ENDOGENOUS CALCIUM BUFFERING CAPACITY OF SUBSTANTIA NIGRAL DOPAMINE NEURONS

Running Head: Calcium Buffering by Dopamine Neurons

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Abstract

Dopamine (DA) containing cells from the substantia nigra pars compacta (SNc) play a major role in the initiation of movement. Loss of these cells results in Parkinson’s disease (PD). Changes in [Ca^{2+}]_i elicit several events in DA cells, including spike afterhyperpolarizations (AHPs) and subthreshold oscillations underlying autonomous firing. Continuous Ca^{2+} load due to Ca^{2+}-dependent rhythmicity has been proposed to cause death of DA cells in PD and normal aging (Surmeier, 2007). Because of the physiological and pathophysiological importance of [Ca^{2+}]_i in DA cells, we characterized their intrinsic Ca^{2+}-buffering capacity (K_s) in brain slices. We introduced a fluorescent Ca^{2+}-sensitive exogenous buffer (200 μM fura-2) and cells were tracked from break-in until steady state by stimulating with a single action potential (AP) every 30s and measuring the Ca^{2+}-transient from the proximal dendrite. DA neurons filled exponentially with a τ of ~5-6 min. [Ca^{2+}]_i was assumed to equilibrate between the endogenous Ca^{2+} buffer and the exogenous Ca^{2+} indicator buffer. Intrinsic buffering was estimated by extrapolating from the linear relationships between the amplitude or time constant of the Ca^{2+} transients vs. [fura-2]. Extrapolated Ca^{2+}-transients in the absence of fura-2 had mean peak amplitudes of 293.7 ± 65.3 nM and τ = 124 ± 13 ms (P13-P17). Intrinsic buffering increased with age in DA neurons. For cells from animals P13-P17, K_s was estimated to be ~110 (n = 20). In older animals (P25-P32), the estimate was ~179 (n = 10). These relatively low values may reflect the need for rapid Ca^{2+} signaling, e.g., to allow activation of sK channels, which shape autonomous oscillations and burst firing. Low intrinsic buffering may also make DA cells vulnerable to Ca^{2+}-dependent pathology.

Key Words: dopamine, calcium, buffering, Parkinson’s disease, fura-2

INTRODUCTION

Dopamine (DA) cells are important for the initiation of movement and the loss of these cells results in Parkinson’s disease (PD: Dauer and Przedborski, 2003). In the mammalian midbrain, DA containing cells are primarily found in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc). They typically fire action potentials (APs) tonically at low rates (<10Hz) in vivo, in either regular or irregular patterns (Wilson et al. 1977; Grace and Bunney 1983b; 1984a; Hylund et al. 2002; Overton and Clark, 1997; Tepper et al. 1995). Burst firing is a third pattern, superimposed upon the background of tonic firing and characterized by a high rate of firing within the burst (Celada et al. 1999; Grace and Bunney, 1984b; Hylund et al. 2002; Tepper et al. 1995). Burst firing of DA cells is temporally locked to reward prediction error (Schultz, 2002), allowing these cells to control reinforcement learning (Dayan and Balline, 2002; Schultz, 2002). In vitro, DA cells typically fire autonomously and tonically in a regular pattern (Fujimura and Matsuda, 1989; Grace and Bunney, 1984a,b; Harris et al. 1989; Kita et al. 1989; Lacey et al. 1987; Nedergaard and Greenfield, 1992; Puopolo et al. 2007; Chan et al. 2007).

Many aspects of firing behavior in DA neurons are Ca^{2+}-dependent and several types of Ca^{2+} channels are expressed in DA neurons (Durante et al. 2004; Nedergaard et
al. 1993; Wolfart and Roeppe, 2002; Cardozo and Bean, 1995), including CaV1.3 L-type channels (Takada et al. 2001; Cahn et al. 2007), which activate at relatively negative potentials (vs. CaV1.2 channels: Koschak et al. 2001; Scholze et al. 2001; Xu and Lipscombe, 2001). The autonomous firing of mature DA cells from SNC reflects underlying oscillations mediated by the low-threshold calcium current through CaV1.3 L-type channels (Fujimura and Matsuda, 1989; Grace and Onn, 1989; Harris et al. 1989; Wilson and Callaway, 2000; Kang and Kitai, 1993a,b; Mercuri et al. 1994). While subthreshold activation of L-type channels results in activation of an apamin-sensitive sK-mediated current (Shephard and Bunney, 1991; Ping and Shephard, 1996; Wilson and Callaway, 2000), spike-induced AHPs are coupled to N-type as well as L-type Ca\(^{2+}\) channels (Papuolo et al. 2007). Blockade of this sK current leads to development of long-duration Ca\(^{2+}\)-dependent plateau potentials (Johnson and Wu, 2004; Shepard and Bunney, 1991). Ca\(^{2+}\)-dependent oscillations in dendrites combine to account for tonic firing in DA neurons (coupled oscillator model: Wilson and Callaway, 2000; Medvedev et al. 2003). Addition of NMDA conductance to the model allows burst firing (Kuznetsov et al. 2006). Ca\(^{2+}\)-dependent sK currents play a key role during burst firing by removing inactivation of Na\(^{+}\) currents to allow high frequency firing (Kuznetsov et al. 2006).

