Spontaneous Activity and Properties of Two Types of Principal Neurons From the Ventral Tegmental Area of Rat

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We investigated the spontaneous activity and properties of freshly isolated ventral tegmental area (VTA) principal neurons by whole cell recording and single-cell RT-PCR. The VTA principal neurons, which were tyrosine hydroxylase-positive and glutamic acid decarboxylase (GAD67)-negative, exhibited low firing frequency and long action potential (AP) duration. The VTA principal neurons exhibited a calretinin-positive and parvalbumin-negative Ca\textsuperscript{2+}-binding protein mRNA expression pattern. The VTA principal neurons were classified into two subpopulations based on their firing frequency coefficient of variation (CV) at room temperature (21–23°C): irregular-type neurons with a large CV and tonic-type neurons with a small CV. These two firing patterns were also recorded at the temperature of 34°C and in nystatin-perforated patch recording. In VTA principal neurons, the AP afterhyperpolarization (AHP) amplitude contributed to the firing regularity and AHP decay slope contributed to the firing frequency. The AHP amplitude in the irregular-type VTA principal neurons was smaller than that in the tonic-type VTA principal neurons. There was no significant difference in the AHP decay slope between the two types of VTA principal neurons. Apamin-sensitive small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (SK) channels contributed to the AHP and the regular firing of the tonic-type neurons but contributed little to the AHP and firing of the irregular-type neurons. In voltage-clamp tail-current analysis, in both conventional and nystatin-perforated whole cell recording, the apamin-sensitive AHP current density of the tonic-type neurons was significantly larger than that of the irregular-type neurons. We suggest that apamin-sensitive SK current contributes to intrinsic firing differences between the two subpopulations of VTA principal neurons.

INTRODUCTION

The ventral tegmental area (VTA; A10) is located in the ventromedial region of the mesencephalon (Paxinos and Watson 1996) and is thought to be involved in motivated behavior (McBride et al. 1999; Spangagel and Weiss 1999). The neurons in the VTA have been classified into two major subclasses: principal neurons, which are dopaminergic (DAergic), and secondary neurons, which are γ-aminobutyric acidergic (GABAergic) (Grace and Onn 1989; Johnson and North 1992; Klink et al. 2001). The VTA principal neurons have been reported to have a slower firing frequency (1–8 Hz) and a longer action potential (AP) duration (2–4 ms) than the VTA secondary neurons (Grace and Onn 1989; Johnson and North 1992; Klink et al. 2001; Neuhoff et al. 2002). Morphologically, VTA principal neurons have been found to have a large cell soma with multi- or bipolar dendrite arborization (Grace and Onn 1989; Momiyama et al. 1996). Anatomically, VTA principal neurons have been found to project their axons mainly to the nucleus accumbens (NAcb; mesolimbic projection) and the prefrontal cortex (PFC; mesocortical projection) (Oades and Halliday 1987). It has been proposed that one mechanism that may be involved in mediating the reinforcement of behavior is an increase in the release of dopamine (DA) in the VTA target brain regions, especially the NAcb (McBride et al. 1999; Spangagel and Weiss 1999). In animal studies, the activity of midbrain DAergic neurons has been found to be preferentially enhanced by a reward or with stimuli that predict a subsequent reward (Hyland et al. 2002; Schultz 1998). The enhanced neuronal activity of the midbrain DAergic neurons has been found to be associated with a significant increase in DA release from the terminal regions of these neurons (Gonon 1988). Thus the excitation of VTA principal neurons is thought to be critical for the mediation of rewarded behavior.

There have been several studies of the mechanisms involved in spike firing in midbrain DAergic neurons. These neurons have been found to have intrinsic pacemaker activity, and several voltage- and Ca\textsuperscript{2+}-dependent ion channels have been found to underlie the generation of spontaneous APs and the regulation of AP intervals (Brodie et al. 1999; Grace and Onn 1989; Kang and Kitai 1993; Liss et al. 2001; Nedergaard et al. 1993; Ping and Shepard 1996; Silva et al. 1990; Wolfart et al. 2001; Yang et al. 2001). Persistent Na\textsuperscript{+} and Ca\textsuperscript{2+} currents have been found to drive a voltage-dependent slow depolarizing subthreshold potential, which enables the neurons to reach spike threshold and generate APs (Grace and Onn 1989; Kang and Kitai 1993; Nedergaard et al. 1993). Both high- and low-threshold Ca\textsuperscript{2+} currents have been found to involve in membrane excitability and spike generation (Grace and Onn 1989; Kang and Kitai 1993; Nedergaard et al. 1993). Apamin-sensitive small-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (SK) channels, which contribute to the AHP, have been found to influence the regularity of firing (Wolfart et al. 2001). A hyperpolarization-activated nonselcetive cation current (I\lambda), that is important for pacemaker-like firing in many cell types, has been found to underlie time-dependent inward rectification (voltage-sag) in midbrain DAergic neurons (Mercuri et al. 1995; Neuhoff et al. 2001; Seutin et al. 2001), and a rapidly inactivating A-type K\textsuperscript{+} current (I\textsubscript{A}) has been found to be activated during the phase

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between the AHP and the subsequent AP and to be involved in regulating spike firing frequency in these neurons (Liss et al. 2001; Silva et al. 1990; Yang et al. 2001).

Although there have been several studies on the mechanisms involved in the spontaneous activity of VTA principal neurons, there have been few studies in which the neurons were free from all synaptic influence. The VTA principal neurons receive glutamatergic synaptic inputs from the PFC (Carr and Sesack 2000) and GABAAergic synaptic inputs from local interneurons within the VTA (Johnson and North 1992) and from the NAcB (Walaas and Fonnum 1980). Thus both excitatory and inhibitory synaptic inputs may regulate the excitability of VTA principal neurons and modulate their spontaneous activity. In the study reported here, we freshly isolated VTA neurons with enzyme treatment to eliminate the influence of synaptic activity on the excitability of these neurons. Then on the basis of the quantitative evaluation of both firing frequency and firing regularity, we classified the VTA principal neurons into two subtypes and examined the membrane currents that contribute to both firing frequency and firing regularity in these two types of VTA principal neurons.

