Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 L-type calcium channels regulate dopaminergic firing activity in the mouse ventral tegmental area

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DOPAMINERGIC (DA) neurons in the ventral tegmental area (VTA) play an important role in cognition and reward processing (Cooper 2002; Ikemoto 2007, 2010; Tobler et al. 2005), and abnormalities in these processes are implicated in diseases such as schizophrenia and addiction (Eyles 2012; Gardner 2011; Iversen and Iversen 2007; Koob and Volkow 2010; Tritsch and Sabatini 2012). DA neurons are spontaneously active and display two major firing patterns: single-spike firing (also called pace-making spiking) and burst firing (Liu et al. 2007; Shi 2009; Zhang et al. 2005). The two firing modes are shown to be involved in different functions: pace-making spiking sets the basic tone to maintain DA levels in terminal areas and behaviorally encodes expected stimulations; however, burst firing enhances DA synaptic transmission and signals unexpected environmental stimuli (Chergui et al. 1994; Romo and Schultz 1990; Schultz 2007, 2010; Tobler et al. 2005). Therefore, it is crucial to understand the transition and regulation of the two firing modes in DA neurons.

Ca\textsuperscript{2+} influx through L-type calcium channels (LTCCs) is an important modulator of neuronal firing activities of DA neurons in the VTA. LTCCs are responsible for approximately one-third of total Ca\textsuperscript{2+} currents in DA cells (Cardozo and Bean 1995; Durante et al. 2004) and contribute preferentially to whole cell Ca\textsuperscript{2+} currents evoked by small depolarizations (Durante et al. 2004; Xu and Lipscombe 2001). They have been shown to be involved in spontaneous firing, spontaneous oscillatory potentials, and cholinergic-driven firing increases that are abolished by dihydropyridine (DHP) LTCC blockers (Mercuri et al. 1994; Nedergaard et al. 1993; Zhang et al. 2005). Also, they are involved in the regulation of burst firing. For example, the cholinergic-driven bursting and associated membrane potential oscillations are blocked by the LTCC blocker nifedipine (Zhang et al. 2005). Furthermore, direct activation of LTCCs with activators acting at different sites, (S)-(-)-Bay K8644 and FPL 64176, induces burst firing in DA neurons (Liu et al. 2007; Zhang et al. 2005).

Four subtypes of LTCCs have been identified: Ca\textsubscript{v}1.1, Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3, and Ca\textsubscript{v}1.4; only Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subtypes are expressed in the midbrain (Chan et al. 2007; Rajadhyaksha et al. 2004; Sinnegger-Brauns et al. 2009; Striessnig et al. 2006; Takada et al. 2001). It has been shown that Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 LTCCs are distributed throughout the entire midbrain including DA neurons (Rajadhyaksha et al. 2004; Takada et al. 2001). Compared with Ca\textsubscript{v}1.2 channels, Ca\textsubscript{v}1.3 has a lower activation threshold and a reduced sensitivity to DHP activators and blockers (Durante et al. 2004; Striessnig et al. 2006; Xu et al. 2001). The role of LTCCs in regulating firing activities of DA neurons has been explored; however, current pharmacological agents cannot differentiate Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subtypes, and thus their specific contribution to functions mediated by LTCCs remains unknown. This study aimed to determine the contribution of the two LTCC subtypes to the firing activity of DA neurons in the VTA by using transgenic mouse models that carry specific defects in LTCC subtypes.
(Platzer et al. 2000; Sinnegger-Brauns et al. 2004, 2009; Striessnig and Koschak 2008): Ca\textsubscript{v1.3}-deficient mice (Ca\textsubscript{v1.3}−/−; knockout mice) as well as a knockin mouse strain (Ca\textsubscript{v1.2}DHP−/−) that expresses Ca\textsubscript{v1.2} LTCCs lacking high sensitivity to DHP LTCC activators and blockers.

**MATERIALS AND METHODS**

All procedures involving animal handling and tissue harvesting were reviewed and approved by the Institutional Animal Care Committees at the Memorial University of Newfoundland and Shenyang Pharmaceutical University. Mice were given food and water ad libitum and housed in a room on a 12:12-h light-dark cycle with temperature maintained at 24°C.