Because of the physiological and pathophysiological importance of [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\)-dependent currents in DA cells from SN, we characterized their endogenous Ca\(^{2+}\)-buffering capacities by addition of an exogenous Ca\(^{2+}\) indicator/buffer that alters native [Ca\(^{2+}\)]\(_i\) dynamics in predictable ways (Neher et al. 1992; Helmchen et al. 1996). We found that intrinsic Ca\(^{2+}\) buffering in DA cells increased with postnatal age but buffering remains unexceptional at both age ranges, potentially placing these cells at risk for Ca\(^{2+}\)-dependent pathophysiology.

**METHODS**

**Brain Slice Preparation.** Thin coronal brain slices containing the SNC were made from brains of Sprague-Dawley rats (P13-P17 or P25-P32). The rats were deeply anesthetized with an IP injection of a mixture of 87 mg/kg ketamine and 13 mg/kg xylazine and perfused intracardially with cold cutting solution. This solution contained (in mM): 250 sucrose, 25 KCl, 1 NaH\(_2\)PO\(_4\), 11 glucose, 4 MgSO\(_4\), 0.1 CaCl\(_2\), and 15 HEPES (pH 7.3–7.4; 300 mOsm/L). Their brains were then removed and the brain was sliced at 200-300 μm (P25-P32) or 300 μm (P13-P17). Slices were maintained in an artificial cerebrospinal fluid (aCSF) mixture of (in mM) 124 NaCl, 2.5 KCl, 2.0 CaCl\(_2\), 2.0 MgCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 D-glucose (bubbled with 95% O\(_2\)-5% CO\(_2\), pH 7.4). Slices were stored at room temperature prior to recording. Recordings were obtained at 32±1°C as Ca\(^{2+}\)-dependent oscillations were much more robust at temperatures approximating those in vivo.

**Patch Clamp Recording.** Slices were transferred to a mesh surface in a chamber containing aCSF at room temperature for at least 1 hour. The brain slice was placed in a recording chamber on the stage of an Olympus BX50WI upright microscope (in carbogenated aCSF at 2 ml/min). Cells were visualized in the slice with IR-DIC optics using a 40X (0.8 NA) water immersion objective and under IR illumination.
(780 ± 30 nm) using the same CCD camera used for Ca\(^{2+}\) imaging. A tilt Polychrome II monochromator was used to change the excitation frequency for fura-2.

Micropipettes had resistances of 5-8 MΩ and were filled with a solution containing (in mM) 135 K-Gluconate, 5 KCl, 4 NaCl, 10 HEPES, 1 Na-ATP, 1 Mg-ATP, 0.3 Na-GTP, and 0.2 fura-2 (pH 7.4). Current-clamp recordings were made using a Neurodata active bridge amplifier. Electrical and optical data were collected synchronously using a single computer using software written by Dr. J. Callaway (Abel et al. 2004; Callaway et al. 1993, 1995a,b, 1997). Electrical records were digitized at 16-bit resolution at 10 kHz, and corrected for a 10-mV liquid junction potential. Cells with overshooting action potentials (APs) and strong fura-2 signals were accepted for further study. At 30 s intervals beginning from cell break-in, a single action potential was elicited by a 10 ms suprathreshold current injection and the fluorescence at 360 nm (isosbestic wavelength) and 380 nm was measured.

**Ca\(^{2+}\) Imaging.** Current clamp data were taken while the membrane potential was held hyperpolarized with DC current (to ~60 mV) to prevent autonomous activity. Optical recordings were obtained using a Sensicam Imago cooled CCD camera at a frame rate of 50 Hz. Changes in fluorescence values were processed and interpreted using a modification of the methods described by Lev-Ram et al. (1992). Ratiometric measurements were converted to calcium concentration (Grynkiewicz et al. 1985) using our measured value for the fura-2 calcium dissociation constant (fura \(k_D\) = 260 nM) and the maximal (\(R_{max} = 7.96\)) and minimal fluorescences (\(R_{min} = 0.42\): Sf380/Sb380 = 10.98) of fura-2 in our electrode filling solution. These values were determined using standards obtained from Molecular Probes. Each trial began with a 1s segment of data gathered at ~60 mV. Resting [Ca\(^{2+}\)]\(_i\) was calculated from the pre-AP fluorescence at 360 nM vs. 380 nM. Subsequent changes in fluorescence at 380 nM then were converted to calcium concentrations using the formula from Wilson and Callaway (2000):

\[
[\text{Ca}]_2 = - \frac{\Delta F/F \cdot KD + [\text{Ca}]_i \cdot ((\Delta F/F - 1) \cdot Sb380 + 1)}{[\text{Ca}]_i \cdot \Delta F/F \cdot Sb380 + (\Delta F/F - 1 + Sb380/Sf380)}
\]

where Sb380/Sf380 is the ratio of fluorescence of bound and free fura-2 as used in Grynkiewicz et al. (1985), \(\Delta F/F\) is the change in fluorescence at 380 nm divided by the fluorescence measured immediately after the opening of the shutter, corrected for autofluorescence as described below. Fluorescence measurements were corrected for photobleaching during the trial by measuring the bleaching that occurred when the cell was held hyperpolarized (~60 mV), filtering the resulting curve at 3 Hz, and subtracting the resulting curve from trials in which the cell was depolarized. An autofluorescence correction was performed by subtraction of measured autofluorescence of a nearby region of the slice from the measured initial value of \(F\).

