Dopamine neurons in the ventral tegmental area fire faster in adolescent rats than in adults

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Methods

Ethical approval. All experiments were approved by the Rosalind Franklin University Institutional Animal Care and Use Committee and conform to methods described in the authors' Animal Care and Use protocols.

Subjects. Male adolescent (postnatal day (P) 37–48; 125–285 g) and adult (P82–100; 350–450 g) Sprague-Dawley rats were used for all experiments. Rats were housed two to three per cage to preserve social interactions, which are particularly important during adolescence (Spear 2000). Rats were allowed to acclimate for 1–2 wk before testing on a 12:12-h light-dark cycle (lights on at 7:00 AM). Rats were acquired from Harlan (Indianapolis, IN) unless otherwise stated. Food and water were available ad libitum.

In vivo electrophysiology. Extracellular recordings of dopamine neurons in the VTA were made under chloral hydrate anesthesia as previously reported (Marinelli and White 2000). A total of 92 neurons were recorded; 19 of these were previously reported in McCutcheon et al. (2006).
and Marinelli (2009). VTA coordinates were 2.4–3.4 mm anterior to lambda, 0.3–0.7 mm lateral from the midline, and 7.5–8.5 ventral from the cortical surface for adolescents and 3.0–3.8 mm anterior to lambda, 0.3–0.7 mm lateral from the midline, and 7.8–8.8 mm ventral from the cortical surface for adults. Dopamine neurons were recorded using glass electrodes (tip 1–2 μm, 1.5–2.5 MΩ) filled with 2 M NaCl solution saturated with 1% fast green dye. Neuronal activity was determined in one to four neurons per rat. Neurons were recorded for between 3 and 5 min to establish a mean firing rate, and only neurons with stable activity (≤5% variation measured over 10-s bins) over this period were included. At the end of the recordings, a negative current was passed through the electrode, resulting in a deposit of fast green dye. Electrode placement within the VTA was confirmed after each experiment by fixing the brains with 4% formaldehyde, sectioning on a microtome, and locating the fast green deposit using light microscopy.

Firing rate was calculated as the total number of spikes occurring over time (3–5 min of stable activity, with ≤5% variation). Bursting activity was also analyzed. Bursts are clusters of spikes occurring at high frequencies and were calculated according to previously established criteria (Grace and Bunney 1984). Briefly, a burst initiates with a pair of spikes having an interspike interval (ISI) of 80 ms or less and terminates when the ISI is 160 ms or greater. We evaluated the amount of bursting activity by calculating the percentage of spikes emitted in bursts, the burst event frequency by calculating the number of burst events over time, characteristics of bursts by measuring the number of spikes/burst and the frequency of the spikes within the bursts, and the postburst interval by measuring the ISIs following each burst. To determine the relative contribution of bursting vs. nonbursting activity on overall changes in firing rate, we analyzed “nonbursting activity” by subtracting burst events from overall firing activity (Mathon et al. 2003). In this analysis, the ISIs that precede and follow each burst event were not considered because their timing could be influenced by conductances that initiate and terminate the burst events.

Dopamine neuron identification for in vivo studies. Dopamine neurons were identified according to standard physiological criteria including all of the following: slow-frequency firing rate (0.5–10 Hz) and irregular firing pattern interspersed with high-frequency burst events. In addition, neurons exhibited triphasic +/-+ waves of long duration recorded with two different filter settings (>2.5 ms from beginning to end using 400- to 500-Hz filters (Grace and Bunney 1983; Wang 1981a; White and Wang 1984) and >1.1 ms from beginning to trough using 50- to 800-Hz filters (Unghess et al. 2004); for review, see Grace et al. (2007) and Marinelli et al. (2006)]. Although such criteria have been questioned for ex vivo recordings (Margolis et al. 2006), these criteria are efficient at identifying dopamine neurons in vivo, as determined with juxtacellular labeling and colocalization with tyrosine hydroxylase (TH; marker of dopamine neurons) (Luo et al. 2008; Unghess et al. 2004; Unghess and Grace 2012). No differences in waveform duration were seen across adolescent and adult rats using 400- to 500-Hz filters [3.02 ± 0.03 and 3.00 ± 0.04, respectively, not significant (NS)] or 50- to 800-Hz filters (1.27 ± 0.02 and 1.35 ± 0.02, respectively, NS).

Ex vivo electrophysiology. Rats were anesthetized with isoflurane and were placed in ice-cold sucrose-based artificial cerebrospinal fluid (aCSF; in mM: 200 sucrose, 20 glucose, 25 NaHCO3, 2.5 KCl, 1 CaCl2, 2.5 MgCl2, 1 NaHPO4, and 10 ascorbic acid) kept at 32°C and perfused at a rate of 20 μl/min for at least 30 min before transfer to the recording chamber. Slices in the recording chamber were constantly perfused with 32–34°C aCSF at a rate of 2 μl/min. All solutions were saturated with 95% O2/5% CO2.