**Animals**

C57BL/6J mice and transgenic strains with a C57BL/6J background were used in this study. Wild-type (WT) mice were purchased from Charles River Canada. The Ca\textsubscript{v1.2}DHP−/− mice carry the T1066Y mutation within the DHP binding domain of the Ca\textsubscript{v1.2} α1-subunit that makes it insensitive to DHP modulators (Striessnig et al. 2006; Zhang et al. 2007). Ca\textsubscript{v1.2}DHP−/− mice show no physiological phenotype because Ca\textsubscript{v1.2} functions normally, while Ca\textsubscript{v1.3}−/− mice have abnormalities such as bradycardia, deafness, and an anti-depressant-like behavior (Busquet et al. 2010; Platzer et al. 2000; Striessnig et al. 2006; Striessnig and Koschak 2008).

Congenic transgenic mice were first bred from heterozygous pairs and maintained with Ca\textsubscript{v1.2}DHP−/−Ca\textsubscript{v1.2}DHP−/− breeding pairs for DHP knockin mice. Ca\textsubscript{v1.3}−/− females were poor breeders, and to increase the yield of Ca\textsubscript{v1.3}−/− pups a breeding group consisting of a heterozygous Ca\textsubscript{v1.3}−/−/− female, a Ca\textsubscript{v1.3}−/−/− female, and a Ca\textsubscript{v1.3}−/− male was established. Mice were genotyped as previously described (Giordano et al. 2006, 2010; Platzer et al. 2000; Zhang et al. 2007).

**Patch-Clamp Recording**

**Slice preparation.** Mice (2–4 mo old) were deeply anesthetized with halothane and killed by chest compression. The skull was quickly opened to expose the brain, which was cooled in situ with ice-cold, carbogenated cutting solution (composition in mM: 250 glycerol, 2.5 KCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 1.2 MgCl\textsubscript{2}, 2.4 CaCl\textsubscript{2}, 26 NaHCO\textsubscript{3}, and 11 glucose, pH 7.4 when bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2}) (Liu and Chen 2008; Ye et al. 2006). The brain was removed, and a block containing the midbrain was cut horizontally on a Leica vibratome (VT 1000, Wetzlar, Germany). Tissue slices (300–400 μm thick) were allowed to recover in a carbogenated artificial cerebrospinal fluid (ACSF, composition in mM: 126 NaCl, 2.5 KCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 1.2 MgCl\textsubscript{2}, 2.4 CaCl\textsubscript{2}, 18 NaHCO\textsubscript{3}, and 11 glucose, pH 7.4 when bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2}) at 31°C for 1 h and then maintained at room temperature (22°C) until use. Slices were further trimmed to fit into a recording chamber and continuously perfused with carbogenated ACSF at a rate of 2–3 ml/min at room temperature.

**Nystatin-perforated patch-clamp recording.** All recordings were made from VTA identified under a dissecting microscope (Leica MZ6). Patch electrodes were prepared from KG-33 glass micropipettes (OD 1.5 mm; Garner Glass, Claremont, CA) on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA). Glass electrodes were filled to the tip with intracellular solution (mM: 120 potassium acetate, 40 HEPES, 5 MgCl\textsubscript{2}, and 10 EGTA, pH adjusted to 7.35 with 0.1 N KO\textsubscript{H}) and then back-filled with the same solution containing 450 μg/ml nystatin and Pluronic F127, yielding a tip resistance of 4–8 MΩ. Gigaohm seals were made with a MultiClamp 700B (Axon Instruments, Foster City, CA) amplifier. Signals were sampled at 5 kHz and digitized by a DigiData 1320A using pCLAMP 9 software (Axon Instruments).

Selection of nystatin-perforated cell recordings in current-clamp mode was determined by the size of the action potential, since many VTA cells were spontaneously active. After adequate partitioning of nystatin into the membrane, action potentials overshoot 0 mV and measured at least 50 mV. Episodic protocols were used to induce I\textsubscript{h} and derive passive characteristics of the cell such as current-voltage relationship and input resistance. Current pulses for I\textsubscript{h} induction were 1 s, and the intervals between pulses were 8 s to allow complete recovery of I\textsubscript{h} channels. Cells that displayed a prominent I\textsubscript{h} and an apparent DA-induced hyperpolarization were identified as “putative DA cells,” because the neurons recorded were not confirmed to be dopaminergic by postrecording immunohistochemistry (Liu et al. 2007; Liu and Chen 2008).

**Chemicals**

Reagents for extracellular and intracellular solutions were purchased from bulk distributors Fisher Scientific (Nepean, ON, Canada) and VWR International (Mississauga, ON, Canada). All other chemicals were obtained from Sigma (St. Louis, MO) and Tocris (Ellisville, MO). Chemicals were dissolved in deionized water or DMSO (0.1% final concentration) as required. Aliquots of stock solutions were kept at −30°C. Prior to application, an aliquot was diluted to working concentration and applied to the ACSF bath. DA solution was made fresh daily with an equimolar concentration of the antioxidant disodium metabisulfite.