METHODS

Region-specific cell isolation

All animals were used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH85-23). Sprague-Dawley rats (12–16 days old, both genders) were decapitated, and the brain was quickly removed. The midbrain was transected at the fourth ventricle level, and the brain slices, at a thickness of 500–600 μm, were made using a Vibratome (Series 1000, St. Louis, MO). The brain slices were incubated in ACSF, which was constantly bubbled with 95% O2–5% CO2, at 34°C. Region-specific cell isolation

Electrophysiological recording

Current- and voltage-clamp recordings were performed using an EPC-7 patch-clamp amplifier (Heka Electronik, Lambrecht, Germany) or an Axopatch-1B patch-clamp amplifier (Axon Instruments, San Rafael, CA). Microelectrodes were fabricated, using a PC-10 puller (Narishige, Tokyo) or a P-87 puller (Sutter Instrument, Novato, CA), from borosilicate glass tubing (1.5 mm OD; World Precision Instruments, Sarasota, FL). The microelectrodes were heat-polished on a microforge (Narishige). The tip resistance of the electrodes was 3–6 MΩ when filled with internal solution [containing, in mM: 120 K-glutamate, 10 KCl, 0.2 ethylene glycol-bis(β-hydroxyethyl)ether-N,N,N′,N′-tetraacetic acid (EGTA), 4 MgATP, 0.3 Na2GTP, 10 N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES); pH 7.2 with KOH, final [K+]i = 139 mM; osmolality was adjusted to 290 mosM with sucrose]. Cell capacitance was determined after the capacitative transients were cancelled. Series resistance was 8–15 MΩ and was periodically monitored. The liquid junction potential (LJP) was estimated using a technique described by Neher (1992). Namely, the offset was set to zero while recording and adjusting the position through the patch pipette and 3 M KCl salt-bridge ground electrode when the chamber was filled with the same intracellular solution used in the pipette; after zeroing, the chamber solution was changed to the HEPES-buffered extracellular solution, and the potential recorded was estimated as the LJP between the intra- and extracellular solutions. By this method, the LJP between the K-glutamate-based internal solution and the HEPES-buffered solution was estimated to be 15 mV, and the results have been corrected by this amount. Membrane input resistance was determined by hyperpolarizing current pulses that induced a voltage shift of 10–15 mV negative to the resting potential, where the voltage-sag was not detected. Membrane current and voltage were filtered at 2 kHz (Model 900, Frequency Devices, Haverhill, MA) and acquired at a sampling frequency of 10 kHz. Data acquisition was performed by a DigiData 1200A interface and pClamp software version 8.0 (Axon Instrument, Union City, CA). Isolated VTA neurons were visualized with Hoffman optics on an inverted microscope (Diaphot-TMD, Nikon, Tokyo). In some experiments, nystatin-perforated patch recording was employed (Akaike and Harata 1994) to minimize intracellular dialysis. The tip resistance of these electrodes was 1.5–2.5 MΩ when filled with internal solution (containing, in mM: 60 K-acetate, 60 KCl, 1 CaCl2, 2 MgCl2, 40 HEPES; pH 7.2 with KOH, final [K+]i = 131 mM; osmolality was adjusted to 290 mosM with sucrose). Nystatin was dissolved in methanol to be 10 mg/ml as a stock solution. The pipette solution containing nystatin with a final concentration of 100–200 μg/ml was backfilled into the electrodes. When cell-attached configuration had been completed, access resistance was periodically monitored by giving hyperpolarizing voltage steps (amplitude, 10 mV; duration, 100 ms). When the access resistance had reached a steady level (7–13 MΩ), the recording was started. LJP between the K-acetate-based internal solution and the HEPES-buffered solution was estimated to be 5 mV and the results have been corrected by this amount.

Experimental solutions

The ACSF contained (in mM) 124 NaCl, 2.5 KCl, 2.4 CaCl2, 1.3 MgSO4, 1.2 KH2PO4, 24 NaHCO3, and 10 glucose. The osmolality was adjusted to 310 mosM with sucrose. The HEPES-buffered extracellular medium was composed of (in mM) 145 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose. The pH was adjusted to 7.3 with NaOH and the osmolality was adjusted to 310 mosM with sucrose. Osmolality of the solutions was measured by a Vapro vapor pressure osmometer (Wescor, Logan, UT).

Drug application and temperature manipulation

Solutions were applied using a linear multi-barrel pipette array (the internal diameter of each pipette was ~300 μm) that was positioned...
Single-cell RT-PCR analysis

Single-cell RT-PCR (scRT-PCR) analysis was conducted in the following manner. After conventional whole cell patch-clamp recording, a cell was lifted up into a stream of the HEPES-buffered extracellular solution, and the cell contents were aspirated into the electrode pipette by applying negative pressure. The cell contents were aspirated into another barrel and moving the barrel array so that the desired solution superfused the cell (SF-77B, Warner Instrument, Hamden, CT). In some experiments, solutions were applied using a multibarrel manifold (the internal diameter of each barrel was ~300 μm), which was composed of six fused tubes glued in a cylindrical pattern and inserted into a silicon rubber tube with one output barrel (580 μm ID). Drug solutions were applied by opening or closing a solenoid valve connected to each barrel. When the temperature of the HEPES-buffered extracellular solution was elevated to 34°C, the medium was warmed by an in-line solution heater, which was connected to a thermostatic temperature circulator (Model 801, Fisher Scientific, Pittsburgh, PA). The temperature of the bathing solution in the culture dish was directly monitored by a digital thermometer (Model BAT-12, Physitemp Instruments, Clifton, NJ).

The electrodes were broken and the contents were ejected into a 500 ml gluconate, 10 KCl, 0.2 EGTA, 10 HEPES, pH 7.2 with KOH, Table 1. The first PCR was performed in a final volume of 50 ml polymerase (Platinum MA) with the following cycling protocol: 1 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 70 s at 72°C followed by 1 min at 72°C.