Recordings were made with glass pipettes (2–5 MΩ) filled with the appropriate internal solution (see below). Voltage-clamp data were collected using an Axopatch 200B (filtered at 1–2 kHz; digitized at 5–10 kHz; Molecular Devices, Palo Alto, CA). Current-clamp data were collected using a Dagan BVC-700A (open filter, digitized at 20 kHz; Minneapolis, MN). The liquid junction potential was not corrected for. Series resistance in whole cell recordings was always below 25 MΩ and was not compensated. Cell-attached recordings were made using both amplifiers, and software filters were used to isolate spikes (0.8 kHz, 300 Hz). All data were collected using pClamp 9.2 software (Molecular Devices). For paired-pulse experiments, a bipolar tungsten stimulating electrode was placed ~300 μm rostral to the recording electrode and was used to evoke excitatory (EPSCs) and inhibitory postsynaptic currents (IPSCs; 0.2 ms pulse every 30 s). Stimulation intensity was selected as the minimum required to evoke EPSCs with <15% trial-to-trial variability (50–300 μA).

Internal solutions. Potassium gluconate internal solution (in mM: 120 K-gluconate, 10 HEPES, 2 KCl, 4 ATP-Na2, 2 ATP-K2, 0.3 GTP-Na2, and 0.13% neurobiotin, pH 7.2–7.3) was used in all recordings except for measurements of AMPA-mediated spontaneous postsynaptic currents (sEPSCs), for which cesium gluconate internal solution (in mM: 140 Cs-gluconate, 10 HEPES, 2 MgCl2, 5 ATP-Na2, 0.3 GTP-Na2, 1 QX-314, 0.1 spermine, and 0.1% neurobiotin, pH 7.2–7.3) was used, and for measurements of GABA-A-mediated spontaneous inhibitory currents (sIPSCs), for which potassium chloride internal solution (in mM: 140 KCl, 10 HEPES, 2 MgCl2, 5 ATP-Na2, 0.3 GTP-Na2, and 0.13% neurobiotin, pH 7.2–7.3) was used. For cell-attached experiments, any one of the above internal solutions, 145 mM NaCl with 0.1% neurobiotin, or aCSF was used.

Dopamine neuron identification for ex vivo studies. Because of recent controversies regarding dopamine cell identification (Chien et al. 2011; Margolis et al. 2006, 2010; Zhang et al. 2010), putative dopamine neurons were identified using a number of criteria. For cell-attached studies, location in midbrain (medial to the medial terminal nucleus of the accessory optic tract, MT), cell somata diameter >20 μm, slow and regular firing rate (0.5–6 Hz), and long-duration action potentials (>1.2 ms) were used (Ford et al. 2006; Mueller and Brodie 1989). For whole cell studies, location in the slice and the presence of a large (>50 pA at ~120 mV) hyperpolarization-activated cation current (i_h) were used. In a subset of cell-attached and whole cell ex vivo recordings, neurons were filled with neurobiotin and identified as dopamine neurons by post hoc immunohistochemical staining for TH (see below). Using this technique, we demonstrated that >85% of neurobiotin-filled neurons fulfilling the above criteria were TH positive. In addition, there was no age-related difference in the ratio of TH-positive to TH-negative neurons (NS for all experiments, numerical values provided in RESULTS). Neurons confirmed as TH negative were excluded from further analysis.

Immunohistochemistry. After recording, slices were fixed in 4% formaldehyde (12–72 h. In pilot studies (data not shown), TH antibody was found to penetrate only 12–20 μm from tissue surface; therefore, slices were resectioned at 40 μm on a freezing microtome. Sections (40 μm) were subsequently placed in blocking solution (3% normal goat serum, 3% Triton, in PBS) for 1–2 h before being incubated with primary antibody (1:500; rabbit anti-TH, AB152; Millipore, Billerica, MA) either overnight at room temperature or for 48–72 h at 4°C. Sections were then incubated with an anti-rabbit fluorescent secondary antibody and a streptavidin-conjugated fluorophor (Alexa 488/594; Invitrogen, Carlsbad, CA) for 2 h. Between each incubation, sections were washed for at least 3 times for 10 min each in PB and were agitated at every step. Sections were mounted onto gelatinized slides and coverslipped using anti-fade mountant. Slides were examined using an upright microscope (E400; Nikon, Melville, NY), and photomicrographs were taken using Neurolucida (MBF
Biosciences, Williston, VT). Resulting images had pseudocolor added and were merged using Photoshop (Adobe, San Jose, CA).

**Protein biochemistry.** Adolescent (P48, n = 6) and adult (P95, n= 6) male Sprague-Dawley rats were decapitated and the brains rapidly removed. The VTA was dissected on ice from a 1-mm coronal slice obtained from a brain matrix. The tissue was placed into ice-cold lysis buffer [25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA, 20 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 0.1% Nonidet-P40 (vol/vol), 1 mM Na2Ov, 1 μM okadaic acid, 1 μM microsystin-LF, and 1:100 protease inhibitor cocktail set I (Calbiochem, San Diego, CA)], sonicated, and stored at −80°C until preparation for SDS-PAGE. Protein concentration was determined using the Bio-Rad protein assay kit (Heracles, CA). Samples were heated (70°C, 10 min) in Laemmli sample treatment buffer with 100 mM DTT, loaded (5 μg of protein), and electrophoresed on 4–15% Tris-HCl gradient gels (Bio-Rad) under reducing conditions, and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). After transfer, PVDF membranes were rinsed in Tris-buffered saline (TBS), blocked for 1 h at room temperature with 1% (vol/vol) goat serum and 5% (wt/vol) nonfat dry milk or 3% (wt/vol) bovine serum albumin (BSA) in TBS-Tween 20 (TBS-T; 0.05% Tween 20, vol/vol), and then incubated overnight on a rocker at 4°C with primary antibody: phospho-(Ser 40)TH (PhosphoSolutions, Aurora, CO) diluted 1:100,000 in TBS or phospho-ERK (Cell Signaling Technologies, Danvers, MA) diluted 1:10,000 in 3% BSA/TBS-T. PVDF membranes were washed in TBS-T, incubated at room temperature for 60 min with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000; Invitrogen), washed with TBS-T, and rinsed with diH2O. Proteins of interest were detected with enhanced chemiluminescence (ECL) substrate and ECL films. The resulting films were analyzed using TotalLab software (BioSystematica, Llandysul, UK).