**Data Analysis**

Data were analyzed off-line with Mini Analysis (Synaptosoft, Decatur, GA) and pCLAMP software. Basal firing frequencies were averaged values of at least 5-min stable baseline recording. I\textsubscript{h} was measured as the difference in voltage or current between instantaneous and steady-state readings. Analysis of firing behavior was based on interspike intervals (ISIs) measured with the Mini Analysis program. Averaged firing frequencies were derived from those intervals. Relative densities of ISIs in 0.1- and 0.5-s bins were plotted to reveal the distribution of a given ISI series. Burst firing was defined as two spikes or more in each bursting cycle at a frequency higher than nonbursting periods and separated by a postburst hyperpolarization, which was visually tagged and quantified. Data are expressed as means and SE. Statistical comparisons of data were performed with two-tailed paired or unpaired Student’s t-test and χ\textsuperscript{2}-test. Values were considered significant when P < 0.05.

**RESULTS**

For all experiments, putative DA cells in the VTA identified according to criteria outlined in MATERIALS AND METHODS were included.

**VTA DA Cells of Ca\textsubscript{v1.2}DHP−/− and WT Mice Have Similar Basic Electrophysiological Properties**

The percentage of spontaneously active cells in the WT group (18 of 31, from 25 mice, 58.1%) was similar to that in Ca\textsubscript{v1.2}DHP−/− mice (32 of 50, from 42 mice, 64.0%; t-test, P > 0.05; Fig. 1C). Both groups displayed single-spike firing with comparable basal firing frequencies (0.68 ± 0.20 Hz for WT, 0.72 ± 0.11 Hz for Ca\textsubscript{v1.2}DHP−/−, unpaired t-test, P > 0.05, Fig. 1; Table 1). The resting membrane potentials (RMPs) were similar between the two groups (−52.69 ± 1.17 mV for WT vs. −51.98 ± 1.03 mV for Ca\textsubscript{v1.2}DHP−/−, P > 0.05, unpaired t-test); however, there was an apparent differ-
Resting membrane potentials (mV) in RMPs of spiking and quiescent cells in both strains. The RMPs of spiking cells [for WT, \(-49.87 \pm 0.59\) mV (\(n = 18\), from 18 mice); for \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\), \(-49.98 \pm 0.88\) mV (\(n = 32\), from 32 mice)] were more depolarized than those of quiescent cells [for WT, \(-56.01 \pm 1.68\) mV (unpaired \(t\)-test, \(P < 0.001\)); for \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\), \(-57.89 \pm 1.70\) mV (unpaired \(t\)-test, \(P < 0.001\))]. There were no differences in RMPs of spiking cells (\(P > 0.05\)) or quiescent cells (\(P > 0.05\)) between WT and \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) groups. In terms of spiking cells, there were no significant differences in spike amplitude (58.00 ± 2.39 mV in WT mice vs. 58.13 ± 2.09 mV in \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) mice, unpaired \(t\)-test, \(P > 0.05\)), threshold (\(-38.91 \pm 0.76\) mV in WT mice vs. \(-38.87 \pm 0.88\) mV in \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) mice, unpaired \(t\)-test, \(P > 0.05\)), half-width (2.97 ± 0.11 ms in WT mice vs. 3.01 ± 0.15 ms in \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) mice, unpaired \(t\)-test, \(P > 0.05\)), or hyperpolarization afterpotentials (\(-13.20 \pm 1.57\) mV in WT mice vs. \(-13.57 \pm 1.30\) mV in \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\), unpaired \(t\)-test, \(P > 0.05\)). In addition, the average hyperpolarization following a brief application of 50 \(\mu\)M DA (within 90 s) was not significantly different between WT (\(-9.01 \pm 1.02\) mV) and \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) (\(-9.46 \pm 1.00\) mV) mice (unpaired \(t\)-test, \(P > 0.05\)). When current injections were adjusted in each cell to result in a peak hyperpolarization of about \(-110\) mV, the \(I_h\) sag was comparable between WT (20.98 ± 1.17 mV) and \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) (20.09 ± 1.10 mV) (unpaired \(t\)-test, \(P > 0.05\)). These results confirm that VTA neurons of \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\) mice and WT mice have indistinguishable electrophysiological properties (Table 1).

