leading to less cumulative inactivation in SNr GABA neurons. These results are consistent with the combination of our qPCR data that SNr GABA neurons express more Na\textsubscript{v}1.6 and less Na\textsubscript{v}1.2 than DA neurons (Fig. 3) and published results that Na\textsubscript{v}1.2 undergo more cumulative inactivation than Na\textsubscript{v}1.6 in peripheral neurons (Rush et al. 2005). The different rate of recovery from inactivation may contribute to the fact that spike firing was not or only slightly adaptive in amplitude in SNr GABA neurons whereas it was strongly adaptive in SNc DA neurons (Fig. 1D, E).
The differences in $I_{\text{NaT}}$ kinetics between fast-spiking SNr GABA neurons and slow-spiking DA were relatively modest (Table 3). This is consistent with published studies that different heterologously expressed Na$_v$ channel subunits or isoforms display only minor or subtle differences in their kinetic properties (Smith and Goldin 1998; Goldin 2001). However, subtle differences in $I_{\text{NaT}}$ kinetics may be sufficient to affect spike initiation and waveform (Engel and Jonas 2005; Hu et al. 2009). Parts of our results on $I_{\text{NaT}}$ ($I_{\text{NaV}}$ density and inactivation time course) are also consistent with a recent study (Seutin and Engel 2010). However, some of our results on $I_{\text{NaT}}$ kinetics are in contrast with Seutin and Engel (2010). For example, Seutin and Engel (2010) indicated a faster rise time for $I_{\text{NaT}}$ in GABA neurons than in DA neurons, whereas our data indicated a similar rise time for $I_{\text{NaT}}$ for both cell types. Seutin and Engel (2010) also indicated that there was no difference in activation, deactivation and recovery from inactivation, whereas we found differences in these three parameters. Specifically, Seutin and Engel (2010) reported that the mid-point potentials of activation were $-9.6 \text{ mV}$ and $-12.6 \text{ mV}$ for DA and GABA neurons respectively. These 2 values are over $10 \text{ mV}$ more positive than the $V_{1/2}$ of $I_{\text{NaT}}$ activation reported for multiple types of neurons (Martina and Jonas 1997; Raman et al. 2000; Colbert and Pan 2002; Maurice et al. 2004; Gittis and Lac 2008; Hu et al. 2009). Further, Seutin and Engel (2010) reported that the mid-point potential of steady-state inactivation was more positive ($-49 \text{ mV}$) in DA neurons than in GABA neurons ($-56 \text{ mV}$), indicating that Na$_v$ channels in DA neurons are less likely to inactivate, whereas we found there was no significant difference in this parameter: $-63.3 \text{ mV}$ for SNr GABA neurons and $-61.3 \text{ mV}$ for nigral DA neurons. $I_{\text{NaT}}$ recovery from inactivation is important and needs to be discussed. Seutin and Engel (2010) also found no difference in time course of recovery from fast inactivation among the two cell types, while we found that $I_{\text{NaT}}$ recovered more quickly from both fast and slow inactivation in SNr GABA neurons than in nigral DA neurons. We also found that there was less cumulative inactivation in $I_{\text{NaT}}$ in SNr GABA neurons than in SNc DA neurons, indicating that more Na$_v$ channels become available after action potential firing and the refractory period is shorter. Another discrepancy between our present study and Seutin and Engel (2010) is the deactivation time course. Our data indicate a faster deactivation in SNr GABA neurons than in nigral DA neurons (Fig. 9), whereas Seutin and Engel (2010) reported no difference. We believe that this discrepancy can be explained by the fact that we used a different deactivation protocol. We first used the same protocol with a 300 μs at 0 mV activation pulse used by Seutin and Engel (2010) and saw no apparent
difference in deactivation (data not shown). However, we noticed that at the end of 300 μs at 0 mV, $I_{NaT}$ had inactivated significantly. Thus, 300 μs at 0 mV activation pulse was too long for studying deactivation under our recording conditions. Based on our activation data, $I_{NaT}$ was at its peak at 200 μs after stepping to 0 mV. Thus, we shortened the activation part to 200 μs in the deactivation protocol and detected the faster deactivation in SNr GABA neurons. In our opinion, our data on $I_{NaT}$ kinetics can better explain the differences in spiking properties in fast spiking SNr GABA neurons and slow spiking DA neurons. For example, Na V channels with a steeper activation slope, faster deactivation, and faster recovery from inactivation can support the fast spiking in SNr GABA neurons. Certainly, independent studies are required to resolve these discrepancies.