**Dugs.** All drugs and reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. Drugs were bath-applied. Stock solutions of drugs were dissolved in water, dimethyl sulfoxide (DMSO), or 0.2 N NaOH, and the final concentration of DMSO in aCSF never exceeded 0.1%. To isolate AMPA receptor-mediated currents, we used picrotoxin (100 μM) and t-2-aminophosphonopentanoate (APV; 50 μM). To isolate GABAergic mediated currents, we used 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and APV (50 μM).

**Statistical analysis.** Electrophysiological traces were analyzed using the software packages Clampfit 9.2 (Molecular Devices) and MiniAnalysis 6.0.7 (Synaptosoft, Fort Lee, NJ). Data were compiled and analyzed using Excel 2003 (Microsoft, Redmond, WA) and Statistica 9 (StatSoft, Tulsa, OK). Unless stated otherwise, comparisons between adolescents and adults were made using two-tailed unpaired Student’s t-tests.

For analysis of firing rate in the ex vivo preparation, spike events were placed in 10-ms bins. Drug-free recordings of at least 2 min were used to determine mean firing rate. Neurons were excluded from analysis if they showed either >5% variation in firing over time or a slope of >0.003 Hz/s. Outliers that were more than two standard deviations from the mean were also excluded (adolescents, 2/51; adults, 3/46).

$I_h$ current analysis was based on Arencibia-Albite et al. (2012). Briefly, $I_h$ amplitude was calculated as the difference between the instantaneous ($I_{inj}$, ~50 ms from the disappearance of the capacitive transient) and steady-state currents ($I_h$, 800 ms from voltage step). Charge transfer ($\Delta I_{inj}$ and $I_h$) was calculated using numerical integration. $I_h$ conductance was calculated using Ohm’s law with $I_h$ reversal potential estimated at −40 mV.

The effect of different concentrations of quinpirole on firing rate and holding current analysis was repeated using repeated-measures ANOVA with age (adolescents vs. adults) and concentration (firing rate, 20 vs. 300 nM; holding current, 300 vs. 1,000 nM) as between-group factors and drug as a within-subjects factor (pre- vs. postapplication). For analysis of sPSCs, threshold for detection was set at four times the maximum root mean square of the noise. Noise did not differ between adolescent and adult groups (P = 0.440 and P = 0.725 for AMPA-mediated and GABAergic sPSCs, respectively). Cumulative distribution of events were analyzed using the Kolmogorov-Smirnov test.

For immunohistochemistry experiments, the ratio of TH-positive to TH-negative neurons in adults and adolescents was compared using the χ² test.

**RESULTS**

**VTA dopamine neurons fire faster in adolescents than in adults: in vivo.** We assessed firing rate of VTA dopamine neurons from adolescent and adult rats under chloral hydrate anesthesia, using in vivo electrophysiology. All results and statistics are provided in Table 1. VTA neurons from adolescent rats fired faster than VTA neurons from adult rats by ~1.5 Hz (~40% faster; Fig. 1). The higher overall firing was mostly reflected in the “nonbursting” firing rate. The amount of bursting (percentage of spikes in burst, time spent bursting, and burst event frequency) did not differ across age groups. The characteristics of the bursts, however, differed with neurons from adolescent rats displaying higher burst duration by ~65 ms (>50 increase in duration) and an increased number of spikes per burst. The intraburst firing rate was lower in adolescents compared with adults, and the mean ISI within each burst was longer. This could partially be accounted for by the greater number of spikes per burst in adolescence (bursts show spike-frequency adaptation, with increasing interspike interval across bursting). However, when only the first two spikes of each burst were considered, interspike interval was still longer by ~6 ms in adolescents compared with adults, suggesting that the mechanisms responsible for determining intraburst firing rate may be separable from those determining nonbursting firing rate. Finally, the postburst interval, i.e., the time it took for firing to resume after a burst had terminated, was shorter by ~215 ms in adolescents compared with adults, reflecting a shorter postburst recovery period in adolescents.

Taken together, these results indicate that activity of the mesolimbic dopamine system changes during development. To explore possible underlying mechanisms, we moved to an ex vivo midbrain slice preparation.

