Resurgent Na Currents in Four Classes of Neurons of the Cerebellum

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Submitted 17 March 2004; accepted in final form 19 June 2004

INTRODUCTION

The upstroke of action potentials in most neurons is mediated primarily by current flow through tetrodotoxin-(TTX-) sensitive voltage-gated Na channels. Upon depolarization, these channels open and inactivate; upon repolarization, channels recover from inactivation, generally without reopening (Bezanilla and Armstrong 1977; Hodgkin and Huxley 1952; Kuo and Bean 1994). The Na channels of cerebellar Purkinje neurons, however, tend to recover from inactivation through open states, giving rise to a “resurgent” current that flows upon repolarization from positive potentials (Raman and Bean 1997). These resurgent kinetics appear to result from channels with at least two mechanisms of rapid inactivation: the conventional or “fast” inactivation gate, corresponding to the cytoplasmic linker between domains III and IV of the Na channel α subunit (Stühmer et al. 1989; Vassilev et al. 1989), as well as an endogenous, voltage-dependent, open-channel blocker (Raman and Bean 2001). Upon repolarization, the open-channel blocker binds more rapidly than the inactivation gate, limiting the extent of fast inactivation, particularly at positive potentials. Upon repolarization, the blocker is expelled, allowing resurgent current to flow and restoring the availability of Na channels. Because the recovery from inactivation associated with resurgent current shortens the refractory period between action potentials, high-frequency firing appears to be facilitated by Na channels with resurgent kinetics (Khaliq et al. 2003; Raman and Bean 2001).

Resurgent Na current was first characterized in cerebellar Purkinje neurons (Raman and Bean 1997), but recently it has also been described in neurons of the subthalamic nuclei (Do and Bean 2003). Several additional observations are also suggestive of the expression of resurgent currents by other classes of neurons. First, in a preliminary report (Mossadeghi and Slater 1998), resurgent current was documented in unipolar brush cells, which are neurons of the vestibulocerebellum that form a system of mossy fibers within the cerebellar cortex (Mugnaini and Floris 1994; Mugnaini et al. 1997). Second, in a study of spontaneous firing in cerebellar nuclear neurons, a subset of cells showed a small resurgent component to their Na currents (Raman et al. 2000). Third, in a modeling study, simulations of action potential firing predicted that cerebellar granule cells might express resurgent current (D’Angelo et al. 2002). These preliminary observations raise the possibility that resurgent Na currents may be more widespread than previously thought.

In this study, we compared the TTX-sensitive, voltage-gated Na currents from three classes of cerebellar neurons—cerebellar nuclear cells, unipolar brush cells, and granule cells—to those of Purkinje neurons, with a focus on testing for the presence of resurgent Na current in each cell type. The results indicate that the non–Purkinje cerebellar neurons indeed express Na currents with resurgent kinetics, although the resurgent component can be quite small in the low concentrations of external Na and high concentrations of divalent calcium-channel blocking ions that are often used for biophysical studies. When recordings are repeated in more physiological ionic species and concentrations, however, the resurgent component of sodium current is increased to a greater extent than the nonresurgent component, such that all four cerebellar cell types produce robust resurgent currents. These results suggest that
the channels with resurgent kinetics may regulate firing rates in several types of neurons.

**METHODS**

**Cell preparation**

Purkinje, granule, and cerebellar nuclear neurons were acutely dissociated from 13- to 17-day-old C57BL/6 mice, and unipolar brush cells were acutely isolated from 17- to 18-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). In accordance with institutional guidelines, animals were anesthetized with halothane (mice) or isoflurane (rats) and decapitated. All procedures were consistent with approved protocols.

Purkinje and granule neurons were isolated as previously described (Raman et al. 1997; Regan 1991). The superficial layers of the cerebellum were removed and minced in ice-cold, oxygenated dissociation solution containing (in mM) 82 Na₂SO₄, 30 K₂SO₄, 5 MgCl₂, 10 HEPES, 10 glucose, and 0.001% phenol red (buffered to a pH of 7.4 with NaOH). The tissue was incubated for 7 min at 31°C in 10 ml of dissociation solution containing 3 mg/ml of protease XXIII (pH readjusted) with oxygen blown over the surface of the fluid. After enzymatic treatment, the tissue was washed in dissociation solution containing 1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin (pH readjusted), in which the meninges were removed. The tissue pieces were then triturated with a series of fire-polished Pasteur pipettes to release Purkinje and granule neurons.