Larger resurgent sodium current ($I_{NaR}$) in SNr GABA neurons than in SN DA neurons

The resurgent Na current ($I_{NaR}$) is generated during membrane repolarization after depolarization and inactivation of $I_{NaT}$ (Raman and Bean 1997). Particularly, Na Vβ4 may act as an open channel blocker, bind to the open Na V channels upon depolarization. Upon repolarization, NaVβ4 peptide unbinds and allows Na$^+$ flow briefly, thereby producing $I_{NaR}$ (Grieco et al. 2005; Bant and Raman. 2010). $I_{NaR}$ is much smaller but longer-lasting than $I_{NaT}$. Evidence indicates that NaV1.6 (α6) and NaVβ4 (β4) are critical for $I_{NaR}$ generation (Raman et al. 1997; Do and Bean 2004; Grieco and Raman 2004; Grieco et al. 2005; Bant and Raman 2010). Since $I_{NaR}$ is induced upon repolarization, it may promote repetitive firing (Khaliq et al. 2003).

Our qRT-PCR data indicated that NaV1.6 and NaVβ4 mRNA are expressed at higher levels in SNr GABA neurons than in nigral DA neurons (Fig. 3). In addition, our nucleated patch clamp recordings showed that $I_{NaR}$ was larger in SNr GABA neurons than in nigral DA neurons (Fig. 10). These results are consistent with reports suggesting that NaV1.6 and NaVβ4 subunits are the key subunits responsible for generating $I_{NaR}$ in other neuron types (Raman et al. 1997; Grieco et al. 2004; Rush et al. 2005; Aman and Raman 2007; Mercer et al. 2007; Bant and Raman 2010).

Larger persistent sodium current in SNr GABA neurons than in SN DA neurons

Although the precise mechanisms generating $I_{NaP}$ are not clear, studies indicate that $I_{NaP}$ may be generated by the same Na V channels that generate the $I_{NaT}$ but via
different gating mechanisms that allow a small fraction of Na\_v channels remain open for long periods of time (Patlak and Ortiz 1986; Alzheimer et al. 1993; Crill 1996; Taddese and Bean 2002; Bant and Raman 2010). Evidence also indicates that Na\_v\_β4 (β4) subunit, when co-expressed Na\_v1.1 (α1) subunit, promotes the generation of I\_Na\_P and I\_Na\_R (Aman et al. 2009; Bant and Raman 2010). Although the amplitude of I\_Na\_P is only a fraction of I\_Na\_T, its duration is much longer than that of I\_Na\_T such that it can enhance neuronal excitability (Crill 1996). Consequently I\_Na\_P is involved in pacemaking activity in many types of neurons (Taddese and Bean 2002; Do and Bean 2003; Jackson et al. 2004; Swensen and Bean 2003; Mercer et al. 2007; Puopolo et al. 2007; Koizumi and Smith 2008; Khaliq and Bean 2010). Previous studies also examined I\_Na\_P in SNr GABA neurons and SNc DA neurons (Atherton and Bevan 2005; Puopolo et al. 2007; Chan et al. 2007), although a direct side-by-side comparison of I\_Na\_P in these two neuron types was lacking. Based on our qRT-PCR results we predicted larger persistent sodium current in SNr GABA neurons than SN DA neurons and our experiment proved this hypothesis. We also found that persistent sodium current took part in maintaining the membrane potential at a relatively depolarized range in addition to pacemaking. Blocking persistent sodium current with 10 µM riluzole caused a larger hyperpolarization in SNr GABA neurons than SN DA neurons. Those results indicated that persistent sodium current played an important role in shaping the different firing patterns between SNr GABA neurons and SN DA neurons.

Our electrophysiological results are consistent with our qRT-PCR data that SNr GABA neurons had higher expression of Na\_v1.1 and Na\_v1.6 than SN DA neurons while SN DA neurons had higher expression of Na\_v1.2 than SNr GABA neurons. These results were also consistent with literature data that heterologously expressed Na\_v1.1 and Na\_v1.6 conduct larger persistent sodium currents than Na\_v1.2 (Smith et al. 1998).