**VTA dopamine neurons fire faster in adolescents than in adults: ex vivo.** We assessed firing rates of putative VTA dopamine neurons from adolescent and adult rats, in horizontal brain slices, using cell-attached recordings to prevent rundown. For these “on-cell” recordings, dopamine neurons were identified by their slow regular firing rate, long-duration action potentials, large-diameter somata, and location medial to the medial terminal nucleus of the accessory optic tract (MT) (Ford et al. 2006; Mueller and Brodie 1989). However, these criteria alone are not sufficient to determine the dopaminergic nature of the neurons (Lammel et al. 2011; Margolis et al. 2011; Stinus et al. 1989). Therefore, we used the same criteria as described above to identify VTA dopamine neurons. The effect of different concentrations of quinpirole on firing rate and holding current analysis was conducted using repeated-measures ANOVA with age (adolescents vs. adults) and concentration (firing rate, 20 vs. 300 nM; holding current, 300 vs. 1,000 nM) as between-group factors and drug as a within-subjects factor (pre- vs. postapplication). For analysis of sPSCs, threshold for detection was set at four times the maximum root mean square of the noise. Noise did not differ between adolescent and adult groups (P = 0.440 and P = 0.725 for AMPA-mediated and GABAergic sPSCs, respectively). Cumulative distribution of events were analyzed using the Kolmogorov-Smirnov test.

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For immunohistochemistry experiments, the ratio of TH-positive to TH-negative neurons in adults and adolescents was compared using the χ² test.
Therefore, a subset of neurons recorded in this manner (37/92) were filled with neurobiotin at the end of the recording for postrecording immunocytochemistry. Of these neurobiotin-filled neurons, 32/37 (i.e., 86.5%) were immunoreactive for TH, indicating that the majority of recorded neurons were indeed dopaminergic. In addition, there was no difference in the proportion of TH-positive neurons between adolescents and adults (adolescent: 16/19, adult: 16/18; NS). An example of a TH-positive neuron is illustrated in Fig. 2. Analysis of all neurons vs. TH-positive neurons did not yield different results.

Table 1. Electrophysiological properties of VTA dopamine neurons recorded under chloral hydrate anesthesia in adolescent and adult rats

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adolescent</th>
<th>Adult</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firing rate, Hz</td>
<td>4.89 ± 0.26 (54)</td>
<td>3.43 ± 0.25 (38)</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Nonbursting firing rate, Hz</td>
<td>4.73 ± 0.24 (54)</td>
<td>3.66 ± 0.24 (38)</td>
<td>0.003†</td>
</tr>
<tr>
<td>CV of firing rate</td>
<td>0.62 ± 0.05 (54)</td>
<td>0.79 ± 0.06 (38)</td>
<td>0.032*</td>
</tr>
<tr>
<td><strong>Amount of bursting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spikes in burst, %</td>
<td>22.72 ± 3.51 (54)</td>
<td>27.62 ± 4.24 (38)</td>
<td>0.375</td>
</tr>
<tr>
<td>Time spent bursting, %</td>
<td>7.44 ± 1.36 (54)</td>
<td>5.10 ± 1.30 (38)</td>
<td>0.232</td>
</tr>
<tr>
<td><strong>Burst event frequency, Hz</strong></td>
<td>0.34 ± 0.05 (54)</td>
<td>0.36 ± 0.06 (38)</td>
<td>0.826</td>
</tr>
<tr>
<td>Interburst interval, s</td>
<td>14.54 ± 4.15 (51)</td>
<td>6.65 ± 1.48 (32)</td>
<td>0.136</td>
</tr>
<tr>
<td>Characteristics of bursts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of spikes per burst</td>
<td>3.25 ± 0.19 (51)</td>
<td>2.70 ± 0.11 (34)</td>
<td>0.031*</td>
</tr>
<tr>
<td>Burst duration, ms</td>
<td>179.86 ± 19.31 (51)</td>
<td>113.83 ± 10.75 (34)</td>
<td>0.011*</td>
</tr>
<tr>
<td>Intraburst frequency, Hz</td>
<td>15.79 ± 0.67 (51)</td>
<td>18.94 ± 1.18 (34)</td>
<td>0.015*</td>
</tr>
<tr>
<td>Burst ISI, ms</td>
<td>71.79 ± 2.19 (51)</td>
<td>61.30 ± 2.62 (34)</td>
<td>0.003†</td>
</tr>
<tr>
<td>ISI of first two spikes, ms</td>
<td>61.01 ± 1.59 (51)</td>
<td>54.40 ± 1.86 (34)</td>
<td>0.009*</td>
</tr>
<tr>
<td>Postburst interval, ms</td>
<td>390.49 ± 32.10 (51)</td>
<td>606.67 ± 66.68 (34)</td>
<td>0.002†</td>
</tr>
</tbody>
</table>

Values are means ± SE with n (no. of cells) in parentheses. P values after 2-tailed Student’s t-test are given for adolescent vs. adult rats for each parameter (*P ≤ 0.05; †P ≤ 0.01; ‡P ≤ 0.001). CV, coefficient of variation; ISI, interspike interval; VTA, ventral tegmental area.

Similar to the in vivo preparation, in this reduced ex vivo preparation, VTA neurons from adolescent rats fired faster than VTA neurons from adult rats (Fig. 2A and Table 2). As previously shown (Grace and Onn, 1989), there were differences between in vivo and ex vivo measures of firing rate data; in the slice, activity was slower and spikes occurred in a regular, pacemaker-like pattern that was devoid of bursts. The elevation in activity in adolescent neurons was reflected in a shorter mean ISI than in adult neurons. The coefficient of variation of firing rate (CV) and action potential width were not different between ages.