Cerebellar nuclei and unipolar brush cells were dissociated with slight modifications to previous methods (Raman and Trussell 1992; Raman et al. 2000). Cerebella were removed in oxygenated Tyrode’s solution containing (in mM) 150 NaCl, 4 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, and 10 glucose, pH adjusted to 7.4 with NaOH (final Na concentration of 155 mM). Parasagittal cerebellar slices (350–400 µm thick) were cut either on a tissue chopper (for cerebellar nuclear cells; McIlwain, UK) or on a Vibratome Series 1000 (for unipolar brush cells, in Tyrode’s solution at 33°C; Vibratome, St. Louis, MO). Slices were incubated for 20 min at 31°C; Vibratome, St. Louis, MO).

**Electrophysiological recordings:**

Neurons were visualized with infra-red differential interference contrast microscopy on a Zeiss FS1 microscope. Slices were bathed in oxygenated ACSF. Borosilicate pipettes (1–4 MΩ) contained (in mM) 108 KCH₃O₃S, 9 NaCl, 1.8 MgCl₂, 9 EGTA, 155 NaCl, 5 TEACl, 10 HEPES, and 0.5 EDTA, pH adjusted to 7.2 with KOH. Purkinje cells were voltage-clamped nominally at −76 mV in high Na (unipolar brush cell experiments). Cells were also allowed to settle in the recording chambers in Tyrode’s solution for ≥1 h before recordings. Recordings were made at room temperature within 6 h after trituration.

For slice experiments, slices were prepared using standard techniques (Telgkamp and Raman 2002). Briefly, mice (P13–P16) were anesthetized with halothane, perfused with ice-cold artificial cerebrospinal fluid (ACSF, 4°C, in mM: 123.75 NaCl, 3.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, and 10 glucose), and rapidly decapitated. The cerebellum was bathed in ice-cold ACSF bubbled with 95% O₂-5% CO₂, and parasagittal cerebellar slices (300 µM) were cut on a Leica VT1000 vibratome. Slices were allowed to recover in oxygenated ACSF at 34°C for 1 h before recording.

**Electrophysiological recordings: isolated cells**

Borosilicate pipettes (1–3 MΩ for Purkinje and cerebellar nuclear cells; 3–4 MΩ for unipolar brush cells, 7–10 MΩ for granule cells) were wrapped with parafilm or coated with Sylgard ( Dow Corning, Midland, MI) and fire polished. For Purkinje, granule, and cerebellar nuclear cells, the pipette solution consisted of (in mM) 108 CsCH₃SO₃, 9 NaCl, 1.8 MgCl₂, 9 HEPES, 0.9 EGTA, 47.7 sucrose, 14 Tris-CreatinePO₄, 4 MgATP, and 0.3 TrisGTP (buffered to a pH of 7.40 with CsOH). For unipolar brush cells, 117 CsCl replaced the CsCH₃SO₃, and NaCl, and 4 Na,ATP replaced the MgATP. Voltage-clamp recordings were made with an Axopatch 200A or 200B (Axon Instruments, Union City, CA). The bath was grounded with a 3 M KCl agar bridge to minimize the junction potential between the pipette and bath solution (see following text in METHODS). Data were recorded with an IntrinsTech ITCl-18 interface (Great Neck, NY) and Pulse software (Heka Electronic, Lambrecht, Germany) or with the Digidata/pCLAMP system (Axon Instruments). After a whole cell recording was established, each cell was positioned in front of a series of gravity-driven flow pipes containing different extracellular solutions. Whole cell series resistances of 5–7 MΩ were routinely compensated by >70%.

Several control extracellular solutions were used for recordings, and each is referred to by Na concentration and primary divalent cation. The “high Na/Ca” solution consisted of Tyrode’s solution with 15 mM TACl and 30 µM CdCl₂, except in Fig. 8. The “high Na/Ca” solution of Fig. 8 and the “high Na/CaO” solution consisted of (in mM) 155 NaCl, 5 TEACl, 10 HEPES, and either 2 CaCl₂, 0.3 CdCl₂ or 2 CoCl₂, buffered to pH 7.4 with TrisOH for a final Na concentration of 155 mM. The “low Na/CaO,” “low Na/Ba,” and “low Na/CaAl” solutions consisted of (in mM) 50 NaCl, 110 TEACl, 10 HEPES, and 2 CoCl₂, 2 BaCl₂, 0.3 CdCl₂, or 2 CaCl₂, 0.3 CdCl₂, buffered to pH 7.4 with TrisOH for a final Na concentration of 50 mM. After recordings were made in any control solution, protocols were repeated in a solution identical to the control but which included 300 mM or 1 µM TTX. TTX-sensitive Na currents in either low Na or high Na were obtained by subtraction. All drugs were from Sigma Aldrich, except for TTX (Alomone).