The nested PCR was carried out for each cDNA, with 2 ml of the first PCR product under similar conditions except that extension time was shortened (45 s at 72°C), using the primer pairs for the nested PCR (Table 1). To examine the PCR product, 10 ml aliquots were separated and visualized in an ethidium bromide-containing agarose gel (1%) by electrophoresis. To exclude the possibility of detecting genomic DNA, negative control studies were carried out following the same RT-PCR procedure without using reverse transcriptase; no PCR product was detected from 10 neurons (Fig. 3Ac).

Source of drugs and chemical agents

The drugs and chemical agents used in this study, apamin, EGTA, HEPES, nystatin, papain, tetraethylammonium chloride (TEA), and tetrodotoxin (TTX) were purchased from Sigma (Saint Louis, MO).

Data analysis

Action potentials were analyzed off-line using Mini-Analysis software (Synaptosoft, Leonia, NJ). Interspike interval (ISI) histograms were created as described by Cocaire-Ziligien and Delcomyn (1992). The number of bins was equal to the square root of the number of ISIs. Bin width was obtained by dividing the ISI range (maximum ISI minus minimum ISI) by the number of bins. Spike density histograms were created to distinguish irregular firing from burst firing, as described by Kaneoke and Vitek (1996). The number of APs within the time duration that equals the mean ISI was counted from the beginning to the end of a continuous recording. The x axis of the histograms indicates AP number in the mean ISI and the y axis of the histograms indicates the number of the mean ISI. The distribution was checked by χ² test from a Poisson distribution. Coefficient of variation (CV) of firing frequency was obtained by dividing the SD of the ISIs by the mean ISIs. Data with AP amplitudes <50 mV were discarded. Firing frequency before and after drug application was examined by non-parametric analysis (Kolmogorov-Smirnov test). All average values are expressed as means ± SE. Statistical comparison was done by Student’s t-test to assess significant differences. Correlation was evaluated by linear regression with P < 0.05 being considered significant.

RESULTS

Two types of VTA principal neurons

We studied 83 isolated spontaneously active VTA principal neurons in conventional whole cell current-clamp recording; they had an average firing frequency of 2.6 ± 0.2 Hz and an average action potential (AP) half-width of 3.5 ± 0.1 ms. Figure 1 illustrates the firing of VTA principal neurons. Because in vivo electrophysiological studies have reported three

TABLE 1. PCR primer sets

<table>
<thead>
<tr>
<th>mRNA</th>
<th>First PCR Primer (5' to 3') position</th>
<th>Sequence</th>
<th>Nested PCR Primer (5' to 3') position</th>
<th>Sequence</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>393</td>
<td>CACCTGGAGTATTTTTTGTCCG</td>
<td>942</td>
<td>TGCAACCGAGTAATCACCGGCA</td>
<td>NM_012740</td>
</tr>
<tr>
<td></td>
<td>1531</td>
<td>GCTCTGGCTGGCTGATCAGAA</td>
<td>1318</td>
<td>TGCCGAGAACAAATGCAAAGAA</td>
<td>M76177</td>
</tr>
<tr>
<td>GAD67</td>
<td>795</td>
<td>TGAATCTAGGCCAATTCCG</td>
<td>825</td>
<td>CATATGGAATTCGACCGCGC</td>
<td>NM_031984</td>
</tr>
<tr>
<td></td>
<td>1899</td>
<td>GGGTTGAGAAATAGCATTGCC</td>
<td>1526</td>
<td>CGTCTACAGTAGGACGTCGCA</td>
<td>M12725</td>
</tr>
<tr>
<td>CB</td>
<td>260</td>
<td>CGCTCTCCTCAGTACGCGAC</td>
<td>337</td>
<td>GAGATGCAGGTCATCAGCGAC</td>
<td>50 mV were discarded. Firing</td>
</tr>
<tr>
<td></td>
<td>1147</td>
<td>CAGGCTCTACTGTTTTAGCGACA</td>
<td>776</td>
<td>AGTCCGAGTCGTCATTGCGCA</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>33</td>
<td>CAGGAACTTAGAAGATGGTGAG</td>
<td>129</td>
<td>GAAGACATTTTGAAGAATGCA</td>
<td>M053988</td>
</tr>
<tr>
<td></td>
<td>1183</td>
<td>CGGAACTTAAATAGCTACGGA</td>
<td>709</td>
<td>GGTATACGGATAGTAGAGCTG</td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td>63</td>
<td>AAGCTTGGCTGGCTGGGCGA</td>
<td>103</td>
<td>GACAGAAACTGAGGGCTAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>606</td>
<td>CCTACAGGGTGTGGCAGATG</td>
<td>554</td>
<td>CAGAGAGGGTGTGGCAGATG</td>
<td></td>
</tr>
</tbody>
</table>

TH, tyrosine hydroxylase; GAD67, glutamic acid decarboxylase; CB, calbindin; CR, calretinin; PV, parvalbumin.

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types of firing patterns in VTA principal neurons; regular, irregular, and burst firing (Chiodo et al. 1984; Kiyatkin and Rebec 1998), we made two types of histograms to classify the firing patterns of the isolated VTA principal neurons. One type of histogram, an ISI histogram, was used for the detection of regular firing pattern (Cocatre-Zilgien and Delcomyn 1992). The other type of histogram, a spike density histogram, was used to distinguish burst firing from irregular firing (Kaneoke and Vitek 1996). The irregular firing pattern illustrated in Fig. 1Aa was analyzed by the histograms in Fig. 1Ab. The ISI histogram in Fig. 1Ab (left) is skewed leftward. In the spike density histogram in Fig. 1Ab (right), the distribution of APs is well fitted by a Poisson distribution. The regular tonic firing pattern illustrated in Fig. 1Ba was analyzed by the histograms in Fig. 1Bb. The ISI histogram in Fig. 1Bb (left) has a peak at 0.6 s. In the spike density histogram in Fig. 1Bb (right), the distribution of APs is not fitted by a Poisson distribution. Figure 1C illustrates the relationship between the firing frequency and the firing coefficient of variation (CV), which is a suitable parameter for firing regularity, for the 83 VTA principal neurons. The firing frequency of all cells was not beyond 8 Hz, and the CV of firing frequency was distributed over a wide range (0.03–1.25). Figure 1D illustrates a histogram of the CV of firing frequency for the 83 VTA principal neurons. This histogram shows a bimodal distribution, suggesting there are two subgroups of VTA principal neurons. On the basis of this histogram, a CV of firing frequency value of 0.405 was chosen to separate these two subgroups. The VTA principal neurons under this value were classified as tonic-type neurons (average CV of firing frequency, 0.19 ± 0.01, n = 40), and those over this value were classified as irregular-type neurons (average CV of firing frequency, 0.79 ± 0.03, n = 43). Table 2 summarizes the membrane properties of the irregular- and the tonic-type VTA principal neurons. The irregular-type VTA principal neurons had a greater AP half-width and a smaller AHP amplitude than the tonic-type VTA principal neurons (Table 2).