Fig. 1. Ventral tegmental area (VTA) dopamine neurons fire faster in adolescents than adults in vivo in chloral hydrate-anesthetized rats. A: firing rates (in Hz) of VTA dopamine neurons from adolescent (black circles) and adult rats (gray circles). Each circle represents 1 neuron, and horizontal lines show means for each age group. B: representative traces from 2 neurons in A. Averaged action potential waveforms after filtering (left), taken from 5-s traces (right), are shown. Dashed lines show measurement of waveform duration. C, D, and E: nonbursting activity, frequency of burst events, and burst duration, respectively. Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001, adolescent (n = 54) vs. adult (n = 38).
Biochemical markers of neuronal activity are higher in adolescents than adults. To further confirm differences in firing rate across ages, we used biochemistry to examine two phosphorylated proteins known to be sensitive to neuronal activity: TH, which in the midbrain is specific to dopamine neurons, and extracellular signal-regulated kinase (ERK). For both proteins, the ratio between the phosphorylated form and total protein was elevated in adolescents compared with adults (pTH/TH, P = 0.037, Fig. 3A; pERK/ERK, P = 0.006, Fig. 3B).

Passive and active membrane properties are similar in adolescent and adult rats. We compared passive and active membrane properties in VTA dopamine neurons in tissue slices from adolescent and adult rats, using whole cell electrophysiology (Table 2). Resting membrane potential, input resistance, and capacitance were similar at both ages. Action potential amplitude, threshold, half-width and AHP amplitude also did not differ between ages. $I_h$ current was elicited using a series of hyperpolarizing steps (from a holding potential of −60 mV to −120 mV in 15-mV steps; Fig. 4A). This voltage-dependent

![Image](image-url)

**Fig. 2.** VTA dopamine neurons fire faster in adolescents than in adults in ex vivo slice preparations. *A* firing rates (in Hz) of VTA dopamine neurons from adolescent (black circles) and adult rats (gray circles). Each circle represents 1 neuron, and horizontal lines show means for each age group. *p < 0.05. *B* representative traces from 2 neurons in A. Averaged action potential waveforms after filtering (left), taken from 10-s traces (right), are shown. Dashed lines show measurement of action potential width (after Ford et al. 2006). *C* example of a neurobiotin-filled neuron colocalized with tyrosine hydroxylase (TH). Low-magnification photomicrograph (far left) shows TH (green) and neurobiotin (red). White outline shows area of magnification for the other 3 images showing TH alone, neurobiotin alone, and merged image (left to right). MT, medial terminal nucleus of the accessory optic tract.

### Table 2. Electrophysiological properties of VTA dopamine neurons recorded in an ex vivo midbrain slice preparation from adolescent and adult rats

<table>
<thead>
<tr>
<th></th>
<th>Adolescent</th>
<th>Adult</th>
<th>P Value</th>
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<tbody>
<tr>
<td><strong>Cell-attached properties</strong></td>
<td></td>
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<tr>
<td>Mean firing rate, Hz</td>
<td>2.22 ± 0.12 (47)</td>
<td>1.82 ± 0.11 (42)</td>
<td>0.020*</td>
</tr>
<tr>
<td>ISI, mean, ms</td>
<td>533.56 ± 36.09 (47)</td>
<td>688.94 ± 69.64 (42)</td>
<td>0.044*</td>
</tr>
<tr>
<td>ISI, mode, ms</td>
<td>518.94 ± 32.93 (47)</td>
<td>672.14 ± 69.07 (42)</td>
<td>0.041*</td>
</tr>
<tr>
<td>CV of firing rate</td>
<td>0.15 ± 0.01 (47)</td>
<td>0.14 ± 0.02 (42)</td>
<td>0.748</td>
</tr>
<tr>
<td>AP width, ms</td>
<td>2.01 ± 0.17 (47)</td>
<td>2.32 ± 0.21 (42)</td>
<td>0.230</td>
</tr>
<tr>
<td><strong>Passive membrane properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>−41.97 ± 1.41 (12)</td>
<td>−42.75 ± 1.73 (12)</td>
<td>0.729</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>264.85 ± 30.84 (12)</td>
<td>219.48 ± 54.82 (12)</td>
<td>0.478</td>
</tr>
<tr>
<td>Capacitance, pF</td>
<td>64.78 ± 6.85 (11)</td>
<td>58.82 ± 7.94 (9)</td>
<td>0.575</td>
</tr>
<tr>
<td><strong>Action potential properties</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>80.06 ± 2.74 (12)</td>
<td>75.35 ± 2.88 (12)</td>
<td>0.249</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−26.25 ± 1.32 (12)</td>
<td>−26.39 ± 1.72 (12)</td>
<td>0.947</td>
</tr>
<tr>
<td>Half-width, ms</td>
<td>1.09 ± 0.14 (12)</td>
<td>1.15 ± 0.15 (12)</td>
<td>0.753</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>−22.03 ± 1.45 (12)</td>
<td>−21.55 ± 1.49 (12)</td>
<td>0.820</td>
</tr>
<tr>
<td>$I_h$ properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{in}$, pA</td>
<td>267.21 ± 36.93 (9)</td>
<td>399.13 ± 82.10 (8)</td>
<td>0.174</td>
</tr>
<tr>
<td>$I_{as}$, pA</td>
<td>593.08 ± 112.06 (9)</td>
<td>762.73 ± 109.56 (8)</td>
<td>0.296</td>
</tr>
<tr>
<td>$I_h$ amplitude ($I_{in} - I_{as}$), pA</td>
<td>325.87 ± 83.43 (9)</td>
<td>363.60 ± 88.44 (8)</td>
<td>0.761</td>
</tr>
<tr>
<td>$I_{h}$ charge transfer, pA</td>
<td>204.02 ± 28.61 (9)</td>
<td>297.56 ± 59.38 (8)</td>
<td>0.186</td>
</tr>
<tr>
<td>$I_h$ activation time, ms</td>
<td>182.79 ± 51.11 (9)</td>
<td>197.43 ± 49.52 (8)</td>
<td>0.840</td>
</tr>
<tr>
<td>$I_{sk}$ properties</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude, pA</td>
<td>166.2 ± 45.6 (10)</td>
<td>160.9 ± 58.5 (6)</td>
<td>0.943</td>
</tr>
<tr>
<td>Decay time, ms</td>
<td>265.6 ± 38.7 (10)</td>
<td>239.9 ± 57.2 (6)</td>
<td>0.704</td>
</tr>
</tbody>
</table>