**Analysis**

Data were analyzed with IGOR 4.02 software (WaveMetrics, Lake Oswego, OR). In isolated cells, for calculation of Na conductance, the reversal potential of the Na current was extrapolated from the peak current-voltage relation for each cell, and conductance was calculated by dividing peak current amplitude by driving force at each potential. Conductance-voltage data were fit with a Boltzmann equation of the form \( G(V) = 1 / [1 + \exp(-V - V_{1/2}/k)] \), where \( G \) is conductance, \( V \) is voltage, \( V_{1/2} \) is the voltage for half-maximal activation, and \( k \) is the slope factor. With the solutions used, and at 25°C, \( E_{Na} \) was predicted to be +44 mV in low Na and +73 mV in high Na (Purkinje, granule, and cerebellar nuclear cell experiments) or +47 mV in low Na and +76 mV in high Na (unipolar brush cell experiments). Because the reversal potentials extrapolated from the transient current-voltage relations were near the predicted \( E_{Na} \) values, the predicted values were used for the analyses of resurgent conductance.

Steady-state availability of Na channels was plotted as peak current evoked at 0 mV following a conditioning step versus the conditioning voltage. Data were fit with a modified Boltzmann equation of the form
\[ III_{\text{max}} = I/(1 + \exp((V - V_{1/2})/k)) \]

where \( III_{\text{max}} \) is relative current, 
\( V_{1/2} \) is the half-maximal voltage for inactivation, and \( k \) is the slope factor.

To estimate the predicted change in current amplitudes in the low Na and high Na solutions at different potentials, predicted currents (as a function of permeability) were calculated with the Goldman-Hodgkin-Katz (GHK) current equation:

\[ I_{\text{Na}} = P_{\text{Na}} z^2 (E/RT) ([\text{Na}]_o - [\text{Na}]_i) \exp(-E/RT)/(1 - \exp(-E/RT)), \]

where \( I_{\text{Na}} \) is Na current, \( P_{\text{Na}} \) is Na permeability, \( z \) is valence, \( E \) is voltage, \( F \) is Faraday’s constant, \( R \) is the gas constant, \( T \) is temperature, \([\text{Na}]_o\) is the extracellular concentration of Na\(^{+} \), and \([\text{Na}]_i\) is the intracellular concentration of Na\(^{+} \). The ratio of currents in high Na relative to those in low Na was calculated for the relevant potentials, assuming \( P_{\text{Na}} \) was constant.

Chord conductances were also calculated by dividing the predicted current by driving force, and the ratio of conductance at \(-30 \text{ mV} \) to conductance at \(+30 \text{ mV} \) was calculated for high Na and low Na (junction potentials accounted for; see following text in METHODS). The GHK-predicted conductance ratio in high Na was 119% of that in low Na, and this was used as a reference for comparison of the resurgent-to-transient conductance ratio in the different solutions in Fig. 4.

Illustrated voltage protocols and the plots of current or conductance versus voltage have not been corrected for the liquid junction potential of \( 3 \text{ mV} \) for \( \text{C}_2\text{H}_2\text{O}_4\text{S} \)-based intracellular solutions, but the junction potential was taken into account in calculations and reports of the parameters of activation and inactivation. For \( \text{CsCl} \)-based solutions, the junction potential was \(-1 \text{ mV} \) and was not corrected. For both pipette solutions, recordings in low Na were corrected for the additional \( 2.5 \text{ mV} \) junction potential that developed between the high Na bath and low Na flow pipe solution. Capacitance has been digitally reduced in most figures. Data are reported as mean \( \pm \) SE. Statistical significance (\( P < 0.05 \)) was assessed using Student’s two-tailed, paired \( t \)-test in all cases where paired data were obtained. In other cases, Student’s two-tailed, two-sample, unequal variance \( t \)-test were used, and these \( P \) values are marked “unpaired.”