Two types of VTA principal neurons at the higher temperature of 34°C

Because the spontaneous firing pattern of central neurons has been reported to be affected by the temperature of the extracellular solution (Womack and Khodakhah 2002), we examined whether the isolated VTA principal neurons exhibit the two patterns of firing at 34°C. As illustrated in Fig. 2, A and B, at 34°C there were two patterns of firing in the isolated VTA neurons.

### TABLE 2. Two types of ventral tegmental area (VTA) principal neurons

<table>
<thead>
<tr>
<th>Action potential (AP)</th>
<th>Irregular-type neurons</th>
<th>Tonic-type neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude, mV</td>
<td>63.6 ± 1.2 (43)</td>
<td>66.0 ± 1.7 (40)</td>
</tr>
<tr>
<td>Half-Width, ms</td>
<td>3.8 ± 0.1 (43)</td>
<td>3.3 ± 0.1 (40)</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−41.2 ± 0.7 (43)</td>
<td>−40.5 ± 0.5 (40)</td>
</tr>
<tr>
<td>Peak AHP, mV</td>
<td>−60.1 ± 0.7 (43)</td>
<td>−67.9 ± 0.9 (40)</td>
</tr>
<tr>
<td>AHP Decay Slope, mV/s</td>
<td>110.6 ± 11.0 (43)</td>
<td>110.2 ± 8.8 (40)</td>
</tr>
</tbody>
</table>

All average values are expressed as means ± SE. Statistical comparison was done by Student’s t-test to assess significant differences. Cell capacitance was determined after the capacitative transients were cancelled. Membrane input resistance was determined by hyperpolarizing current pulses that induced a voltage shift of 10–15 mV negative to the resting potential, where the voltage-sag was not detected. AP half-width was measured half way between AP threshold and AP peak. AHP slope was measured by fitting a line from the AHP peak to the rising phase toward a subsequent AP spike. Numbers of neurons in parentheses.
principal neurons. Figure 2Aa illustrates an irregular firing pattern that was analyzed by the histograms in Fig. 2Ab. The ISI histogram in Fig. 2Ab (left) is skewed leftward. In the spike density histogram in Fig. 2Ab (right), the distribution of APs is reasonably well fitted by a Poisson distribution. In four VTA principal neurons with irregular firing at 34°C, the average firing frequency was 3.2 ± 0.7 Hz, and the CV of firing frequency was 0.49 ± 0.08. Figure 2Ba illustrates a tonic firing pattern that was analyzed by the histograms in Fig. 2Bb. The ISI histogram in Fig. 2Bb (left) has a peak at 0.26 s. In the spike density histogram in Fig. 2Bb (right), the distribution of APs is not fitted by a Poisson distribution. In four VTA principal neurons with regular tonic firing at 34°C, the average firing frequency was 4.2 ± 0.3 Hz and the CV of firing frequency was 0.07 ± 0.01.

**Two types of VTA principal neurons in nystatin-perforated patch recording**

To evaluate the possibility that the two types of firing might be due to an effect of intracellular dialysis, we examined the firing pattern of the isolated VTA principal neurons using nystatin-perforated patch recording. As illustrated in Fig. 2, C and D, using nystatin-perforated patch recording there were two patterns of firing in VTA principal neurons. Figure 2Ca illustrates an irregular firing pattern using nystatin-perforated patch recording that was analyzed by the histograms in Fig. 2Cb. The ISI histogram in Fig. 2Cb (left) is skewed leftward. In the spike density histogram in Fig. 2Cb (right), the distribution of APs is reasonably well fitted by a Poisson distribution. In eight VTA principal neurons with irregular firing recorded by nystatin-perforated patch recording, the average firing frequency was 1.4 ± 0.1 Hz and the CV of firing frequency was 0.46 ± 0.06. Figure 2Da illustrates a regular tonic firing pattern using nystatin-perforated patch recording that was analyzed by the histograms in Fig. 2Db. The ISI histogram in Fig. 2Db (left) has a peak at 0.52 s. In the spike density histogram in Fig. 2Db (right), the distribution of APs is not fitted by a Poisson distribution. In eight VTA principal neurons with regular tonic firing recorded by nystatin-perforated patch recording, the average firing frequency was 2.3 ± 0.4 Hz and the CV of firing frequency was 0.12 ± 0.01.