Values are means ± SE with n in parentheses. P values after 2-tailed Student’s t-test are given for adolescent vs. adult rats for each parameter (*P ≤ 0.05). AP, action potential; AHP, afterhyperpolarization; $I_h$, hyperpolarization-activated cation current; $I_{in}$, instantaneous current; $I_{as}$, steady-state current; SK, small-conductance calcium-activated potassium current.
current did not differ between adolescents and adults in amplitude, time of activation, or time to decay (Table 2). SK-mediated currents were evoked by depolarizing neurons (from a holding potential of $-60$ mV to $+80$ mV) for 100 ms to induce unclamped action potentials (Fig. 4B). We blocked the apamin-sensitive SK component with apamin (1 μM). This apamin-sensitive current did not differ between adolescent and adult rats in either its amplitude or its time to decay (Table 2).

All of these neurons were filled with neurobiotin and processed for TH immunoreactivity. Of this population, 85.7% ($I_h$ experiment) and 86.7% (SK experiment) of neurobiotin-positive neurons were immunoreactive for TH, again supporting the idea that the majority of recorded neurons were dopaminergic. As before, no difference was found in the proportion of TH-positive neurons between adult and adolescent (adult, 12/14; adolescent, 17/21; NS). Analysis of all neurons vs. TH-positive neurons did not yield different results.

**Dopamine D2 receptor function in adolescent and adult rats.** Dopamine D2-class autoreceptors (D2Rs) regulate basal firing activity by hyperpolarizing the neuron in response to somatodendritically released dopamine (Wang 1981b). Their downregulation, or decreased functional sensitivity, leads to elevated firing rates (White and Wang 1984). Hence, we assessed whether differences in D2R function in these age groups could account for differences in firing rate. Using cell-attached recordings, we found that, as expected, the D2R agonist quinpirole inhibited firing in dopamine neurons and that this inhibition was greatest at the highest of the two concentrations tested (Fig. 5A; 300 vs. 20 nM; main effect of concentration, $F_{1,34} = 11.7, P = 0.002$). Quinpirole had a similar magnitude of effect at both ages (no effect of age, $F_{1,34} = 0.230, P = 0.634$, or age × concentration interaction, $F_{1,34} = 0.260, P = 0.614$). However, because firing rate in adolescents was elevated at baseline, quinpirole was less effective at shutting down firing. Furthermore, in whole cell voltage-clamp recordings (holding potential, $-60$ mV), bath-applied quinpirole induced an outward current that was greatest at the highest of the two concentrations tested (1,000 vs. 300 nM; main effect of concentration, $F_{1,26} = 6.1, P = 0.020$), which did not differ between ages (Fig. 5B; no effect of age, $F_{1,26} = 0.129, P = 0.723$, or age × concentration interaction, $F_{1,26} = 0.509, P = 0.482$). Finally, in current-clamp mode, the membrane hyperpolarization caused by quinpirole did not differ in adult and adolescent rats (300 nM: $11.5 \pm 1.7$ vs. $10.6 \pm 2.3$ mV, $n = 7–8, P = 0.750$). Hence, although D2R function seems similar across ages, activation of these receptors is less effective at silencing dopamine neurons in the adolescent, a feature that is likely to have important ramifications for intracellular signaling and neurotransmission.
activity, which was the major contributor of elevated dopamine neuron firing during adolescence, in vivo, in chloral hydrate anesthetized rats. The most pronounced effect was on nonbursting firing during adolescence, in vivo, in chloral hydrate anesthesia.

We initially observed an elevation of dopamine neuron firing in adolescent rats. Neurons were held in voltage-clamp mode (holding potential, −60 mV) with either picrotoxin or CNQX in the bath to record sEPSCs and sIPSCs, respectively. The frequency of AMPA receptor-mediated sEPSCs was lower by 26.9% in adolescents relative to adults (1.79 vs. 2.45 Hz, \( P = 0.003 \)), although amplitude did not differ (20.81 vs. 21.23 pA, \( P = 0.626 \)). The paired-pulse ratio of electrically evoked EPSCs was not different at any interval tested (20, 50, and 100 ms; Fig. 6A; all \( P > 0.05 \)). When GABA\(_A\)-mediated sIPSCs were examined, their frequency was lower by 20.5% (5.51 vs. 6.93 Hz) and their amplitude was lower by 16.7% (30.21 vs. 36.26 pA, \( P = 0.025 \)) in adolescents relative to adult rats. Paired-pulse ratio of GABA\(_A\)-mediated electrically evoked IPSCs was similar at both ages for all ratios tested (Fig. 6B; all \( P > 0.05 \)).