### Results

#### Identification of cells

The four classes of cerebellar neurons studied in this series of experiments were identified by the region of the cerebellum from which they originated (see METHODS), as well as by their size and morphology. Purkinje neurons were large (>20 \( \mu \text{m} \) diam), with a pear-shaped soma and an apical dendritic stump, and were therefore easily distinguishable from the smaller (5–10 \( \mu \text{m} \) diam), round granule cells released by the same dissociation procedure (Fig. 1A) (Regan 1991). Unipolar brush cells were also small (5–10 \( \mu \text{m} \) diam) but had a single, thick dendritic stem that terminated in a brush-like formation of branchlets (Fig. 1B) (Mugnaini and Floris 1994; Rossi et al. 1995). “Large” cerebellar nuclear cells had somata that were 10–20 \( \mu \text{m} \) in diameter and generally had two to four dendrites (Fig. 1C) (Raman et al. 2000).

#### Na currents measured in low external Na\(^{+} \)

To compare the properties of Na currents in the various cerebellar neurons, we recorded TTX-sensitive currents evoked by depolarization (“transient currents”) as well as by repolarization (“resurgent currents”). To decrease the amplitude of Na currents and to improve the quality of voltage clamp, these recordings were made in solutions in which the external Na\(^{+} \) concentration had been reduced from physiological levels to 50 mM. Also, to reduce currents through Ca and K channels, 2 mM Ca\(^{2+} \) was replaced either by 2 mM Co\(^{2+} \) or by 2 mM Ba\(^{2+} \) with 0.3 mM Cd\(^{2+} \). Mg\(^{2+} \) was omitted from the solution, and osmolarity was compensated with TEACl. Although transient currents in Purkinje cells were on average two to three times larger than those of cerebellar nuclear and unipolar brush cells, the transient currents had a similar voltage-dependence of activation in all three cell types, consistent with previously reported values (Raman et al. 1997, 2000) (Fig. 2, A and B). In granule cells, transient currents were too small to be reliably recorded in low Na, and recordings in higher Na were discussed later in the RESULTS.

When Purkinje cells were repolarized after a brief step to a positive potential, resurgent Na currents were consistently evoked (Raman and Bean 1997). Figure 2C (top) shows the resurgent current elicited by a repolarizing step to \(-30 \text{ mV} \). A similar voltage protocol applied to cerebellar nuclear and unipolar brush cells also elicited currents that appeared to have a small resurgent component (Fig. 2C, middle and bottom; \( n = 7/7 \) cerebellar nuclear cells, \( n = 8/8 \) unipolar brush cells). The resurgent currents were occasionally obscured by the noise, however, particularly in cells with small transient current amplitudes (quantified below in the RESULTS).

#### Na currents measured in quasi-physiological ionic conditions

To improve our resolution of resurgent currents, as well as to allow us to compare resurgent currents across cell types, we repeated the recordings in a quasi-physiological, modified Tyrode’s solution (“high Na/Ca\(^{+} \)”): This solution included physiological concentrations of Na\(^{+} \) (155 mM), K\(^{+} \) (4 mM), Ca\(^{2+} \) (2 mM), and Mg\(^{2+} \) (2 mM), but included 30 \( \mu \text{M} \) Cd\(^{2+} \) and 5 mM TEA\(^{-} \) to reduce Ca and K currents.