**mRNA expression patterns in two types of VTA principal neurons**

Figure 3A illustrates an irregular-type VTA principal neuron. The firing pattern of this neuron is shown in Fig. 3Aa (left). The AP of this neuron is shown on a faster time scale in Fig. 3Aa (right); the AP half-width was 3.7 ms. After recording, the cell contents of this neuron were harvested and analyzed by single-cell RT-PCR (scRT-PCR). As shown in Fig. 3Ab, this irregular-type VTA principal neuron exhibited a tyrosine hydroxylase (TH)-positive and GAD<sub>67</sub>-negative mRNA expression pattern, indicating that it was DAergic. Because midbrain DAergic neurons have been reported to express different types of Ca<sup>2+</sup>-binding proteins (Gonzalez-Hernandez and Rodriguez 2000), we also used scRT-PCR to examine the expression of three Ca<sup>2+</sup>-binding proteins: calbindin (CB), calretinin (CR), and parvalbumin (PV). As shown in Fig. 3Ab, this irregular-type VTA principal neuron exhibited a tyrosine hydroxylase (TH)-positive and GAD<sub>67</sub>-negative mRNA expression pattern, indicating that it was DAergic. Because midbrain DAergic neurons have been reported to express different types of Ca<sup>2+</sup>-binding proteins (Gonzalez-Hernandez and Rodriguez 2000), we also used scRT-PCR to examine the expression of three Ca<sup>2+</sup>-binding proteins: calbindin (CB), calretinin (CR), and parvalbumin (PV). As shown in Fig. 3Ab, this irregular-type VTA principal neuron exhibited a tyrosine hydroxylase (TH)-positive and GAD<sub>67</sub>-negative mRNA expression pattern, indicating that it was DAergic.
neuron exhibited a TH-positive and GAD_{67}-negative mRNA expression pattern, indicating that it was DAergic. In addition, as shown in Fig. 3b, this neuron expressed both CB and CR, but PV was not detected. Figure 3C summarizes the results of scRT-PCR analysis from 9 VTA principal neurons. These VTA principal neurons were all TH-positive and GAD_{67}-negative. In all of these neurons, CR was detected but PV was not detected. Six of the nine VTA principal neurons (67%) were CB-positive. Among these six CB-positive neurons, three were irregular-type neurons; the other three were tonic-type neurons (Fig. 3D).

**Firing frequency and AHP of VTA principal neurons**

Because it has been proposed that the AHP controls the ISI and subsequent AP generation (Hille 2001), as illustrated in Fig. 4, we investigated whether there is a relationship between firing frequency and AHP in VTA principal neurons. Figure 4A illustrates the spontaneous firing (Fig. 4A, left) and AHP (Fig. 4A, middle) of a slowly firing irregular-type neuron (Fig. 4Aa) and a more rapidly firing irregular-type neuron (Fig. 4Ab). Figure 4Ac shows the relationship between firing frequency and AHP parameters of the irregular-type neurons. The average firing frequency of the irregular-type neurons was 2.0 ± 0.2 Hz (n = 43). Peak AHP did not correlate with firing frequency (r = 0.075, P > 0.6, n = 43; Fig. 4Ac, left), whereas the AHP decay slope, which was measured by fitting a line from the AHP peak to the rising phase toward a subsequent AP spike, positively correlated with firing frequency (r = 0.613, P < 0.0001, n = 43; Fig. 4Ac, right). Figure 4B illustrates the spontaneous firing (Fig. 4B, left) and AHP (Fig. 4B, middle) of a more slowly firing tonic-type neuron (Fig. 4Ba) and a rapidly firing tonic-type neuron (Fig. 4Bb). Figure 4Bc shows the relationship between firing frequency and AHP parameters of the tonic-type neurons. The average firing frequency of the tonic-type neurons was 3.1 ± 0.2 Hz (n = 40). Peak AHP did not correlate with firing frequency (r = -0.003, P > 0.9, n = 40; Fig. 4Bc, left), whereas the AHP decay slope positively correlated with firing frequency (r = 0.796, P < 0.0001, n = 40; Fig. 4Bc, right).

**Firing regularity and AHP of VTA principal neurons**

Ca^{2+}-dependent small conductance K^{+} (SK) channels have been reported to contribute to the AHP of VTA principal neurons (Brodie et al. 1999) and to be selectively blocked by the bee venom toxin, apamin (Sah 1996). In view of this, we used apamin to examine the role of SK channels in the AHP and firing properties of the two subclasses of VTA principal neurons (Fig. 5). In irregular-type neurons, 200 nM apamin had little apparent effect on either the firing pattern (Fig. 5Aa, left) or AHP (Fig. 5Aa, right), and the distribution of ISIs for 60 s before and after apamin treatment was not significantly different (P > 0.2, Kolmogorov-Smirnov [K-S] test, 173 spikes in control and 146 spikes with apamin; Fig. 5Ab). Figure 5Ac shows a summary of the effect of apamin in the irregular-type VTA principal neurons. In six irregular-type neurons, 200 nM apamin did not significantly affect either the firing frequency (1.2 ± 0.4 Hz in control, 1.4 ± 0.3 Hz with apamin; paired Student’s t-test, P > 0.4) or the CV of firing frequency (0.78 ± 0.03 in control, 0.89 ± 0.10 with apamin; paired Student’s t-test, P > 0.4; Fig. 5Ac).
In tonic-type neurons, on the other hand, 200 nM apamin altered the firing pattern (Fig. 5Ba, left) and reduced the AHP amplitude (Fig. 5Ba, right). The distribution of ISIs for 60 s before and after apamin treatment was significantly different (P < 0.0001, K-S test, 164 spikes in control and 94 spikes with apamin; Fig. 5Bb). Figure 5Bc shows a summary of the effect of apamin in the tonic-type VTA principal neurons. In five tonic-type neurons, 200 nM apamin significantly increased the CV of firing frequency (0.23 ± 0.02 in control, 0.77 ± 0.10 with apamin; paired Student’s t-test, P < 0.01; Fig. 5Bc). However, 200 nM apamin decreased the firing frequency of three and increased the firing frequency of two of the five tonic-type neurons; on average, apamin had no significant effect on the firing frequency of the tonic-type neurons (2.5 ± 0.5 Hz in control, 2.1 ± 0.4 Hz with apamin; paired Student’s t-test, P > 0.6).

In addition, to minimize possible effects on AP shape of membrane potential change after apamin treatment, we also continuously injected hyperpolarizing current to stop spontaneous AP generation, held membrane potential to the initial level, and injected a small depolarizing current to evoke APs before and after apamin treatment (Fig. 5, C and D). As illustrated in Fig. 5Ca, 200 nM apamin had little apparent effect on the AHP of an irregular-type neuron. Figure 5Cb summarizes apamin’s effect on the peak AHP of the irregular-type VTA principal neurons. Apamin did not significantly change the peak AHP of the irregular-type neurons (−59.4 ± 2.6 mV in control; −58.2 ± 2.8 mV with apamin; paired Student’s t-test, P > 0.2, n = 5; Fig. 5Cb). In a tonic-type neuron, 200 nM apamin appeared to reduce the peak AHP and increase the number of evoked APs (Fig. 5Da). Figure 5Db summarizes apamin’s effect on the peak AHP of the tonic-type VTA principal neurons. Apamin significantly shifted the peak AHP of the tonic-type neurons to the positive direction (−66.0 ± 0.9 mV in control; −62.3 ± 0.8 mV with apamin; paired Student’s t-test, P < 0.001, n = 4; Fig. 5Db).