DISCUSSION

Dopamine neurons fire faster in adolescent rats than in adults. We show this in three distinct preparations: 1) in vivo, under chloral hydrate anesthesia, 2) ex vivo, in a midbrain slice preparation, and 3) using biochemical markers of neuronal activation. Furthermore, using the slice preparation, we examined a number of electrophysiological properties that can contribute to firing rate and identified decreased GABA tone in the adolescent as a potential underlying mechanism.

Dopamine neurons fire faster in adolescent rats than in adult rats. We initially observed an elevation of dopamine neuron firing during adolescence, in vivo, in chloral hydrate anesthetized rats. The most pronounced effect was on nonbursting activity, which was the major contributor of elevated dopamine neuron activity. With respect to burst events, no difference was found in the frequency of burst events or the time spent bursting across ages. However, the characteristics of the bursts were different. Burst duration was longer and the number of spikes per burst was higher in adolescents compared with adults. Interestingly, we did not see an elevation of intraburst activity in adolescents; in fact, we found the opposite, lower intraburst firing frequency in adolescents than in adults. These findings suggest different mechanisms determine nonbursting and intraburst firing frequency, as has been suggested by others (Morikawa and Paladini 2011).

We followed our in vivo findings with a set of ex vivo experiments in the slice. Cell-attached recordings were used to assay firing rate in the slice, because these recordings are not affected by potential rundown often exhibited in whole cell recordings. As previously shown (Grace and Onn 1989), we saw a difference in firing pattern between in vivo and ex vivo preparations with bursting activity being absent in the slice. Even in this reduced preparation, and in the absence of bursting activity, dopamine neurons from adolescent rats fired faster than neurons from adult rats. Next, we reasoned that if dopamine neuron activity is elevated throughout adolescence, then biochemical markers known to be sensitive to the level of neuronal activity should also be elevated in this period. Thus we probed for levels of pTH and pERK. Levels of pTH are correlated with dopamine neuron firing (Witkovsky et al. 2004); levels of pERK are known to be elevated after behavioral manipulations that lead to increased dopamine neuron firing such as stress exposure (Iñiguez et al. 2010). We found that both pTH and pERK were expressed at a higher level, relative to their nonphosphorylated counterparts, in adolescent VTA compared with adult VTA. Because VTA pTH/TH is restricted to dopamine neurons, this result, combined with the high proportion (～85%) of TH staining found in recorded
neurons, provides confirmation that the elevated firing rate is found in dopamine neurons, rather than another cell population.

Mechanisms underlying faster firing in adolescent rats relative to adult rats. To further probe the mechanism, we performed further whole cell recordings in ex vivo slices. We demonstrated that passive membrane properties (input resistance, resting membrane potential, and capacitance) did not differ across ages. Equally, active membrane properties underlying action potential threshold (amplitude, half-width, rise time, and afterhyperpolarization amplitude) also did not differ. Next, we assayed $I_h$ and apamin-sensitive SK current, both of which may, under certain circumstances, contribute to firing rate (Neuhoff et al. 2002; Wolfart et al. 2001). We found no difference in the profile of $I_h$ between adolescent and adult rats. In addition, the apamin-sensitive SK current was similar in both age groups. Our results do contrast with a previous report showing an increase in SK3 mRNA in the VTA in “adolescent” rats (Sarpal et al. 2004). However, this discrepancy is easily resolved, given the rats used in this earlier study were much younger than the ones used presently (<P21 vs. P37–P45).

Dopamine D2Rs in the VTA are a key regulator of dopamine neuron firing (Wang 1981b; White and Wang 1984). Release of somatodendritic dopamine hyperpolarizes neurons via acti-

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Fig. 6. AMPA receptor- and GABA <sub>A</sub> receptor-mediated synaptic transmission differs in VTA dopamine neurons from adolescent and adult rats. A, top: sample traces showing AMPA receptor-mediated spontaneous excitatory postsynaptic currents (sEPSCs) recorded at −60 mV. Averaged spontaneous events from each neuron are shown below traces. Middle, graphs showing cumulative probability of interevent intervals (upper) and amplitudes (lower) of AMPA receptor-mediated sEPSCs in adolescent (black line) and adult rats (gray line). Respectively insets show average frequency and average amplitude of sEPSCs. Bottom, paired-pulse ratio (PPR) of evoked current at 20-, 50-, and 100-ms intervals. Inset shows representative traces from adolescents (black) and adults (gray) at 50-ms interval. Scale bars, 50 ms, 50 pA. B: same information as described in A but for GABA <sub>A</sub> receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) and evoked IPSCs. Scale bars, 50 ms, 100 pA. Values are means ± SE. *$p < 0.05$; **$p < 0.01$. 