As expected, transient currents were larger in the high Na/Ca solution (quantified below). More strikingly, resurgent current
increased greatly in all three cell types (Fig. 3, A and B), such that all cerebellar nuclear neurons (n = 9) and all unipolar brush cells (n = 5), like Purkinje cells, unequivocally produced robust resurgent currents. An enhancement of all components of Na current at potentials negative to the reversal is expected because of the increased extracellular Na concentration and the increased driving force. Based on the Na concentration change alone, the GHK current equation predicts that, in the solutions used, the ratio of the current in 155 mM Na to that in 50 mM Na (I_{155 Na}/I_{50 Na}) will be 4.6 at nominally +30 mV, dropping to 3.1 at −30 mV, as the asymptotic value (155/50) is approached at negative voltages that are distant from the reversal potential (junction potentials considered; see METHODS). Measuring transient currents at −30 mV gave an I_{155 Na}/I_{50 Na} of 4.7 ± 0.5 for Purkinje cells (n = 15 paired observations) and 5.2 ± 0.94 for cerebellar nuclear cells (n = 7 paired observations). The observed ratios were quite close to the GHK prediction (P = 0.8 for Purkinje and P = 0.6 for cerebellar nuclear cells). As shown in Fig. 4A, deviations from the predicted ratio (dotted line) were most noticeable in Purkinje cells with the largest transient currents (I_{155 Na}/I_{50 Na}), which were most likely to be subject to some clamp error in high Na. In the same set of cells, however, the amplitude ratio of resurgent currents at −30 mV increased rather than decreased. I_{155 Na}/I_{50 Na} became 6.9 ± 0.7 for Purkinje cells and 6.4 ± 0.9 for cerebellar nuclear cells, considerably greater than the predicted value of 3.1 (Fig. 4B; P < 0.05 for both cell types). The population of unipolar brush cells showed the same
trend; the mean $I_{\text{Na}}/I_{\text{Na}}$ for transient current at $+30 \text{ mV}$ was 3.2 and for resurgent current at $-30 \text{ mV}$ was 5.8 ($n = 8$ in $50 \text{ Na}/\text{Ba}$ and $n = 5$ in $155 \text{ Na}/\text{Ca}$). To quantify the extent to which the resurgent component was preferentially increased, we calculated the ratio of resurgent conductance at $-30 \text{ mV}$ to the transient conductance at $+30 \text{ mV}$.

### TABLE 1. Activation and inactivation parameters for PKJs, CBNs, and UBCs

<table>
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<tr>
<th></th>
<th>PKJ Low Na/Co</th>
<th>PKJ High Na/Co</th>
<th>CBN Low Na/Co</th>
<th>CBN High Na/Co</th>
<th>UBC Low Na/Ba</th>
<th>UBC High Na/Co</th>
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<tr>
<td>$V_{1/2}$ (mV)</td>
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<td>$-37.2 \pm 2.1$</td>
<td>$-38.0 \pm 1.0$</td>
<td>$-38.0 \pm 1.0$</td>
<td>$-53.0 \pm 0.2$</td>
<td>$-16.2 \pm 1.3$</td>
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<td>$k$ (mV)</td>
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<td>$6.4 \pm 0.5$</td>
<td>$5.3 \pm 0.2$</td>
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<tr>
<td>$G_{\text{max}}$ (nS)</td>
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<td>$29 \pm 7$</td>
<td>$16 \pm 10$</td>
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<tr>
<td>$V_{1/2}$ (mV)</td>
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<td>$-52.5 \pm 1.2$</td>
<td>$-51.9 \pm 1.0$</td>
<td>$-51.9 \pm 1.0$</td>
<td>$-58.7 \pm 0.5$</td>
<td>$-46.2 \pm 0.8$</td>
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<tr>
<td>$k$ (mV)</td>
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<tr>
<td>$G_{\text{res}}/G_{\text{trans}}$ ratio</td>
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<td>$0.017 \pm 0.002$</td>
<td>$0.039 \pm 0.005$</td>
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<td>$P$ value $k$</td>
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Values are means ± SE. PKJ, Purkinje neuron; CBN, cerebellar nuclear cells; UBC, unipolar brush cell. Reported $P$ values are from paired $t$-tests unless indicated.

FIG. 3. Enhancement of the resurgent component of Na current in quasi-physiological solutions. A: voltage protocol, top. Currents evoked by step repolarizations in 1 Purkinje cell (left), 1 cerebellar nuclear cell (middle), and 2 unipolar brush cells (right) in 2 solutions, as labeled. Note that scale for bottom traces is one-half that for top traces. B: current-voltage relation for peak resurgent currents. $n = 10$ Purkinje cells, (paired observations), 8 cerebellar nuclear cells (paired observations), and 8 and 5 unipolar brush cells in low Na/Ba and high Na/Ca, respectively.
+30 mV in the two different extracellular solutions. Based solely on the change in Na\(^+\) concentration, the GHK equation predicts that this conductance ratio in high Na\(^+\) should be 119% of that in low Na\(^+\) (METHODS). As shown in Fig. 4C, however, paired observations indicated that the resurgent component increased to a greater extent than the transient component in high Na/Ca. The mean resurgent-to-transient conductance ratio in high Na/Ca was 270 ± 20% of that in low Na/Co in Purkinje cells (n = 15 pairs), 264 ± 60% in cerebellar nuclear cells (n = 7 pairs), and 234% for unipolar brush cells (n = 8 in low Na/Ba and n = 5 in high Na/Ca; see also Table 1). Figure 4D summarizes the resurgent to transient conductance ratios in the different solutions, showing the disproportionate enhancement of resurgent conductance in high Na/Ca relative to low Na/Co or low Na/Ba for the different cell types.