Functional implications of differences in Na\_v currents in fast-spiking SNr GABA neurons and slow-spiking nigral DA neurons

According to our present study, I\_Na\_T has a higher density and a steeper voltage-dependent activation in SNr GABA neurons than in nigral DA neurons. These two properties can contribute to the faster rise rate and the larger amplitude of action potentials in SNr GABA neurons than in nigral DA neurons (Fig. 1C-F). Equally important, compared with nigral DA neurons, I\_Na\_T in SNr GABA neurons recovered more quickly from inactivation and resisted cumulative inactivation, thus enabling or
supporting the sustained high frequency firing in SNr GABA neurons. The large $I_{\text{NaP}}$ in SNr GABA neurons that activates at or below –60 mV may drive the membrane potential toward the $I_{\text{NaT}}$ activation threshold potential around –50 mV, triggering action potentials. We have previously indicated that SNr GABA neurons have constitutively active TRPC3 cation channels that can depolarize SNr GABA neurons even when they are at very negative membrane potentials (Zhou et al. 2008). On the hand, DA neurons have a prominent $I_h$ cation current that can depolarize the neurons to ~ –65 mV, the threshold for $I_{\text{NaP}}$ activation (Fig. 1B; Fig. 10A). SNr GABA neurons also have a robust expression of Kv3-like channels that can quickly repolarize the membrane after each action potential (Ding et al. 2011). Combination of differential expression of these non-selective cation channels, Kv channels and NaV channels can support the strikingly different spike waveforms and spiking patterns in SNr GABA neurons and nigral DA neurons.
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Figure legends

Fig. 1. Electrophysiological characteristics of SNr GABA neurons and nigral DA neurons.


B: Electrophysiological properties of nigral DA neurons. Current-clamp recordings show spontaneous low frequency pacemaker activity and prominent I_h-mediated depolarizing sag (arrow) in response to hyperpolarizing current injection. 

C: Overlay of a GABA neuron spike and a DA neuron spike, showing the clear differences in the spike waveform between the two cell types. 

D: SNr GABA neurons show little firing frequency and amplitude adaptation to depolarizing current injection (150 pA). 

E: nigral DA neurons show prominent firing frequency and spike amplitude adaptation to depolarizing current injection (100 pA). Recordings were made with a 135 mM KCl-based intracellular solution.

Fig. 2. scRT-PCR detection of Na_v mRNAs in SNr GABA neurons and nigral DA neurons.

A: Na_v mRNAs detected in electrophysiologically identified SNr GABA neurons. No TH mRNA was detected in any of these neurons. Numbers in the parentheses are the expected amplicon sizes in base pair. 

B: Na_v mRNAs detected in electrophysiologically identified nigral DA neurons. The expected amplicon sizes are the same as in A. 

C: Summary of scRT-PCR detection ratio of Na_v channel mRNAs in SNr GABA neurons and nigral DA neurons. For each Na_v mRNA, 5-10 TH mRNA-positive DA neurons and 5-10 GAD1 mRNA-positive neurons were tested. In this figure and Fig. 3, the Na_v α subunits are denoted as α1, α2, α3 and α6 while the standard nomenclature (NaV1.1, NaV1.2, NaV1.3 and NaV1.6) is used in the main text (Catterall et al. 2005).

Fig. 3. qRT-PCR analysis of Na_v channel mRNAs in laser microdissection-captured, immunofluorescence-identified SNr GABA neurons and nigral DA neurons. β-actin mRNA was used as internal control. mRNA semi-quantification was performed using the comparative Cp method in the form of 2^{-(delta)C_p}. 2^{-(delta)C_p} values for Na_v mRNAs were normalized to those in SNr GABA neurons. NaV1.1, NaV1.6, NaVβ1, and NaVβ4 mRNAs were higher in SNr GABA neurons than in nigral DA neurons, whereas NaV1.2, NaVβ2 and NaVβ3 mRNAs were lower in SNr GABA neurons than in nigral DA neurons. The
difference in the expression of these NaV genes between SNr GABA neurons and nigral DA neurons was significant with p<0.005.