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vation of D2Rs, which in turn are coupled to a G protein-activated inwardly rectifying potassium channel (GIRK)-mediated potassium conductance (Beckstead et al. 2004; Lacey et al. 1987). D2Rs are known to be dynamically regulated in a number of situations, including cocaine withdrawal (Marinelli et al. 2003, 2006); furthermore, in striatum and prefrontal cortex, they are developmentally regulated during the periaadolescent period (Andersen et al. 2000; Tarazi and Baldessarini 2000). However, we found no evidence to indicate that D2R number or function differs across ages. To this end, quinpirole application produced the same amount of firing inhibition and the same amount of outward current at both ages. Furthermore, because we found a clear effect of quinpirole concentration, we do not believe that either result was due to the presence of a ceiling effect. All of these effects were blocked by the dopamine D2R antagonist sulpiride, showing that the effects are most likely specific to D2Rs. However, despite the lack of difference between groups, there are two reasons why D2Rs could still differentially contribute to regulation of firing in adolescence compared with adulthood. First, because adolescent neurons have an elevated baseline firing, more receptor activation is required to reduce their firing to a low rate and/or shut it down completely. This may have important consequences on the ability of dopamine neurons to reduce their firing activity and may give rise to profound differences in cell signaling. Second, here we activated D2Rs with bath application of an agonist, but physiologically these receptors respond to somatodendritically released dopamine. The differences in intraburst parameters that we identified in the in vivo preparation, e.g., shorter ISI in adults relative to adolescents, will affect the manner and pattern of such somatodendritic dopamine release, especially since the relationship between dopamine release and firing frequency is nonlinear (Chergui et al. 1994). Thus, despite D2R activation due to the application of an exogenous ligand causing similar magnitude effects, we cannot rule out that D2R activation due to endogenously released dopamine could participate in firing rate differences across ages.

The VTA receives dense glutamate and GABA projections from numerous different brain regions (Carr and Sesack 1999; Geisler et al. 2007; Jhout et al. 2009; Kalivas and Alesdatter 1993; Omelchenko and Sesack 2009), and the balance between these neurotransmitters is essential for determining both the firing rate and the firing pattern of dopamine neurons in vivo (Marinelli et al. 2006; Mathon et al. 2003; Morikawa and Paladini 2011; White 1996). We assayed AMPA- and GABA_A- mediated sPSCs and found that the frequency of both was higher in adults than in adolescents and that the amplitude of GABA_A-mediated sPSCs was also lower in adolescents. Of these findings, those concerning GABA_A are of particular interest, because recent work has shown that the “bombardment” of dopamine neurons by GABA_A afferents provides a tonic inhibitory tone and is a strong contributor to nonbursting firing rate (Lobb et al. 2010). In addition, although many inputs to the VTA are severed during horizontal slice preparation, several key sites containing GABA_A cell bodies are likely preserved, including the rostromedial tegmental nucleus (RMTg) and the substantia nigra pars reticulata (SNpr), as well as local VTA interneurons; thus some GABA_A tone is preserved. In contrast, although a glutamatergic projection from the lateral hypothalamus may be spared in this preparation, most of the other major sources of glutamatergic input, the prefrontal cortex and subcortical nuclei such as the pedunculopontine tegmental nucleus (PPTg) and the lateral dorsal tegmental nucleus (LDTg), are not present. No changes in paired-pulse ratio of evoked AMPA- and GABA_A-mediated PSCs were observed, suggesting that the increases in sPSCs in the adult may reflect a presynaptic alteration such as an increase in synaptic release sites or an increase in spontaneous activity, rather than a change in probability of release. The most likely sources of input underlying our effects are those with cell bodies preserved in the slice, for example, for GABA_A, local interneurons, the RMTg, and the SNpr, and for glutamate, local neurons and the lateral hypothalamus (Dobi et al. 2010; Jhout et al. 2009; Johnson and North 1992; Saitoh et al. 2004).

Functional implications. The elevation of dopamine neuron firing during adolescence is likely to have numerous consequences. As mentioned in the Introduction, elevated dopamine neuron firing has been associated with increased susceptibility to drug-taking in several addiction models (Marinelli et al. 2006). Our results would therefore predict that adolescent rats would differ from adults on a number of behavioral tasks related to drug-taking; specifically, they should show traits indicating increased addiction liability. Indeed, this is the case in a number of reports (Anker et al. 2011; Badanich et al. 2006; Caster et al. 2005; Perry et al. 2007; Shahbazi et al. 2008; Tirelli et al. 2003; but also see Frantz et al. 2007; Kerstetter and Kantak 2007; Li and Frantz 2009). Other traits also emerge during the adolescent period and could be linked to the elevated firing rate. These include an increase in social play, risk-taking behavior, and impulsivity, behaviors thought necessary for the transition from infancy to adulthood (Spear 2000; Vanderschuren et al. 1997). Adolescence is also a time that sees the onset or exacerbation of many psychiatric disorders (Paus et al. 2008), which have been associated with elevated dopaminergic transmission (Carlsson 1978; Diehl and Gershon 1992; Wise and Rompre 1989).

In conclusion, we present results demonstrating an elevation of VTA dopamine neuron activity during adolescence in the rat. We identify increased GABA_A tone in adult rats as a potential mechanism to explain this difference, although future work is necessary to confirm this. Furthermore, determining how this change in dopamine neuron physiology contributes to the unique behavioral complement present during adolescence remains the ultimate goal.

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DISCLOSURES

The authors report no biomedical financial interests or conflicts of interest.
VTA DOPAMINE NEURONS FIRE FASTER IN ADOLESCENTS THAN ADULTS


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