Before considering factors that might be responsible for the high sensitivity of resurgent current to external ionic conditions, we recorded Na currents in granule cells, with a goal of testing the prediction of D’Angelo et al. (2002) that the Na currents of cerebellar granule cells might have a resurgent component. Since the total Na currents are small in these tiny cells, we made recordings only in high Na/Ca. Even under these recording conditions, Na currents, particularly those evoked by repolarizing steps, were often difficult to resolve. In each cell, therefore, we applied a single voltage protocol repeatedly to evoke transient currents at 0 and –30 mV and resurgent currents at –30 mV, and averaged the resulting traces (Fig. 5A). All cells that were tested showed robust resurgent currents (n = 10; Fig. 5, A and B), giving a mean ratio of resurgent to transient conductance of 6.8 ± 0.7%, similar to that in Purkinje neurons (Fig. 4B).

Next, we considered factors that might lead to the apparently preferential increase in resurgent relative to transient current in physiological solutions. Previous studies suggest that, upon repolarization to moderately negative potentials, voltage-dependent open-channel block is relieved, allowing the channels to carry resurgent current. The current flows until binding of

**FIG. 4.** Preferential increase in resurgent current by quasi-physiological solutions. A and B: plots of Na current in high Na/Ca vs. low Na/Co for transient current at +30 mV (A) and resurgent current at –30 mV (B). Dotted lines are predictions of the Goldman-Hodgkin-Katz (GHK) equation based solely on increases in Na concentration. C: ratio of resurgent to transient conductance in high Na/Ca vs. ratio in low Na/Co. Dotted line is the prediction of the GHK equation for the change in conductance ratio in the 2 solutions. Plots in A–C include all cells for which paired observations were made. D: summary plot of the ratio of resurgent to transient conductance in different solutions, as labeled, for Purkinje, cerebellar nuclear, unipolar brush, and granule (GRN) cells. PKJ, n = 15 paired (same data as in A); CBN, n = 7 paired (same data as in A); UBC, n = 8, n = 5 unpaired; GRN, n = 10 (high Na/Ca only). *P < 0.05. Values and statistics are given in Table 1.
the conventional fast inactivation gate causes the channels to stop conducting (Grieco et al. 2002; Raman and Bean 2001). It seemed possible, therefore, that manipulations that increase resurgent current might also increase channel availability at moderately negative potentials.

We therefore tested the effects of changing extracellular solutions on steady-state inactivation of Na currents by comparing the steady-state availability curves measured in low Na/Co and high Na/Ca. Channels were conditioned with 200-ms steps to potentials between −90 and 0 mV, a protocol that is likely to favor binding of the fast inactivation gate rather than block by the endogenous blocker (Raman and Bean 2001). After each conditioning step, the availability of channels was assessed with a test step to 0 mV. In Purkinje cells, switching from low Na/Co to high Na/Ca significantly shifted the steady-state inactivation curves by an average of +9.5 mV and also increased their steepness (Fig. 6, A–C; Table 1). Not surprisingly, transient currents evoked during the 200-ms conditioning pulses sometimes showed signs of voltage escape in high Na/Ca, particularly at moderately negative potentials. Inadequate voltage clamp at the beginning of the conditioning step in high Na, however, is likely to lead to underestimation of the depolarizing shift in the inactivation curve, since any loss of voltage control will tend to promote rather than to relieve inactivation.

Measurement of the steady-state inactivation curves of cerebellar nuclear and unipolar brush cells revealed a change in Na channel availability that was even more pronounced than in Purkinje cells (Fig. 7, A and B). The $V_{1/2}$ of inactivation was shifted positive by about 14.5 mV in cerebellar nuclear cells and by about 12.5 mV in unipolar brush cells; in the nuclear cells, the inactivation curve also became steeper (Fig. 7C; Table 1). Thus quasi-physiological solutions not only increased resurgent current amplitudes but also increased the availability of transient current at moderately negative potentials.