**Measurement of fura-2 filling.** After membrane breakthrough, the loading of fura-2 was followed by measuring the isosbestic fluorescence (\(\lambda=360\) nm) over time. Measurements were made at the main proximal dendrite where it connects to the soma. Fluorescence was measured every 30s until steady-state filling of the dye fura-2 was observed. The time course of fura-2 filling was then fitted with a single exponential using a macro in Igor. Concentration of fura-2 in the cell was assumed to be zero just
before breaking into the cell and 200 μM (concentration in electrode) at steady-state. The concentration at any point in time could then be extrapolated from the filling curve.

**Ca**\(^{2+}\)-binding capacity. The Ca\(^{2+}\) transient is buffered by endogenous mechanisms (Ca\(^{2+}\)-binding proteins, internal stores, extrusion mechanisms, etc.) and by the exogenous indicator/buffer (in this case, fura-2). Neher (1995) measured the Ca\(^{2+}\) transient in chromaffin cells in response to different exogenous Ca\(^{2+}\)-indicator/buffer concentrations. By extrapolation to zero exogenous buffer, the endogenous component of Ca\(^{2+}\) buffering was estimated (“added buffer method”). Using a single compartment model of Ca\(^{2+}\) transient buffering (Neher and Augustine 1992; Helmchen et al. 1996), the [Ca\(^{2+}\)] transient evoked by an action potential can be described by the balance in partitioning of free Ca\(^{2+}\) among different Ca\(^{2+}\) buffers and Ca\(^{2+}\) removal mechanisms, expressed together collectively as the endogenous Ca\(^{2+}\) buffering capacity, \(K_S\).

In general, the differential Ca\(^{2+}\)-binding capacity (or binding ratio) of Ca\(^{2+}\) buffer X is defined as the ratio of buffer bound Ca\(^{2+}\) to total free Ca\(^{2+}\): \(K_X = \delta[Ca^{2+}_X]/\delta[Ca]\). An incremental Ca\(^{2+}\)-binding capacity \(K'_X\) can also be defined for significant changes from resting Ca\(^{2+}\): 

\[
\kappa'_X = \frac{K'_D[X]}{(K'_D + [Ca^{2+}_{rest}]) (K'_D + [Ca^{2+}_peak])}
\]

We used the single cell variant of the added buffer method (Helmchen et al. 1996) and a single compartment model (dendrite adjacent to the soma) in DA neurons from SNC. Before patch break-in, buffering is entirely due to endogenous buffers. After break-in, the concentration of exogenous buffer (fura-2) increases until it equilibrates with the concentration in the pipette (200 μM). For brief small changes in [Ca\(^{2+}\)], such as those produced by single action potentials, the total Ca\(^{2+}\) influx, \(\Delta[Ca^{2+}_T]\) will be partitioned between the exogenous buffer capacity, \(K_{B'}\), and endogenous buffer capacity \(K_S\). The incremental buffering capacity of fura-2 (\(K_{B'}\)) was calculated using the following equation:

\[
K_{B'} = \frac{[B]K_D}{([Ca^{2+}_{rest} + K_D])([Ca^{2+}_peak + K_D])}
\]  

Where [B] and K_D are the concentration and dissociation constant for fura-2, respectively (Helmchen et al. 1996). [Ca\(^{2+}\)_peak] is the difference between resting [Ca\(^{2+}\)] and peak [Ca\(^{2+}\)] due to an AP.

We evoked a single AP every 30s after break-in and measurements of fura-2 fluorescence were made at 380 nM to allow estimation of [Ca\(^{2+}\)]i. The Ca\(^{2+}\)-transient evoked by an AP can be described as an instantaneous step function with amplitude A and an exponential decay time constant \(\tau\) for the change in [Ca\(^{2+}\)]i. If AP-induced Ca\(^{2+}\) influx is constant over time during the recording, the time integral of the Ca\(^{2+}\) transient should remain constant and be independent of \(K_{B'}\). Assuming that the Ca\(^{2+}\) influx from the action potential and the endogenous Ca\(^{2+}\) buffering capacity do not change over time, any changes in the Ca\(^{2+}\) transient will be due to the increasing concentration of the exogenous Ca\(^{2+}\) buffer over time. A and \(\tau\) can then be related to the buffering capacity of the endogenous buffer (\(K_S\)) by equations 3 and 4 (Helmchen et al. 1996):

\[
A = \Delta[Ca^{2+}_T]/(1 + K_S + K_B)
\]  

\[
\tau = \frac{\Delta[Ca^{2+}_T]}{K_S}
\]
\[ \tau = \frac{(1 + K_S + K_B)}{\gamma} \]  

(4)

Where \( \Delta[Ca^{2+}]_T \) is the increase in total \( Ca^{2+} \) (free and bound) and \( \gamma \) is the \( Ca^{2+} \) extrusion rate. Both \( \tau \) and the inverse of \( A \) depend linearly on \( K_B \), allowing estimates of endogenous \( Ca^{2+} \) binding ration \( (K_S) \) as the negative X intercept of plots of \( 1/A \) or \( \tau \) vs. \( K_B \) (or \( K_B' \): Helmchen et al. 1996).