**Fig. 4.** Electrophysiological identification of SNr GABA neurons and nigral DA neurons with 135 mM CsCl-based intracellular solution and nucleated patch formation. **A:** Electrophysiological properties of SNr GABA neurons. Current-clamp recordings within 10s after obtaining access to the cell interior show spontaneous high frequency activity and no Ih-mediated sag in response to hyperpolarizing current injection (-200 pA). Due to Cs+ infusion, the spikes were broadened and the firing frequency was increased. **B:** Electrophysiological properties of nigral DA neurons. Current-clamp recordings within 10s after obtaining access to the cell interior show spontaneous low frequency activity and prominent Ih-mediated sag in response to hyperpolarizing current injection. The spikes were broadened and the firing frequency was increased due to Cs+ infusion. Despite Cs+ infusion, the differences in membrane properties are still striking within the first 10 s. **C, D, E:** are sequential images demonstrating the formation of nucleated somatic membrane patch. **C** shows conventional whole cell patch clamp to electrophysiologically fingerprint SNr GABA and nigral DA neurons. **D** shows that gentle negative pressure was being applied and the patch pipette was being withdrawn slowly to form nucleated patches. Arrow indicates the remaining part of the neuron. Arrowhead points to the round nucleated membrane patch. **E:** Spherical nucleated patches free of axon and dendritic processes was formed as demonstrated by including 20 μM fluorescent Alexa 594 in the pipette solution.

**Fig. 5.** Higher INaT density in SNr GABA neurons than in nigral DA neurons. INaT was evoked from a holding potential of −100 mV by a test potential ranging −80 mV to +30 mV in 5 mV steps. INaT started to appear at −55 mV. For example current traces, see Fig. 6A, because **Fig. 5** and **Fig. 6A** were derived from the same data set. The reversal potential was obtained by linear-fitting the data points between 0 mV and 30 mV (dotted lines), according to the method of Safronov and Vogel (1995) and Raman et al. (2000). The estimated reversal potential was 49.8 mV for GABA neurons and 49.9 mV for DA neurons.

**Fig. 6.** Voltage-dependent activation and steady-state inactivation of INaT in SNr GABA and nigral DA neurons. **A, A1:** Activation voltage waveform to evoke INaT. **A2, A3:**
Representative traces of $I_{\text{NaT}}$ evoked in nucleated membrane patches isolated from a SNr GABA neuron (A2) and a nigral DA neuron (A3). A4: Activation curves of $I_{\text{NaT}}$ in SNr GABA neurons (filled circles) ($n = 12$) and nigral DA neurons (open circle) ($n = 9$). Continuous lines are Boltzmann fits. Data points below –60 mV and above 5 mV are not displayed for showing the difference between the two curves more clearly. B, B1: Voltage waveform to induce and determine $I_{\text{NaT}}$ steady-state inactivation. B2, B3: Representative traces of $I_{\text{NaT}}$ evoked by the steady-state inactivation protocol in nucleated membrane patches isolated from a SNr GABA neuron (B2) and a nigral DA neuron (B3). B4: Steady-state inactivation curves of $I_{\text{NaT}}$ in SNr GABA neurons (filled circles) ($n = 12$) and nigral DA neurons (open circle) ($n = 9$). Continuous lines are Boltzmann fits.

**Fig. 7.** $I_{\text{NaT}}$ recovered more rapidly from inactivation in SNr GABA neurons than in SNc DA neurons. A: Voltage protocol for recovery from a 300 ms-induced inactivation. B, C: Representative traces of recovery from inactivation in a SNr GABA neuron nucleated patch (B) and a SNc DA neuron nucleated patch (C). D: Pooled data on $I_{\text{NaT}}$ recovery time course from inactivation by a 300-ms conditioning pulse. Curves represent sums of two exponential fits. Filled circles are nucleated patches from SNr GABA neuron ($n = 5$). Open circles are nucleated patches from SNc DA neuron ($n = 5$).

**Fig. 8.** Less cumulative $I_{\text{NaT}}$ inactivation in SNr GABA neurons than in SN DA neurons. A: Voltage protocol: holding potential, -70 mV; 3 ms test pulses to 0 mV at 100 ms interval. B, C: Representative traces of cumulative $I_{\text{NaT}}$ inactivation in nucleated membrane patches from a SNr GABA neuron (B) and SNc DA neuron (C). D: Pooled data on $I_{\text{NaT}}$ cumulative inactivation. Peak $I_{\text{NaT}}$ was normalized to the $I_{\text{NaT}}$ evoked by the first test pulse.

**Fig. 9.** $I_{\text{NaT}}$ deactivation is faster in SNr GABA neurons than in SNc DA neurons. A, B: Representative traces of $I_{\text{NaT}}$ deactivation in nucleated membrane patches from a SNr GABA neurons (A) and a SNc DA neuron (B). The two traces at –40 mV are displayed in A’ and B’ to show more clearly $I_{\text{NaT}}$ deactivation at –40 mV. The smooth gray curves are single exponential fits. C: Pooled data for deactivation time constants plotted against deactivation potentials. Filled circle: SNr GABA neurons. Open circle: SNc DA neurons.
The time constants were shorter at –80 to –20 mV, p< 0.05. 