### Table 1. Basic electrophysiological properties of dopaminergic cells in wild type (WT), \(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-}\), and \(\text{Ca}_{\text{v}1.3}^{-/-}\) mice

<table>
<thead>
<tr>
<th></th>
<th>wild type (WT)(_1)</th>
<th>(\text{Ca}_{\text{v}1.2}\text{DHP}^{-/-})_0</th>
<th>(\text{Ca}_{\text{v}1.3}^{-/-})_0</th>
<th>(P_{1,2})</th>
<th>(P_{1,3})</th>
</tr>
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<tbody>
<tr>
<td>Number of spontaneously active cells</td>
<td>18 of 31, 58.1%</td>
<td>32 of 50, 64.0%</td>
<td>7 of 26, 26.9%</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Resting membrane potentials (mV)</td>
<td>(-52.69 \pm 1.17)</td>
<td>(-51.98 \pm 1.03)</td>
<td>(-52.13 \pm 1.18)</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Basal firing frequencies (Hz)</td>
<td>0.68 ± 0.20</td>
<td>0.72 ± 0.11</td>
<td>0.25 ± 0.07</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>58.00 ± 2.39</td>
<td>58.13 ± 2.09</td>
<td>54.62 ± 2.31</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Firing threshold (mV)</td>
<td>(-38.91 \pm 0.76)</td>
<td>(-38.87 \pm 0.88)</td>
<td>(-41.03 \pm 1.71)</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
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<tr>
<td>Spike half-width (ms)</td>
<td>2.97 ± 0.11</td>
<td>3.01 ± 0.15</td>
<td>2.97 ± 0.20</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hyperpolarizing afterpotentials (mV)</td>
<td>(-13.20 \pm 1.57)</td>
<td>(-13.57 \pm 1.30)</td>
<td>(-13.81 \pm 1.51)</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hyperpolarization following an application of 50 (\mu)M dopamine (mV)</td>
<td>(-9.01 \pm 1.02)</td>
<td>(-9.46 \pm 1.00)</td>
<td>(-7.96 \pm 1.07)</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>(I_h) sag (mV)</td>
<td>20.98 ± 1.17</td>
<td>20.09 ± 1.10</td>
<td>19.20 ± 1.00</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE.
in firing rate following application of nifedipine in Fig. 2 is specific. In Ca\textsubscript{v}1.2DHP\textsuperscript{−/−} mice in which modulatory effects of nifedipine can only be mediated by Cav1.3, bath application of 5 \textmu M nifedipine for 10 min reduced firing rates by 13.8 ± 6.3% from 0.61 ± 0.17 Hz to 0.52 ± 0.13 Hz (n = 6, from 6 mice, paired t-test, P < 0.05) (Fig. 2). A peak reduction of 20.7 ± 6.0% from 0.61 ± 0.17 Hz to 0.50 ± 0.15 Hz was recorded after nifedipine was washed out for 6 min (paired t-test, P < 0.05) (Fig. 2). Nifedipine at 10 \textmu M reduced basal firing at greater percentages [20.3 ± 8.1% from 0.85 ± 0.17 Hz to 0.70 ± 0.21 Hz at 15 min (n = 5 from 5 WT mice, paired t-test, P < 0.05); 24.9 ± 7.7% from 0.77 ± 0.21 Hz to 0.63 ± 0.20 Hz at 15 min (n = 5 from 5 Ca\textsubscript{v}1.2DHP\textsuperscript{−/−} mice, paired t-test, P < 0.05)] (Fig. 2C). There was no significant difference in the reduction of basal firing by nifedipine between WT and Ca\textsubscript{v}1.2DHP\textsuperscript{−/−} mice (nifedipine 5 \textmu M, unpaired t-test, P > 0.05; nifedipine 10 \textmu M, unpaired t-test, P > 0.05) (Fig. 2C), indicating that the Ca\textsubscript{v}1.3 subtype drives basal firing in VTA DA cells.