Using a modification of the multivariate curve fitting method of Jackson and Redman (2003), the concentration of the endogenous buffer \( [B_T] \) and its dissociation constant \( K_D \) can be estimated (assuming a single, lumped buffer). In this experiment, the amplitude of the \( Ca^{2+} \)-transient is dependent on the concentration of exogenous buffer (fura-2) introduced into the cell and the endogenous buffering capacity remains constant \( (K_S) \):

\[
\frac{1}{A} = \frac{1}{A_T} + \frac{1}{A_T} (K_S) + \frac{1}{A_T} \left( \frac{[B_T]K_B}{([Ca^{2+}]_{resting} + K_B) ([Ca^{2+}]_{peak} + K_B)} \right) 
\]  

(5)

From the experiment, the values of \( A, A_T, [Ca^{2+}]_{rest} \) and \( [Ca^{2+}]_{peak} \) are known. Therefore, the only free parameters are \( [B_T] \) and \( K_B \). Using a multivariate fit by minimizing the sum-of-squares error, solutions to \( [B_T] \) and \( K_B \) were obtained. We compared solutions to our estimate of \( K_S \) from the previous analysis and only solutions that provide a similar estimated \( K_S \) were accepted.

Similarly,

\[
\tau = \tau_T + \tau_T (K_S) + \tau_T \left( \frac{[B_T]K_B}{([Ca^{2+}]_{resting} + K_B) ([Ca^{2+}]_{peak} + K_B)} \right) 
\]  

(6)

The values of \( \tau, \tau_T, [Ca^{2+}]_{resting} \) and \( [Ca^{2+}]_{peak} \) are known. Again, the only free parameters are \( [B_T] \) and \( K_B \). The previous analysis for \( K_S \) provides an estimate of the acceptable \( K_S \) solution space.

The multivariate fits and estimates of errors in the fitting parameters for \( [B_T] \) and \( K_B \) were obtained using Origin. Summary data are presented as mean ± standard error of the mean (SEM). Linear curve fitting was performed using Kaleidograph. Summary statistics and unpaired t-tests were performed using Prism and Excel.

**RESULTS**

All recordings were obtained from DA cells in slices from SNC of Sprague–Dawley rats (P13-P17 or P25-P32) using internal solutions that included 200 \( \mu M \) fura-2. DA cells fire autonomously in slices (Fig. 1D). In mature neurons, the subthreshold oscillations underlying this firing persist after blockade of Na+ channels with tetrodotoxin (TTX: 1 \( \mu M \); Fig. 1D) and are due to \( Ca^{2+} \) entry through L-type channels (Nedargaard et al. 1993; Wilson and Callaway, 2000; Chan et al. 2007). This subthreshold \( Ca^{2+} \) entry also activates sk channels leading to an apamin-sensitive mAHP (Shephard and Bunney, 1991; Ping and Shephard, 1996; Wilson and Callaway, 2000; Wolfart et al. 2001; Wolfart and Roeper, 2002; Puopolo et al. 2007). In this study, all cells were hyperpolarized to ~ -
60 mV with DC current and action potentials were elicited with brief (10 ms) current injection (APs: Fig. 1). Mean values for “resting” membrane potential, AP amplitude, AP width at half-amplitude (from RMP) for the sample of neurons at P13-P17 or P25-P32 are included in Table 1. Following a single AP, DA cells expressed a prominent AHP (Fig. 1B; Table 1).

Table 1. Data for membrane potentials and action potentials. AP and AHP amplitude were measured from “resting” potential to the peak voltage change. AP half-width was measured at ½ of the amplitude (measured from rest).

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>AP (mV)</th>
<th>AP Half-width (ms)</th>
<th>AHP Amplitude (mV)</th>
<th>AHP duration (ms)</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13-P17</td>
<td>-63 ± 0.9</td>
<td>70 ± 2</td>
<td>2.1 ± 0.1 ms</td>
<td>10 ± 1</td>
<td>622 ± 99</td>
<td>24</td>
</tr>
<tr>
<td>P25-P32</td>
<td>-67 ± 1.3</td>
<td>73 ± 1.8</td>
<td>1.8 ± 0.1 ms</td>
<td>12 ± 2.1</td>
<td>522 ± 62</td>
<td>15</td>
</tr>
</tbody>
</table>

Our initial experiments were on young animals (P13-P17) to take advantage of the lack of myelin and advantageous optical conditions. This facilitated imaging at early times after break-in, when fura-2 concentrations in the cell are low. Every 30s, we measured single AP-induced changes in fura-2 fluorescence (excitation at 380 nM). Prior to the AP, data was also obtained at the isosbestic wavelength (360 nm). Figure 1A shows typical transient fura-2 responses corresponding to a single AP. Records at 380 nM reflect Ca²⁺-dependent quenching of fura-2 fluorescence (Fig. 1A, C). All records were corrected for autofluorescence (see Methods) and data are presented as %ΔF/F to correct for the intensity of F just prior to the stimulus. These data were converted to estimates of [Ca²⁺]ᵢ using Equation 1 (Methods: Grynkiewicz et al. 1985; Wilson and Callaway, 2000). %ΔF/F was measured at a basal dendritic location (< 25 μm from soma: boxes in Fig. 1C).

Figure 1 also shows changes in the intensity of fura-2 fluorescence, %ΔF/F, and the AHP with time during the recording (P17 animal). Just after initial break-in, fura-2 concentration was low and fluorescence (measured at 360 or 380 nM) weak (Fig. 1C, upper). Fura-2 fluorescence increased with time after break-in (Fig. 1C, lower). There were also changes in %ΔF/F (Fig. 1A). In this cell, the response at 1 min shows a sharp peak and exponential decay. At 24 min, the peak response was attenuated but the decay was prolonged. AP amplitude and half-width changes little over this time (Fig. 1B). In contrast, the AHP was reduced in amplitude at 24 min (Fig. 1B).