**D:** Voltage waveform used to evoke $I_{NaT}$ deactivation. Holding potential, -90 mV; 50 ms pulse to –120 mV; 200 μs pulse to 0 mV; 50 ms test pulse from –100 mV to –20 mV at 10 mV increase.

**Fig. 10.** $I_{NaR}$ is larger in SNr GABA neurons than in SNc DA neurons. 

A, B: Representative traces of $I_{NaR}$ in nucleated membrane patches from a SNr GABA neuron (A1) and a SNc DA neuron (B1). The boxed areas in A1 and B1 are expanded and displayed in A2 and B2, respectively. 

C: polled data from SNr GABA neurons (n = 5) and SNc DA neurons (n = 4). The difference was significant (p < 0.05). 

D: Voltage waveform used to generate $I_{NaR}$. Holding potential between the pulses was –90 mV.

**Figure 11.** $I_{NaP}$ is larger in SNr GABA neurons than in SNc DA neurons. 

A: Voltage clamp data on $I_{NaP}$. 

A1: diagram showing the voltage ramp waveform consisting of a 4-s ramp from –80 mV to 0 mV. 

A2, A3: Representative current traces before (black) and after (red) perfusion of 1 μM TTX evoked by the voltage ramp, in GABA neurons (A2) and DA neurons (A3). TTX-sensitive $I_{NaP}$ (blue) was obtained by subtraction traces before after perfusion of 1 μM TTX. 

A4: Pooled data of peak $I_{NaP}$ in SNr GABA (n = 7) and SNc DA (n = 6) neurons. 

B: Current clamp data on $I_{NaP}$. 

B1, B2: Representative traces showing the effects of 10 μM riluzole in SNr GABA neurons (B1) and SNc DA neurons (B2). Bath perfusion of 10 μM riluzole induced a stronger hyperpolarization in GABA neurons than in DA neurons. Spike amplitudes were truncated due to slow re-sampling (1 kHz) for reducing the large data size of long recording segments. 

B3: Pooled data on 10 μM riluzole-induced hyperpolarization in SNr GABA neurons (n = 6) and SNc DA neurons (n = 5). The difference was significant (p < 0.05).

**Fig. 12.** Voltage-dependent activation of $I_{NaT}$ in SNr GABA and nigral DA neurons at room temperature (25 °C). 

A, B: Representative traces of $I_{NaT}$ evoked in nucleated membrane patches isolated from a SNr GABA neuron (A) and a nigral DA neuron (B). The same voltage protocol used in Fig. 6A was used. 

C: current-voltage plots for $I_{NaT}$ in SNr GABA neurons (n = 9) and nigral DA neurons (n = 11). 

D: $I_{NaT}$ activation curves in SNr GABA neurons (n = 9) and nigral DA neurons (n = 11). Continuous lines are Boltzmann fits.
Fig. 13. $I_{\text{NaT}}$ deactivation is faster in SNr GABA neurons than in SNc DA neurons at room temperature (25 °C). A, B: Representative traces of $I_{\text{NaT}}$ deactivation in nucleated membrane patches from a SNr GABA neurons (A) and a SNc DA neuron (B). The traces at –40 mV are displayed in A′ and B′ to show more clearly $I_{\text{NaT}}$ deactivation at –40 mV. The smooth gray curves are single exponential fits. C: Pooled data for deactivation time constants plotted against deactivation potentials. Filled circle: SNr GABA neurons (n = 4). Open circle: SNc DA neurons (n = 5). The time constants were shorter at –80 to –40 mV, p < 0.05. D: Voltage waveform used to evoke $I_{\text{NaT}}$ deactivation. Holding potential, –90 mV; 50 ms pulse to –120 mV; 200 μs pulse to 0 mV; 50 ms test pulse from –100 mV to –40 mV at 10 mV increase.
A GABA neuron

B DA neuron

C

D GABA neuron

E DA neuron

Membrane potential (mV)

Time (s)

-200 pA, 40 pA/step

-200 pA, 40 pA/step

2 ms

20 mV

Membrane potential

Time (s)