**Spontaneously Active DA Cells in Ca\textsubscript{v}1.3\textsuperscript{−/−} Mice Are Fewer and Have a Lower Basal Firing Frequency**

To further confirm the involvement of Ca\textsubscript{v}1.3 in basal firing, we studied the properties of VTA DA neurons from Ca\textsubscript{v}1.3-deficient mice. Although Ca\textsubscript{v}1.3\textsuperscript{−/−} mice displayed an overall similarity in basic electrophysiological characteristics to WT, they exhibited a significantly lower number of spontaneously active cells and a significantly lower basal firing rate (Table 1). Seven of 26 cells from 26 mice (26.9%, \chi\textsuperscript{2}-test, P < 0.05 compared with that of WT group; Fig. 1C) were spontaneously active at a significantly lower basal firing frequency of 0.25 ± 0.07 Hz (unpaired t-test, P < 0.05 compared with that of WT group) (Fig. 1). Compared with WT, there were no differences in RMP (−52.13 ± 1.18 mV, unpaired t-test, P > 0.05), RMP of spiking (−47.25 ± 1.56 mV (n = 7, from 7 mice)) or quiescent (−53.82 ± 1.23 mV (n = 19, from 19 mice)) cells, spike amplitude (54.62 ± 2.31, unpaired t-test, P > 0.05), firing threshold (−41.03 ± 1.71 mV, unpaired t-test, P > 0.05), half-width (2.97 ± 0.20 ms, unpaired t-test, P > 0.05), or hyperpolarizing afterpotentials (−13.81 ± 1.51 mV, unpaired t-test, P > 0.05). Furthermore, compared with the WT group, Ca\textsubscript{v}1.3\textsuperscript{−/−} mice had similar average hyperpolarization following a brief application of 50 \textmu M DA (−7.96 ± 1.07 mV, unpaired t-test, P > 0.05) and I\textsubscript{h} sag (19.20 ± 1.00 mV, unpaired t-test, P > 0.05).

**LTCC Activators Convert Single Spiking to Bursting in Mice**

LTCC activators induce firing pattern conversion in rats (Liu et al. 2007; Zhang et al. 2005). We first set out to confirm that bursting could be induced by LTCC activators in mice. Bath application of 5 \textmu M (S)-(−)-Bay K8644, a DHP LTCC activator, for 7–11 min induced burst firing in 7 of 12 treated cells from 12 WT mice regardless of their original basal firing activity being quiescent or single spiking (Table 2; Fig. 3A), the same as we reported in rats (Liu et al. 2007; Zhang et al. 2005). The lag between the start of drug application and burst firing ranged from 9 to 37 min with an average of 22 ± 4.5 min, while in control experiments (n = 6) with vehicle no bursts developed in 1 h. Bursts showed a typical action potential clustering (3–6 spikes/burst, average 4.14 ± 0.46 spikes/burst) followed by a postburst hyperpolarization. The firing frequency in the bursts (1.17 ± 0.27 Hz; Fig. 3C) was much higher than that before bursts (0.11 ± 0.08 Hz, paired t-test, P < 0.05), and the frequency of bursting cycles was 0.06 ±
was much higher than that before the bursts (0.45 Hz; Fig. 4) was much higher than that before the bursts (0.45 Hz; Fig. 4). Similarly, bath application of 1–4 μM FPL 64176, a benzoylpyrrole site LTCC activator, for 8–24 min converted firing patterns from quiescent state or single spiking to burst firing in 11 of 16 treated cells from 16 mice (Table 2; Fig. 4). The time course was similar to that after (S)-(−)-Bay K8644, a depolarization and an increase in firing rates followed by a conversion of firing patterns 11–40 min (average: 24 ± 3.4 min) after the start of application. The burst firing was long lasting and could be reverted to single-spike firing or no firing by nifedipine (10 μM, 5–10 min, n = 6 from 6 mice; Fig. 4A). The bursts themselves had 2–9 spikes/burst (average 4.28 ± 0.79 spikes/burst). The firing frequency of spikes in the bursts (2.01 ± 0.44 Hz; Fig. 4C) was much higher than that before the bursts (0.75 ± 0.17 Hz, paired t-test, P < 0.05), and the frequency of bursting cycles was 0.12 ± 0.04 Hz.

The DHP LTCC Activator Converts Firing Patterns in Ca\(_{\text{v}} \)1.2DHP\(^{−/−}\) Mice

To further explore which of the two channel isoforms mediates drug-induced burst firing, Ca\(_{\text{v}} \)1.2DHP\(^{−/−}\) mice were used in which (S)-(−)-Bay K8644 selectively activates only Ca\(_{\text{v}} \)1.3 but not Ca\(_{\text{v}} \)1.2 (Sinnegger-Brauns et al. 2004). Bath application of 5 μM (S)-(−)-Bay K8644 for 8–20 min induced burst firing in 7 of 14 cells tested in 14 Ca\(_{\text{v}} \)1.2DHP\(^{−/−}\) mice (Fig. 5), similar to WT. The lag between the start of drug application and burst firing was longer but not significantly different (range 16–40 min with the average time of 26 ± 3.5 min, unpaired t-test, P > 0.05 compared with WT group). As in WT, burst firing was long lasting in six spontaneously firing cells but only lasted for 6 min in a quiescent cell. Nifedipine (10 μM for 10 min) reverted (S)-(−)-Bay K8644-induced burst firing to single-spike firing (n = 6, from 6 mice) (Fig. 5A). The bursts had 2–13 spikes/burst with an average of 4.39 ± 1.18 spikes/burst. The intraburst firing frequency (1.38 ± 0.32 Hz; Fig. 5C) was much higher than that before the bursts (0.45 ± 0.16 Hz, paired t-test, P < 0.05), and the frequency of bursting cycles was 0.10 ± 0.05 Hz.