Fluorescence at the isosbestic wavelength (360 nM) was used to estimate the rate of filling of the cell with fura-2 (Fig. 2). The amplitude of the 360 nM response increased with time and these data were well fit by a single exponential function. Figure 2 shows a representative cell (filling τ = 3.69 min) and the histogram summarizes data from 27 cells (τ = 4.8 ± 0.4 min: P13-P17). Deviations from exponential filling may be due to changes in pipette access or cell leakage thus cells exhibiting such deviations were not studied further. Concentration of fura-2 in the cell was assumed to be zero just before
breaking into the cell and 200 μM (concentration in electrode) at steady-state. The concentration at any point in time could then be extrapolated from the filling curve.

We used the methods of Helmchen et al. (1996) to estimate $K_S$ by extrapolation of plots of $K_B$ (determined from estimated [fura-2]: Methods). Figure 3 shows determination of $K_S$ in a representative cell: P15). Fura-2 transients (%$\Delta F/F$) in response to a single AP changed with time after break-in (Figs. 1A, 3A). These transients were fit with a single exponential function to determine $\tau_{\text{decay}}$ and amplitude (extrapolate to time zero: Fig. 3A). Similar estimates of $K_S$ were obtained from transient amplitude obtained by extrapolation or measured directly from the peak of the transient. $K_S$ can be estimated from either the change in the amplitude of %$\Delta F/F$ (Fig. 3B) or from the decay $\tau$ (Fig. 3C). We plotted either the reciprocal of the Amplitude (1/A) or $\tau_{\text{decay}}$ against $K_B$. (determined for each time from the filling curve: see above). The negative of the X intercept corresponds to $K_S$ (Methods).

For the cell in Figure 3, the plots of 1/A vs. $K_B$ estimated $K_S$ to be ~ 65 and the estimate from $\tau_{\text{decay}}$ was ~ 104. Typically there was closer agreement between these two measures in a given cell (P13-P17) and overall there were no significant differences between estimates of $K_S$ from 1/A (110 ± 12, n=20 cells) and $\tau_{\text{decay}}$ (117 ± 21, 11 cells). We also obtained similar values for $K_S$ from 1/A (101 ± 13; n = 10) and $\tau$ (96 ± 5; n= 3) for layer II/III neocortical pyramidal cells (P13-P17: data not shown).

Extrapolations also permitted estimates of the amplitude and $\tau_{\text{decay}}$ that would be expected in DA cells in the absence of exogenous buffer. We estimated transient amplitude in the absence of exogenous buffer to be 269 ± 32 nM (n = 20) and $\tau_{\text{decay}}$ as 124 ± 13 ms (n = 19). These values are similar to those obtained for pyramidal neurons (Helmchen et al. 1996; Maravall et al. 2000; Kaiser et al. 2001) and neocortical bitufted interneurons (Kaiser et al. 2001). We used multivariate curve fitting methods (Methods; Jackson and Redman, 2003) to estimate values of total buffer ($B_T$), buffer $K_D$, and resting $[\text{Ca}^{2+}]_i$. For $B_T$ we estimated 2.8 ± 0.5 μM from transient amplitude (n = 10) and 2.1 ± 0.5 μM from $\tau_{\text{decay}}$ (n=7). Estimates for $K_D$ were 271 ± 64 nM from amplitude (n = 10) and 197 ± 75 nM from $\tau_{\text{decay}}$ (n = 7).

An assumption with this method is that Ca$^{2+}$ entry and changes in $[\text{Ca}^{2+}]_i$ are constant with time (constant stimulus so that changes with time reflect only changes in exogenous buffer: Helmchen). To test this, we measured the integral of $[\text{Ca}^{2+}]_i$ (A*t) and plotted this against $K_B$ (Fig. 3D). This slope of this relationship was not significantly different from zero in any cell measured (n = 21), indicating that Ca$^{2+}$ entry did not change significantly over time.

For technical reasons (see above), most of our data were obtained from very young rats (P13-P17). Because Ca$^{2+}$-dependent autonomous firing is developmentally labile in DA cells (Chen et al. 2007) and adult DA cells become impaired and die in PD, we tested whether intrinsic Ca$^{2+}$ buffering changes with age in DA neurons. We thus recorded from DA cells from animals at P25-P32. Attempts to study older animals were limited by the development of large myelinated axons adjacent to and above the DA neurons in SNc. The increased light scatter in animals > P32 resulted in our being unable to detect early stages of cell filling with fura-2 and limited resolution of cell dendrites. We were able to obtain filling curves from 10 cells in the older age group, with $\tau_{\text{filling}} = 6.0 ± 0.7$ min (Fig. 2, inset). From plots of 1/A, our estimate of $K_S$ was 179 ± 33 (n=10).
for the older animals (Fig. 3B, inset). This was significantly greater than in P13-P17
animals (p < 0.02, unpaired t-test). We could only obtain reliable measurements of $\tau_{\text{decay}}$
to determine $K_S$ in 4 cells (120 ± 44). There was no relationship between $K_B$ and $A^*\tau$
in the older animals (not shown).