150 pA for 2 s

100 pA for 2 s
The image shows a bar graph comparing the expression levels of various Na\textsubscript{\text{v}} subunits in GABA neurons and DA neurons. The x-axis represents the subunits \(\alpha_1, \alpha_2, \alpha_6, \beta_1, \beta_2, \beta_3, \beta_4\), and the y-axis represents the mRNA expression normalized to GABA neuron level. The bars indicate higher expression in GABA neurons compared to DA neurons for some subunits.
A  Activation
A1  Activation voltage waveform
  30 mV 20 ms

A2  GABA neuron

A3  DA neuron

A4  Activation curve
  $G/G_{max}$ vs. Command voltage, mV

B  Steady-state inactivation
B1  S-S inactivation voltage waveform
  50 ms 0 mV 20 ms
  Pre-pulses

B2  GABA neuron

B3  DA neuron

B4  S-S inactivation curve
  $|I/I_{max}|$ vs. Pre-pulse, mV
A Recovery voltage protocol

B GABA neuron

C DA neuron

D Graph showing normalized current (I/\bar{I}) over time (\Delta t) for GABA and DA neurons.
A Voltage waveform
3 ms at 0 mV

B GABA neuron

C DA neuron

D Pooled data

\[
\frac{L_i}{L_f}
\]

Pulse number
1 2 3 4 5
**A** Voltage clamp

**A1** Ramp waveform

- $-80\,\text{mV}$
- $0\,\text{mV}$
- $4\,\text{s}$

**A2** GABA neuron

- $\text{TTX}$
- $\text{Pre-TTX}$

![Current vs. Potential Graph](A2_graph.png)

**A3** DA neuron

- $\text{Ramp potential, mV}$
- $\text{Current, pA}$

![Current vs. Potential Graph](A3_graph.png)

**A4**

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<tr>
<th>Peak current (pA)</th>
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**B** Current clamp

**B1** GABA neuron

- riluzole

![Current vs. Time Graph](B1_graph.png)

**B2** DA neuron

- riluzole

![Current vs. Time Graph](B2_graph.png)

**B3**

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<th>Hyperpolarization (mV)</th>
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A  GABA neuron

A'  Deactivation at -40 mV

\[ \tau = 0.14 \text{ ms} \]

B  DA neuron

B'  Deactivation at -40 mV

\[ \tau = 0.25 \text{ ms} \]

C

\begin{itemize}
  \item DA neuron
  \item GABA neuron
\end{itemize}

D  Deactivation waveform

0 mV 0.2 ms

-120 mV

-100 mV

Deactivation pulses
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<td>Parameters</td>
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<td>DA neuron</td>
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<td>Spontaneous firing rate (Hz)</td>
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<td>Amplitude (mV)</td>
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<td>Average rise rate (mV/ms)</td>
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<td>71.8±6.8</td>
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\[
l_{\text{H}}
\]
- Peak \( l_{H} \) density (at -10 mV) (pA/pF) | 148.9±17.8 | 86.0±14.1 | <0.01 |
- Activation 10-90% rise time (at 0 mV) (µs) | 85±5 | 84±5 | >0.05 |
- Activation \( V_{1/2} \) (mV) | -30.2±0.2 | -26.7±0.5 | <0.05 |
- Activation slope K | 6.2±0.2 | 7.3±0.3 | >0.05 |
- Inactivation 10-90% decay time (at 0 mV) (µs) | 191±12 | 195 ± 15 | >0.05 |
- Steady-state inactivation \( V_{1/2} \) (mV) | -63.3±1.3 | -61.3±2.5 | >0.05 |
- Steady-state inactivation slope K | 8.1±0.5 | 10.1±1.6 | >0.05 |

\[
l_{\text{K}}
\]
- Recovery from inactivation
  - \( \tau_{i} \) (ms) | 0.59±0.07 | 1.15±0.1 | <0.05 |
  - \( \tau_{s} \) (ms) | 35.1±6.4 | 79.7±13.1 | <0.05 |

\[
l_{\text{Na}}
\]
- Peak density (at -40 mV) (pA/pF) | 3.3±0.5 | 1.4±0.6 | <0.01 |

\[
l_{\text{Eh}}
\]
- Whole-cell peak amplitude (at -40 mV) (pA) | 185.4±17.5 | 121.3±12.7 | <0.01 |
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<th>Parameter</th>
<th>GABA neurons</th>
<th>DA neurons</th>
<th>Significance</th>
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