Effect of apamin on tail current in irregular- and tonic-type neurons

To measure the apamin-sensitive current components of the two types of VTA principal neurons, we examined tail currents (I_{AHP}) elicited by a step from a holding potential of −50 mV to +10 mV in voltage-clamp configuration, using conventional whole cell recording with a K-gluconate-based pipette solution (reversal potential of K^+ [E_K] = −101 mV by the Nernst equation). The duration of the depolarizing voltage-step was selected to be 100 ms because the I_{AHP} reached approximately maximal amplitude with this duration. In these experiments, TTX (1 μM) and TEA (1 mM) were included in the extracellular solution to block Na^+ channels and large-conductance Ca^{2+}-activated K^+ (BK) channels. In an irregular-type VTA principal neuron, 200 nM apamin does not appear to affect the tail current amplitude (Fig. 6Aa). The apamin-sensitive I_{AHP} component was obtained by digital subtraction of the apamin-resistant current from the control current (Fig. 6Ab). On average, the apamin-sensitive I_{AHP} component in the irregular-type neurons was 17.1 ± 4.0 pA (n = 7); this was too small to adequately analyze the deactivation kinetics. On the other hand, in a tonic-type VTA principal neuron, 200 nM apamin appears to reduce the tail current amplitude (Fig. 6Ba). As in the preceding text, the apamin-sensitive I_{AHP} component was obtained by digital subtraction of the apamin-resistant current from the control current (Fig. 6Bb). On average, the apamin-sensitive I_{AHP} component in the tonic-type neurons was 108.2 ± 23.6 pA and exhibited deactivation kinetics with a single-exponential decay time constant (52.7 ± 5.5 ms; n = 5). To compare the size of the apamin-sensitive current components in the two types of VTA principal neurons, average current density was determined (Fig. 6C). The average density of the apamin-sensitive I_{AHP} component in the tonic-type neurons was 4.2 ± 0.6 pA/µF (n = 5), which was significantly larger than that of the irregular-type

FIG. 4. Firing frequency and AHP in VTA principal neurons. A, left: slow (a) and fast (b) spontaneous firing of 2 irregular-type neurons; (middle) average of 20 spontaneous APs from the slow firing neuron (a) and the fast firing neuron (b). Ac, left: relationship between peak AHP and firing frequency of the irregular-type neurons; right: relationship between AHP decay slope, which was measured by fitting a line from the AHP peak to the rising phase toward a subsequent AP spike, and firing frequency of the irregular-type neurons. B, left: slow (a) and fast (b) spontaneous firing of 2 tonic-type neurons; (middle) average of 20 spontaneous APs from the slow firing neuron (a) and the fast firing neuron (b). Bc, left: relationship between peak AHP and firing frequency of the tonic-type neurons; right: relationship between AHP decay slope and firing frequency of the tonic-type neurons. Correlation was examined by linear regression with P < 0.05 being considered significant.
neurons (0.7 ± 0.2 pA/pF, n = 7; Student’s t-test, P < 0.01).

Because it has been reported that intracellular gluconate can reduce a slow \( I_{\text{AHP}} \) in hippocampal neurons (Zhang et al. 1994), we measured the \( I_{\text{AHP}} \) in VTA principal neurons, using nystatin-perforated patch recording and an extracellular solution. The voltage protocol was used, and 1 \( \mu \)M TTX and 1 mM TEA were added to the extracellular solution. In an irregular-type VTA principal neuron, using nystatin-perforated patch recording 200 nM apamin had only a small effect on the tail current amplitude (Fig. 6Da). The apamin-sensitive \( I_{\text{AHP}} \) component was obtained by digital subtraction of the apamin-resistant current from the control current; on average, the apamin-sensitive \( I_{\text{AHP}} \) component in the irregular-type neurons using nystatin-perforated patch recording was 21.0 ± 1.8 pA (n = 4). There was no significant difference between the apamin-sensitive \( I_{\text{AHP}} \) recorded using the K-gluconate-based pipette solution and that recorded using nystatin-perforated patch recording (Student’s t-test, P > 0.4). In a tonic-type VTA principal neuron, using nystatin-perforated patch recording 200 nM apamin reduced the tail current amplitude (Fig. 6Db). The apamin-sensitive \( I_{\text{AHP}} \) component was obtained by digital subtraction of the apamin-resistant current from the control current; on average, the apamin-sensitive \( I_{\text{AHP}} \) component in the tonic-type neurons using nystatin-perforated patch recording was 88.8 ± 14.5 pA (n = 4). There was no significant difference between the apamin-sensitive \( I_{\text{AHP}} \) recorded using the K-gluconate-based pipette solution and that recorded using nystatin-perforated patch recording (Student’s t-test, P > 0.5). However, the deactivation of the apamin-sensitive \( I_{\text{AHP}} \) recorded using nystatin-perforated patch recording had a slower decay time constant (fitted by a single exponential function: 353.9 ± 69.3 ms, n = 4) than that recorded using the K-gluconate-based pipette solution (52.7 ± 5.5 ms, n = 5; Student’s t-test, P < 0.05). In addition, in the nystatin-perforated patch recording, the average density of the apamin-sensitive \( I_{\text{AHP}} \) component in the tonic-type neurons was 3.1 ± 0.4 pA/pF (n = 4), which was significantly larger than that of the irregular-type neurons (0.8 ± 0.1 pA/pF, n = 4; Student’s t-test, P < 0.01).
FIG. 6. Apamin-sensitive tail current of VTA principal neurons. Aa: tail current (I_{AHP}) of an irregular-type neuron (firing frequency, 2.1 Hz; CV of firing frequency, 0.94) recorded before treatment with 200 nM apamin (black line) and after treatment with 200 nM apamin (gray line); traces are superimposed. Ab: recording of the I_{AHP} in Aa with a higher time resolution; bottom: apamin-sensitive current obtained by subtracting apamin-resistant current from the control current. B: I_{AHP} of a tonic-type neuron (firing frequency, 4.8 Hz; CV of firing frequency, 0.16) recorded before treatment with 200 nM apamin (black line) and after treatment with 200 nM apamin (gray line); traces are superimposed. Bb: top: recording of the I_{AHP} in B with a higher time resolution; bottom: apamin-sensitive current obtained by subtracting apamin-resistant current from the control current. The apamin-sensitive tail current deactivation time constant was 42.5 ms and was well fitted by a single exponential function. C: average apamin-sensitive current density in 7 irregular-type neurons and 5 tonic-type neurons. Da: I_{AHP} of an irregular-type neuron (firing frequency, 1.4 Hz; CV of firing frequency, 0.43) recorded in nystatin-perforated patch voltage-clamp configuration before (black line) and after apamin treatment (gray line) with subtracted apamin-sensitive current component at the bottom; traces are superimposed. Db: I_{AHP} of a tonic-type neuron (firing frequency, 2.2 Hz; CV of firing frequency, 0.14) recorded in nystatin-perforated patch voltage-clamp configuration before (black line) and after apamin treatment (gray line) with subtracted apamin-sensitive current component at the bottom; traces are superimposed. E: average apamin-sensitive current density in 4 irregular-type neurons and 4 tonic-type neurons. The asterisks indicate significant difference on Student’s t-test (P < 0.01).