**DISCUSSION**

In DA neurons from SNc, changes in $[\text{Ca}^{2+}]_i$ elicit several $\text{Ca}^{2+}$-dependent events,
including AHPs and subthreshold oscillations. Notably, the tonic firing of mature DA
cells reflects underlying pacemaker oscillations mediated by a low-threshold calcium
current through L-type channels (Fujimura and Matsuda, 1989; Grace and Onn, 1989; 
Harris et al. 1989; Wilson and Callaway, 2000) and subsequent activation of an apamin-
sensitive $\text{sK}$-mediated current (Shephard and Bunney, 1991; Wilson and Callaway,
2000). $\text{Ca}^{2+}$-dependent oscillations in dendrites combine to account for tonic firing in
DA neurons (Wilson and Callaway, 2000; Medvedev et al. 2003) and $\text{Ca}^{2+}$-dependent $\text{sK}$
currents play a key role in removing inactivation of $\text{Na}^+$ currents to allow high frequency
firing during bursting (Kuznetsov et al. 2006).

Because of the physiological and pathophysiological importance of $[\text{Ca}^{2+}]_i$ in DA
cells (Surmeier, 2007), we characterized the effectiveness of intrinsic $\text{Ca}^{2+}$-buffers by
calculating the intrinsic $\text{Ca}^{2+}$ binding ratio ($K_S$) in brain slices of the SN by the “method
of added buffer”. We introduced a $\text{Ca}^{2+}$-sensitive indicator / buffer at a known
concentration (200 $\mu$M fura-2). DA neurons filled exponentially with fura-2, with a time
constant of ~5-6 min. The concentration of fura-2 at any time during the recording was
then estimated from the filling curve. $K_S$ was estimated by extrapolating from the linear
relationships between the amplitude or time constant of the $\text{Ca}^{2+}$ transients vs. combined
exogenous and endogenous buffering. At P13-P17, $K_S$ was 110-117. At P25-P32, $K_S$
was significantly greater (~179), but still modest. Thus, despite the potential for a
sustained $\text{Ca}^{2+}$ load due to subthreshold $\text{Ca}^{2+}$ entry and autonomous firing, DA cells
display a modest level of intrinsic $\text{Ca}^{2+}$ buffering, similar to many cell types that do not
exhibit $\text{Ca}^{2+}$-dependent pacemaker firing (see below; Neher and Augustine, 1992;

**Measurements of intrinsic $\text{Ca}^{2+}$ buffering ($\text{Ca}^{2+}$ binding ratio).** Only a small
percentage of $\text{Ca}^{2+}$ entering the cytosol remains as free $\text{Ca}^{2+}$ (Gorman and Thomas,
1980; Berridge et al. 2000; Tank et al. 1995) because multiple mechanisms collectively
regulate $[\text{Ca}^{2+}]_i$ within narrow limits. There are a bewildering number of possible $\text{Ca}^{2+}$-
binding reaction partners (including mitochondria, internal stores, calcium binding
proteins), thus an overall estimate of $K_S$ is a reasonable initial step towards understanding
the role of buffering of $\text{Ca}^{2+}$ in different types of cells (Neher, 1995). The basic strategy
of extrapolating relationships between exogenous buffers plus endogenous to reveal
intrinsic buffering was developed by Neher and coworkers (Zhou and Neher, 1993; 
Neher and Augustine, 1992; Neher, 1998). Briefly, $\text{Ca}^{2+}$ buffering capacity can be
quantified by measuring changes in $\text{Ca}^{2+}$ bound buffer divided by the free $\text{Ca}^{2+}$ increase
(calcium binding ratio, $K_S$) using a single compartment model. In chromaffin cells, $K_S$
(bound $\text{Ca}^{2+}$/free $\text{Ca}^{2+}$) was estimated at 40-75 (Neher and Augustine, 1992; Zhou and
Neher, 1993). That is, at steady-state only ~1.3-2.5% of $\text{Ca}^{2+}$ ions that enter, remain free.
Helmchen et al. (1996) and Lee et al. (2000) found excellent agreement between estimates of $K_S$ derived from many cells, each with a single [buffer] to estimates using a single cell method where the dye filling-curve was used to estimate [dye] (see also Kaiser et al. 2001).

$K_S$ provides an estimate of the ability of a cell to handle Ca$^{2+}$ loads during physiological and pathophysiological activation, with high $K_S$ values associated with greater ability to handle a Ca$^{2+}$ load. Estimates of $K_S$ vary approximately 50-fold across the cell types tested to date. We found that despite the potential for nearly continual Ca$^{2+}$ entry in vivo, DA cells do not have especially high intrinsic buffering capacity ($K_S \sim 110$ (P13-P19) or 179 (P25-P32). That is, 0.5-1% of Ca$^{2+}$ that enters remains free at steady-state). Several other neuron types that do not exhibit Ca$^{2+}$-dependent autonomous firing have similar intrinsic Ca$^{2+}$ buffering capacity to DA cells ($K_S$: 100-200). These include neocortical pyramidal cells (our results; Helmchen et al. 1996; Kaiser et al. 2001), basal forebrain neurons (Tatsumi and Katayama, 1993), and hippocampal granule cells (Stocca et al. 2008). In contrast, motoneurons (Lips and Keller, 1998; Palacek et al. 1999) and CA1 hippocampal pyramidal neurons had $K_S$ of 30-60 (Maravall et al. 2000; Lee et al. 2000; Sabatini et al. 2002; Powell et al. 2008; but see Helmchen et al. 1996). Other neuron types have higher intrinsic Ca$^{2+}$ buffering capacity. $K_S$ estimates were 200-300 in cortical GABAergic interneuron’s (Kaiser et al. 2001; Aponte et al. 2008; but see Lee et al. 2000) and $\sim$500-600 in snail neurons (Muller et al. 1993; Belan et al. 1993), crayfish neuromuscular junction (Tank et al. 1995), and mammalian sympathetic neurons (Wanaverbecq et al. 2003). The highest value measured to date was for Purkinje cells (~2000: Fiero and Llano, 1996).