DHP Site LTCC Blocker Inhibits Burst Firing Induced by a Non-DHP Site LTCC Activator in Ca\(_{\text{v}} \)1.2DHP\(^{−/−}\) Mice

Similarly to WT mice, bath application of 2–4 μM FPL 64176 for 13–24 min converted firing patterns from quiescent state or single spiking to burst firing in 7 of 14 treated cells from 14 Ca\(_{\text{v}} \)1.2DHP\(^{−/−}\) mice (Fig. 6). The responses were dose dependent: 4 μM FPL 64176 induced burst firing in three of six cells that did not respond to 2 μM. The time course was similar to that in WT mice, a depolarization and an increase in firing rates followed by a conversion of firing patterns 13–42 min (average: 21 ± 3.6 min, unpaired t-test, P > 0.05) after the start of application. The induced burst firing was long lasting except for one in the quiescent cell that lasted for only 9 min. Nifedipine (10 μM for 5–13 min, n = 6 from 6 mice; Fig. 6) inhibited the induced burst firing, being converted back into single-spike firing or into protracted and slow bursting resembling irregular firing. The bursts had 2–20 spikes/burst, (average 4.45 ± 1.12 spikes/burst). The intraburst firing frequency (5.91 ± 0.95 Hz; Fig. 6B) was much higher than that before the bursts (0.29 ± 0.12 Hz, paired t-test, P < 0.05), and the frequency of bursting cycles was 0.22 ± 0.07 Hz.
**Dose dependent: 4 μM FPL 64176 induced burst firing in 6 of 11 cells that did not respond to 2 μM.** The time course was a little bit different but not significantly: the lag between the start of drug application and appearance of burst firing was 14–50 min (average: 29 ± 2.5 min, unpaired t-test, P > 0.05 compared with that of WT group). The induced burst firing was not all long lasting (>30 min, Fig. 7B), and among 10 bursting cells 4 lasted only for 8, 13, 15, and 22 min (χ²-test, P < 0.05 compared with that of WT group). The bursts had 2–19 spikes/burst (average 5.06 ± 1.46 spikes/burst). The intraburst firing frequency (2.26 ± 1.08 Hz) was much higher than that before the bursts (0.0014 ± 0.0030 Hz, paired t-test, P < 0.05), and the frequency of bursting cycles was 0.04 ± 0.01 Hz. Nifedipine (10 μM for 5–21 min; Fig. 7A) reverted the induced burst firing (n = 6, from 6 mice) back to single-spike firing or no firing. These data clearly demonstrate that activation of Cav1.2 in the absence of Cav1.3 channels is sufficient to induce bursting.

**DISCUSSION**

The firing of DA cells in the VTA dictates the strength of DA transmission that is associated with normal or abnormal expression of motivation and reward processing. The LTCCs promote burst firing of these cells (Liu et al. 2007; Zhang et al. 2005), which is essential for salient stimuli (Chergui et al.
The novel finding of this study is that, by using two mouse models that allow the selective study of the two LTCC subtypes expressed in the brain, Cav1.2 and Cav1.3, we have uncovered their specific contributions to firing regulation. Cav1.3 LTCCs are crucial to regulate basal single-spike firing, while for burst firing both Cav1.2 and Cav1.3 LTCCs are involved.