D I S C U S S I O N

In the present study, we investigated the properties of isolated VTA principal neurons in which the excitatory and inhibitory synaptic inputs onto these neurons had been eliminated. The use of isolated neurons and enzyme treatment enabled us to analyze the pacemaker activity of the VTA principal neurons without the influence of synaptic activity on the excitability of these neurons. It has been reported that VTA principal neurons receive extensive glutamatergic excitatory synaptic inputs from the PFC (Carr and Sesack 2000) and that glutamatergic excitatory synaptic transmission onto VTA principal neurons can regulate the excitability of these neurons (Jones and Kauer 1999; Koga and Momiyama 2000). It has also been reported that glutamatergic excitatory postsynaptic potentials modulate the firing pattern of midbrain DAergic neurons (Kang and Futami 1999). One study, using a mouse brain slice preparation, has reported that Ca^{2+}-dependent small-conductance K\(^+\) (SK) current contributes to the firing pattern of substantia nigra pars compacta (SNc) DAergic neurons but contributes little to the firing pattern of VTA DAergic neurons (Wolfart et al. 2001). Using isolated VTA principal neurons, we have found some important differences with that study. We have found different SK current contributions to spontaneous intrinsic firing between two subpopulations of VTA principal neurons; the neurons were classified by analysis of both their firing frequency and their firing regularity. Consistent with our observations, a topological difference in the density of SK channel mRNA expression in VTA principal neurons has recently been reported (Sarpal et al. 2004). Although our isolated VTA principal neurons have truncated distal dendrites, this would not appear to explain the different SK current contribution to the two subpopulations of VTA principal neurons that we observed.

Membrane properties and Ca^{2+}-binding proteins of VTA principal neurons

In the present study, the VTA principal neurons exhibited a relatively slow firing frequency and a relatively wide AP duration, which is consistent with previous studies (Grace and Onn 1989; Johnson and North 1992; Klink et al. 2001; Momiyama et al. 1996; Mueller and Brodie 1989; Neuhoff et al. 2002; Wolfart et al. 2001). In addition, the VTA principal neurons had a very large input resistance (R_{in}) and a relatively large cell capacitance. The very large R_{in} of the VTA principal neurons is not likely to be due to the elimination of dendritic processes by cell isolation because cultured VTA principal neurons, which had three to five large dendrites, have been reported to have a very large R_{in} (average 1,340 M\(\Omega\)) (Yang et al. 2001).

In scRT-PCR analysis, we found that all VTA principal neurons examined expressed TH but not GAD_{67}, indicating that they were D\(\alpha\)ergic. We also found that CR was expressed in all of the VTA principal neurons examined, whereas PV was
not detected in any of the VTA principal neurons. These results are consistent with a previous immunohistochemical study that found that PV is selectively expressed in midbrain GABAergic neurons (Gonzalez-Hernandez and Rodriguez 2000). In our study, CB was detected in 67% of the VTA principal neurons. In one previous study, CB was found in 50% of the VTA DAergic neurons (Neuhoff et al. 2002), and in another study, 51–72% of the VTA DAergic neurons expressed CB (Barrot et al. 2000). It has been proposed that CB regulates the spontaneous firing in neurons by buffering intracellular Ca$$^{2+}$$ (Li et al. 1995). However, our results are not consistent with that proposal because we found that 50% of CB-positive VTA principal neurons were classified as irregular-type neurons and the other 50% of CB-positive VTA principal neurons were classified as tonic-type neurons.

**Irregular-type VTA principal neurons**

We characterized one subpopulation of VTA principal neurons (52%) by spontaneous AP generation with an irregular pattern at room temperature. The spontaneous irregular firing of these neurons is likely to be an intrinsic characteristic because a similar firing pattern was also recorded at the higher temperature of 34°C and in nystatin-perforated patch configuration; the nystatin-perforated patch recording does not produce an intracellular dialysis effect. In addition, the irregular firing pattern does not appear to be due to recording from unhealthy neurons damaged during the cell isolation process because the $$R_{m}$$ of these neurons was extremely high (average $$R_{m}$$, 896 M$$\Omega$$). In the irregular-type VTA principal neurons, apamin-sensitive SK current was found to be very small, and it appeared to contribute little to the firing pattern or AHP amplitude of these neurons. This is consistent with a previous report that SK3 protein expression is low in VTA DAergic neurons (Wolfart et al. 2001). The irregular-type VTA principal neurons that we observed may correspond to the previously described VTA DAergic neurons with a small AHP (Neuhoff et al. 2002).