**Basis for $K_S$**. The $K_S$ measurement is thought dominated by fixed buffers, as the value does not decrease substantially, even during long-lasting dialysis of the cell by whole cell recording (Neher and Augustine, 1992; Steunkel, 1994; Helmchen et al. 1996). The buffer also shows little sign of saturation over the range of [Ca$^{2+}$]i tested. In addition, comparison of wash-in and wash-out of two different concentrations of fura-2 in a single cell (two successive patch recordings) provide similar estimates of $K_S$, suggesting no wash out of endogenous buffer (Helmchen et al. 1996; Lee et al. 2000). Popular candidates for fixed buffers include various calcium binding proteins (e.g., calmodulin, calbindin, calretinin, and parvalbumin) that are distributed in a cell-type-specific manner in the nervous system (Baimbridge et al. 1992). Neher and Augustine (1992) favored calmodulin as the endogenous buffer in chromaffin cells. Calbindin has been suggested as an important buffer in CA1 pyramidal cells (Muller et al. 2005) and hippocampal granule cell terminals (Jackson and Redman, 2003).

Although it is not clear which proteins underlie measured $K_S$ in rodent SNc DA neurons, immunocytochemical data suggest that DA cells express traditional CaBPs. Many SNc neurons express calretinin in squirrel monkeys (Fortin and Parent, 1996) and rodents (Jacobowitz and Winsky, 1991; Resibois and Rogers 1992; Gonzalez-Hernandez and Rodriguez, 2000; Nemoto et al. 1999). In rats and humans, 40-50% of DA cells in the dorsal medial part of SNc contain calbindin (especially rostral sections) but not parvalbumin (McRitchey et al. 1996; Alfaheh-Kakunda and Silverman, 1997). Calbindin is absent from ventrally located SNc DA cells (Gerfen et al. 1987; Gonzalez-Hernandez and Rodriguez, 2000; Nemoto et al. 1999). With PD in humans, there is relative sparing...
of DA melanin-negative cells containing calbindin (Yamada et al. 1990). The bulk of our recordings were from cells ventral and medial within the SNc.

Surmeier and colleagues proposed that DA cells are at risk for Ca$^{2+}$-dependent mitochondrial failure and cell death (e.g., in Parkinson’s disease PD and aging) because of the persistent Ca$^{2+}$ load from subthreshold Ca$^{2+}$ entry (Chan et al. 2007; Surmeier, 2007). An intriguing possibility is that mitochondria play an important role in determining $K_S$. Mitochondrial polymorphisms are associated with PD (Kazuno et al. 2006) and loss of mitochondrial function has been proposed as a mechanism for Ca$^{2+}$-dependent cell death in DA cells in PD (Chan et al. 2007; Surmeier, 2007).

**Technical Limitations.** Several assumptions are required to estimate $K_S$. First, these methods assume instantaneous Ca$^{2+}$ entry, which is approximated by the brief, steep rising transients elicited by a single AP (Helmchen et al. 1996). The decay of $[Ca^{2+}]_i$ was well fit by a single exponential. Ca$^{2+}$ entry in response to an AP must also be constant with time. This was confirmed by the lack of relationship between the integral of Ca$^{2+}$ entry vs. time or $A*t$ vs. $K_B$. The concentration of the intrinsic buffer is also assumed constant. We found no evidence for loss of highly mobile buffers lost within the first 10-20 minutes after break-in. In some cells at longer times (>20 min), we observed deviations from a linear relationship between either A or $\tau$ vs. $K_B$. These deviations may reflect slow wash-out of buffers. All of our estimates were therefore confined to data taken during the time before such deviations. A recent study suggests that nearly all of the calbindin in CA1 pyramidal cells is mobile but washes out with a $\tau$ of ~10 minutes (Muller et al. 2005), as compared to a $\tau$ of ~5-6 min for wash in of fura-2 in DA cells. The $K_S$ estimates correspond to steady-state Ca$^{2+}$, a condition that may never fully exist in cells with multiple Ca$^{2+}$ reaction partners differing in affinity or kinetics (Markram et al. 1998).

**Development of $K_S$.** Most of our data were obtained from rats aged P13-P17 to facilitate imaging of DA neurons, however, $K_S$ has been shown to be age-sensitive in other cell types (Maravall et al. 2000; Fiero and Llano, 1996; Murchison and Griffith, 1998; Stocca et al. 2008). To test whether $K_S$ was developmentally labile in DA neurons, we also recorded from cells at P25-P32. We found that buffering capacity increased significantly with age, but remains modest in DA neurons. Chan et al. (2007) reported that in mice, the Ca$^{2+}$-dependence of subthreshold oscillations and autonomous firing was developmentally regulated, with DA cells from P12-P17 mice showing Na$^+$-dependent and P28-P32 animals showing Ca$^{2+}$-dependent pacemaking behavior. Thus, the increase in $K_S$ with age may correspond to a change in the basis for pacemaking behavior.