Cav1.3 LTCCs Play an Important Role in Regulating Basal Single-Spike Firing

LTCCs are often assumed to be high voltage activated (e.g., activate between $-40$ and $-20\,\text{mV}$ membrane potentials) (Ertel et al. 2000), and Cav1.2 LTCCs display this typical voltage dependence (Mori et al. 1993). However, Cav1.3 LTCCs activate at relatively hyperpolarized membrane potentials (between $-60$ and $-40\,\text{mV}$ at physiological extracellular Ca$^{2+}$ concentrations) (Xu and Lipscombe 2001), which is near the RMP of DA neurons in the VTA. The difference in activation thresholds between these two subtypes implies that they may be coupled to different molecular signaling pathways (Giordano et al. 2010; Schierberl et al. 2011; Zhang et al. 2006) so as to mediate different physiological roles in neuronal electrical tasks. For example, Cav1.3 channels are likely to be important in mediating Ca$^{2+}$ influx in response to relatively small membrane depolarizations, and such properties may be important for sustaining spontaneous rhythmic firing in neurons. Consistent with this, the DHP blocker nimodipine partially suppresses spontaneous intracellualr calcium oscillations and slows rhythmic firing in postnatal cerebellar Purkinje cells (Liljelund et al. 2000). More direct evidence for the involvement of Cav1.3 channels in driving rhythmic activities in excit able cells comes from our previous study of Cav1.3-deficient mice whose phenotype includes compromised sinoatrial node function (Platzer et al. 2000). In line with this, the present study shows that Cav1.3 channels mediated rhythmic firing in DA neurons in the VTA because 1) Cav1.3-deficient mice, in which there are no Cav1.3 but Cav1.2 LTCCs expressed, have significantly lower basal firing frequencies; and 2) compared with WT mice, the DHP blocker nifedipine slowed spontaneous rhythmic firing to a similar extent in Cav1.2 knockin mice, in which Cav1.2 channels were not sensitive to DHP blockers and only Cav1.3 channels were blocked by nifedipine. The essential role for Cav1.3 channels to mediate spontaneous firing of DA neurons in the substantia nigra has been shown (Chan et al. 2007); other reports showed that LTCC blocker at higher concentrations (20 $\mu\text{M}$) affects channels other than the LTCCs and has differing actions on dendritic calcium oscillations and firing (Chan et al. 2007; Guzman et al. 2009; Khaliq and Bean 2010). Our results with nifedipine at different concentrations, consistent results with LTCC activator and blocker, and reduced basal firing from

![Image of LTCC activator FPL 64176 converting firing pattern in WT mice.](image-url)
slices where no activator or blocker was present indicate that basal firing of putative DA neurons in the VTA is driven by LTCCs, as reported previously by Mercuri et al. (1994), and is not due to nifedipine’s nonspecific actions on other channels.

Both Cav1.2 and Cav1.3 LTCCs Play a Role in VTA DA Burst Firing

Different from pacemaker firing, burst firing is a combination of subthreshold depolarization and action potential clustering, which is likely to be mediated by both Cav1.3 (activated at relatively hyperpolarized membrane potentials) (Xu and Lipscombe 2001) and Cav1.2 (high voltage activated) (Mori et al. 1993). It is reasonable to presume that Cav1.3 LTCCs mediate the subthreshold depolarization that raises the membrane potential to activate sodium channels initiating firing and then Cav1.2 LTCCs take over to maintain sequential firing. Our results suggest that Cav1.3 LTCCs are sufficient for bursting when both subtypes are present, while in the absence of the Cav1.3 subtype Cav1.2 subtype can drive bursting on its own.

Cav1.3−/− mice in which only Cav1.2 LTCCs exist. The explanations of the discrepancy between results from Cav1.2DHP−/− mice and Cav1.3−/− mice might be compensatory upregulation of Cav1.3 LTCCs to maintain the same level of firing.

Fig. 5. DHP site LTCC activator (S)-(−)-Bay K8644 converts firing pattern in Cav1.2DHP−/− mice. A: continuous current-clamp recording from a representative cell showing that (S)-(−)-Bay K8644 (5 μM) converted regular firing to burst firing that could be blocked by nifedipine (10 μM). B: density plot of ISIs in 0.1-s (<2 s) or 0.5-s (>2 s) bins from the same cell as in A in control conditions (209 events) and after (S)-(−)-Bay K8644 application (576 events). (S)-(−)-Bay K8644 dramatically shifted the peak to the left and gave rise to a secondary peak corresponding to the frequency of burst firing cycles. C: the firing frequency in the bursts was much higher than that before bursts (left), and the frequency of bursts was low (right), representing long pauses of firing between adjacent bursts.