**Tonic-type VTA principal neurons**

We characterized the other subpopulation of VTA principal neurons (48%) by spontaneous AP generation with a regular firing pattern at room temperature. The spontaneous regular firing of these neurons is likely to be an intrinsic characteristic because a similar firing pattern was also recorded at the higher temperature of 34°C and in nystatin-perforated patch configuration. In the tonic-type VTA principal neurons, we found that apamin-sensitive SK current contributed significantly to both the regularity of firing and the prominent AHP. Because apamin increased the firing CV of the tonic-type neurons, without significantly affecting firing frequency, apamin-sensitive SK current appears to be critical for firing pattern rather than firing rate. Apamin treatment has been reported to induce burst firing in midbrain DAergic neurons (Ping and Shepard 1996); however, in the present study, apamin was observed to induce irregular firing but not bursting. These disparate observations may result from differences in the preparations used. Because the apamin-induced burst firing (Ping and Shepard 1996) was recorded in brain slice preparations, synaptic effects combined with SK channel blockade may account for the burst firing behavior. The tonic-type VTA principal neurons that we observed may correspond to previously described VTA principal neurons with regular firing (Mueller and Brodie 1989) or VTA DAergic neurons with a large AHP (Neuhoff et al. 2002).

In the tonic-type neurons, in voltage-clamp configuration we recorded the apamin-sensitive component of the AHP as an apamin-sensitive tail current ($$I_{\text{AHP}}$$). Previous studies on various types of neurons have divided the AHP into three main components: a fast hyperpolarization that typically lasts 1–10 ms; a medium-duration AHP that usually lasts 50 ms to several hundred milliseconds; and a slow AHP that lasts for several seconds (Sah 1996). Although the apamin-sensitive $$I_{\text{AHP}}$$ amplitude was similar using K-glutamate pipette solution and nystatin-perforated patch recording, the deactivation of the apamin-sensitive $$I_{\text{AHP}}$$ was significantly different. The apamin-sensitive $$I_{\text{AHP}}$$ in the tonic-type VTA principal neurons decayed with an average time constant of 53 ms in conventional whole cell recording using a K-glutamate-based pipette solution and 354 ms using nystatin-perforated patch recording. Thus the apamin-sensitive $$I_{\text{AHP}}$$ in these neurons would correspond to a medium-duration $$I_{\text{AHP}}$$. The previously reported value for the deactivation time constant of the apamin-sensitive $$I_{\text{AHP}}$$ recorded in conventional whole cell recording with a K-glutamate-based pipette solution was 50 ms (Savic et al. 2001) and that recorded in gramicidin-perforated patch recording was 108 ms (Wolfart et al. 2001).

**AHP and firing frequency of VTA principal neurons**

It is generally assumed that large amplitude AHPs result in a slow rate of spontaneous AP generation and that small amplitude AHPs result in a faster rate of spontaneous AP firing. We found, however, that the firing frequency of VTA principal neurons was positively correlated with the AHP decay slope, but not with the AHP peak. These observations raise the possibility that the AHP may trigger some voltage-dependent ion channels, which control the inter-AP trajectory. Midbrain DAergic neurons have been found to exhibit both a hyperpolarization-activated nonselective cation current ($$I_{\text{h}}$$) (Mercuri et al. 1995; Neuhoff et al. 2002; Seutin et al. 2001) and a rapidly inactivating K$$^{+}$$ current ($$I_{\text{A}}$$) (Liss et al. 2001; Silva et al. 1990; Yang et al. 2001). In midbrain DAergic neurons, the activation threshold of both $$I_{\text{h}}$$ and $$I_{\text{A}}$$ has been reported to be −60 to −50 mV (Liss et al. 2001; Mercuri et al. 1995; Silva et al. 1990; Yang et al. 2001). Because we found that peak AHP was −60.1 mV in the irregular-type neurons and −67.9 mV in the tonic-type neurons, both $$I_{\text{h}}$$ and $$I_{\text{A}}$$ could be activated during the inter-AP interval in VTA principal neurons. In midbrain DAergic neurons, the $$I_{\text{A}}$$ blocker, heteropodatoxin3, was found to increase firing frequency and the charge density of $$I_{\text{A}}$$ correlated negatively with firing frequency (Liss et al. 2001). In addition, the $$I_{\text{h}}$$ antagonist, ZD7288, has been found to decrease firing frequency in midbrain DAergic neurons (Neuhoff et al. 2002; Seutin et al. 2001). These observations suggest that the firing frequency of VTA principal neurons may depend on the activation of both $$I_{\text{h}}$$ and $$I_{\text{A}}$$.  

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Physiological implications

Because VTA principal neurons receive both excitatory and inhibitory synaptic inputs (Carr and Sesack 2000; Johnson and North 1992; Walaa and Fonnum 1980), in vivo the two types of VTA principal neurons described here may exhibit further variation in their firing properties. Consistent with this, in vivo electrophysiological studies have found that VTA principal neurons exhibit a spontaneous bursting mode, in addition to tonic and irregular firing patterns (Chiodo et al. 1984; Kiyatkin and Rebec 1998). Anatomically, VTA DAergic neurons project predominantly to the NAc (mesoaccumbal DA system) and the PFC (mesocortical DA system) (Oades and Halliday 1987). VTA DAergic neurons with irregular firing have been reported to be located predominantly in the ventromedial VTA and to project their axons to the PFC, whereas VTA DAergic neurons with regular firing have been reported to be located predominantly in the dorsolateral VTA and are likely to project to the NAc (Chiodo et al. 1984; Neuhoff et al. 2002). These observations suggest that the irregular-type VTA principal neurons may have a physiological role in the mesocortical DAergic projection, and the tonic-type VTA principal neurons may have a physiological role in the mesoaccumbal DAergic projection. However, further studies are needed on the functional properties of VTA principal neurons labeled retrogradely from both the PCF and the NAc.

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