**Functional Consequences.** Given the autonomous firing and low threshold for Ca$^{2+}$ entry in DA cells from SNc, the modest $K_S$ in these cells would be expected to make these cells vulnerable to Ca$^{2+}$-dependent pathophysiology, such as occurs with aging and in PD (Surmeier, 2007). This low safety factor may be necessitated by the need for sufficient activation of the sK channels important to the oscillations underlying autonomous firing (Wilson and Callaway, 2000; Chan et al. 2007) as well as to prevent Na$^+$ inactivation to allow burst firing (Medvedev et al. 2003). The unknown molecules contributing to $K_S$ would play a major role in determining the dimensions of microdomains for Ca$^{2+}$-dependent activation of sK channels (Muller et al. 2005; Fakler and Muller 2008; Abel et al. 2004). All else being equal, a low $K_S$ for a given Ca$^{2+}$ influx would result in faster Ca$^{2+}$ dynamics (large amplitude, fast decay, local), which would be
advantageous for rhythmic oscillatory activity. This has been previously proposed for the
very low (~40) \( K_s \) in motoneurons (Lips and Keller, 1998; Palecek et al. 1999). This is
also consistent with observations that blockade of sK current with apamin or intracellular
EGTA leads to development of bursting and prolonged \(Ca^{2+}\)-dependent plateau potentials
(Johnson and Wu, 2004; Shepard and Bunney, 1991).
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Shepard PD and Bunney BS. (1991) Repetitive firing properties of putative dopamine-containing neurons in vitro: regulation by an apamin-sensitive Ca\textsuperscript{2+}-activated K\textsuperscript{+} conductance. Exp. Brain Res. 86: 141–150.


Figure Legends.

Figure 1. Fura-2 signals in response to a single action potential (from P17 animal). A. Per cent ΔF/F (380 nm) in response to an AP at 1 min (red) and 24 min after break-in (blue). As fura-2 (200 μM in pipette) diffuses into the cell, the peak %ΔF/F becomes smaller and the time course is prolonged. B. Single AP at 1 min (blue) and 24 min (red) for the same cell as data in A. The AP was 82 mV in amplitude and the half-width was 1.7 ms. Note the large mAHP (19 mV, 220 ms duration). Inset: expanded view of APs at 1 min to show no change in peak amplitude. C. Fluorescent image of the cell at 1 min and 24 min after break-in. The signal (380 nM) becomes much more intense with time. D. Autonomous firing and underlying oscillations in a different DA neuron from a P14 rat. This cell fired spontaneously in a regular pattern. After application of 1 μM TTX, spiking was blocked but the underlying oscillations remained (in this case ~1 pA DC current was injected).

Figure 2. Filling curve for fura-2 in dopamine cell from the substantia nigra pars compacta (SN). Data from a typical cell (P17) are illustrated. The intensity of the fura-2 signal was measured at the isosbestic point (360 nm) at one minute intervals from initial break-in. Data were fit by a single exponential function. In this cell the time constant (τ) was 3.69 ± 0.01 min. The inset is a box plot showing summary data from 27 cells at P13-P17 and 10 cells at P25-P32. In the box plots, the horizontal line within the box represents the median value, the edges of the box are the inner quartiles, and the whiskers represent the outer quartiles.

Figure 3. Determination of Ks in a representative cell (P15). A. With time, the amplitude of the fura-2 transients decreased and the time constants for decay were prolonged. Transients from several times after break-in are indicated by color of the trace and exponential fit. The black trace was at 0.5 min, dark blue at 2.5 min, light blue at 4 min, green at 5.5 min, gold at 8 min and red at 9.5 min. B. Plot of the reciprocal of the amplitude (1/A) vs. KB' (see Methods). An estimate of Ks is obtained as the negative of the X intercept (64.7 in this case; same cell as in A). Solid line is linear best fit to data. Inset: summary data for Ks estimates from 1/A for P3-P17 (P13: n = 20) vs. P25-P32 (P25: n = 10). The difference was significant (p < 0.03). C. Plot of tau vs. KB' for cell in A and B. Ks is estimated from the negative of the X intercept (103.7 in this cell). D. Plot of A*tau vs. KB'. There was no significant relationship, indicating the integral was constant with time.
Table 1. Data for membrane potentials and action potentials. AP and AHP amplitude were measured from “resting” potential to the peak voltage change. AP half-width was measured at $\frac{1}{2}$ of the amplitude (measured from rest).

<table>
<thead>
<tr>
<th></th>
<th>RMP (mV)</th>
<th>AP (mV)</th>
<th>AP Half-width (ms)</th>
<th>AHP Amplitude (mV)</th>
<th>AHP duration (ms)</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13-P17</td>
<td>-63 ± 0.9</td>
<td>70 ± 2</td>
<td>2.1 ± 0.1</td>
<td>10 ± 1</td>
<td>622 ± 99</td>
<td>24</td>
</tr>
<tr>
<td>P25-P32</td>
<td>-67 ± 1.3</td>
<td>73 ± 1.8</td>
<td>1.8 ± 0.1</td>
<td>12 ± 2.1</td>
<td>522 ± 62</td>
<td>15</td>
</tr>
</tbody>
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Figure 1.