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of Cav1.2 in these neurons in CaV1.3 knockouts. Although we cannot rule out this possibility, it appears unlikely because such upregulation has not been observed in any of the cellular systems investigated so far in these mice (Marcantoni et al. 2010; Platzer et al. 2000). A more likely explanation has recently been provided in medium spiny neurons (Olson et al. 2005). In these cells CaV1.3 stabilizes upstate potentials by means of inward current at threshold potentials allowing prolonged firing of these cells. This effect is absent in CaV1.3/−/− neurons but can be rescued by treatment with (S)-(−)-Bay K8644. This can be rationalized by the fact that (S)-(−)-Bay K8644 and FPL 64176 shift activation voltage to more negative voltages, thereby promoting CaV1.2 activity at threshold voltages (Bargas et al. 1994; Fan et al. 2000). However, such CaV1.2 activation by activators might be a purely pharmacological finding, and whether it exists in the real cells remains unclear. Because of the ability of these LTCC activators to modulate channel kinetics in ways that may not occur under physiological conditions, their effects may not necessarily predict functional changes involved during physiological phenomena. FPL 64176 and (S)-(−)-Bay K8644 open LTCC channels by altering gating, prolonging opening, and increasing conductance of the channels (Fan et al. 2001; McDonough et al. 2005), probably different from physiological stimuli on LTCCs. However, it is possible that, for example, receptor-mediated protein kinase A (PKA) phosphorylation of LTCCs, which also enhances open probability and allows activation at lower voltages (Kamp and Hell 2000), can induce activity similar to pharmacological calcium channel activation.

Therefore, we believe that when both LTCC subtypes are present within the VTA CaV1.3 plays a more prominent role than CaV1.2 in regulating DA cell firing and the resulting spike-dependent DA release in its terminals. This understanding of subtype roles was further supported when we blocked the LTCC subtype CaV1.3 in the CaV1.2DHP/−/− mice, as bursting was completely abolished and was returned to control levels. So given the outcomes in both WT and CaV1.2DHP/−/− mice, this strongly indicates that LTCC subtype CaV1.3 has a more active and crucial role in bursting than CaV1.2. CaV1.2 LTCCs are not necessary for burst firing under normal conditions but might aid it in some way, because our data showed that the latency to bursting (lag time) was slightly longer in CaV1.2DHP/−/− slices than in WT slices, and in a few cases induced bursting did not last as long. Also, in CaV1.3/−/− mice significantly fewer bursts induced by activation of LTCCs lasted as long as in WT mice. The mechanism for this might be related to contributions to the hump potential, which is already shown to be mediated by LTCCs (Zhang et al. 2005), similar to the plateau potential underlying bistable membrane behavior in motor neurons in which CaV1.3 LTCCs are involved (Carlin et al. 2000; Hsiao et al. 1998).
Cav1.3 LTCCs are Functionally Important

Burst firing of VTA DA neurons and phasic dopamine release at their terminals in the nucleus accumbens have been shown to be crucial for drug seeking behavior in rodent models of addiction (Gardner 2011; Koob and Volkow 2010). The precise molecular mechanisms involved in VTA DA neuron burst firing that underlies drug seeking behavior remain largely unknown; however, our finding in this study that Cav1.3 channels are important for the transition from spontaneous to burst firing strongly supports a role for Cav1.3 channels. This is further supported by our previous finding that Cav1.3 channels and their Ca\(^{2+}\)-activated pathways, in VTA DA neurons, are recruited after repeated amphetamine treatment (Giordano et al. 2006; Rajadhyaksha et al. 2004) and additionally play a critical role in cocaine’s long-term behavioral effects (Schierberl et al. 2011). In addition to reward seeking behavior, increased burst firing of VTA DA neurons has also been found in rodent models of depression (Cao et al. 2010; Krishnan et al. 2007; Razzoli et al. 2011). Our finding of a role for Cav1.3 channels in VTA DA neuron burst firing supports a potential role for Cav1.3-mediated burst firing in depression-related behaviors. This hypothesis is further supported by our previous finding that Cav1.3 channels modulate depression-like behaviors, as identified in the Cav1.3 knockout mice that we have used in this study (Busquet et al. 2010). Taken together, LTCCs, especially Cav1.3 subtypes, might play a significant

Fig. 7. LTCC activator FPL 64176 converts firing pattern in Cav1.3\(^{-/-}\) mice. A: continuous current-clamp recording from a representative cell showing that FPL 64176 (4 \(\mu\)M) converted regular firing to burst firing that could be blocked by nifedipine (10 \(\mu\)M). B: continuous current-clamp recording from a representative cell showing that the induced burst firing by FPL 64176 in Cav1.3\(^{-/-}\) mice was shorter in duration (<30 min).
role in central DA transmission and its related pathologies. Further experiments on the role of LTCC subtypes would have benefits for the therapies of these DA-related diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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REFERENCES


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