This preferential increase in resurgent current amplitude as well as the shift in the availability curve may result from at least two factors: the increase in Na concentration and/or the differences in divalent cations in the two solutions. Regarding the former, in other preparations, as the external Na concentration is raised, recovery from fast inactivation is accelerated (Kuo and Liao 2000) and entry into slow inactivated states is reduced (Townsend and Horn 1997). Additionally, in preparations in which the fast inactivation gate of Na channels has been removed or mutated, high concentrations of external Na can relieve channel block by exogenous intracellular blockers, such as strychnine, tetra-alkylammonium ions, and peptides that mimic the inactivation gate, by expulsion, or "knock-off," of the blocker bound in the pore (O’Leary et al. 1994; Shapiro 1977; Tang et al. 1996).

Regarding the effects of divalent cations, changes in divalent ion species and concentrations can change surface charge, resulting in shifts of measured voltage-dependence. While these shifts are likely to influence the measurements of steady-state inactivation, they are not large enough to account for the experimental results; for Ba$^{2+}$, the shift is also of the wrong sign (see DISCUSSION). In the context of the present results, a more significant effect of divalent species may result from a direct blocking action on the Na currents themselves. Specifically, substitution of 2 mM Co$^{2+}$ for 2 mM Ca$^{2+}$ can reduce peak Na currents in isolated Purkinje cells by 40–50% (Swensen and Bean 2003), and this Co$^{2+}$ block is most effective at negative potentials (AM Swensen and BP Bean, unpublished observations). A relief of block by external diva-

![Fig. 5](http://jn.physiology.org/)

**FIG. 5.** Transient and resurgent Na currents in granule cells. A: examples of currents evoked by the voltage protocol shown in 3 different granule cells. Conductance ratios of the resurgent component at −30 mV to the transient component at +30 mV were 4.7 (top), 5.0 (middle), and 9.7% (bottom). B: plot of resurgent current at −30 mV vs. transient current at +30 mV, showing that resurgent current was present in 10 of 10 cells tested. Dotted line is a linear fit to the data.
lent cations might therefore contribute both to the larger resurgent currents and to the positively shifted availability curves measured in Purkinje and cerebellar nuclear cells in more physiological solutions.

To distinguish the effects of changing divalent ions from the effects of changing Na concentration, we repeated the experiments in Purkinje cells with a new series of solutions. Recordings were made first in low Na/Co and then were repeated in a high Na/Co solution that contained 155 mM Na⁺ with 2 mM Co²⁺ (see METHODS), so that the two solutions differed only in the Na⁺ and TEA⁺ concentration. Even with divalents held constant, the $V_{1/2}$ of the inactivation curve shifted by +6 mV when the concentration of Na⁺ was increased (from $-63.5 \pm 1.3$ to $-57.5 \pm 1.3$ mV, $n = 10$ paired observations, $P < 0.001$), whereas the slope factor did not change (from $k = 6.7 \pm 0.1$ to $6.5 \pm 0.1$, $P = 0.11$; data not shown). Additionally, the ratio of resurgent conductance at $-30$ mV to transient conductance at $+30$ mV measured in high Na/Co was $180 \pm 20\%$ of that in low Na/Co, considerably larger than the GHK prediction of $119\%$ ($P < 0.05$). These results are suggestive of a significant effect on resurgent current and channel availability that is independent of divalent species in the extracellular solution.

Five of the 10 Purkinje cells were also exposed to the high Na/Ca solution used for previous experiments. Consistent with a relief of voltage-dependent block by external Co²⁺ ions (Swensen and Bean 2003), the resurgent current amplitude at $-30$ mV in high Na/Ca was $2.1 \pm 0.2$-fold larger than that in high Na/Co, whereas the transient current at $+30$ mV increased only to $1.13 \pm 0.16$ times the amplitude in high Na/Co. Moreover, the $V_{1/2}$ of inactivation shifted further positive by $2.5 \pm 0.6$ mV ($P < 0.01$) and the slope became steeper (from $k = 6.4 \pm 0.2$ to $5.9 \pm 0.2$ mV, $P < 0.0001$). Thus the changes in resurgent current and availability curves originally seen with switches from low Na/Co to high Na/Co may be in part attributed to a block of Na current by Co²⁺.

Nevertheless, as mentioned above, even with divalents held constant, the increase in resurgent-to-transient conductance ratio with increasing Na⁺ concentration deviated significantly from the GHK prediction. To test the effect of increasing external Na⁺ with Ca²⁺ as the primary divalent, we repeated the experiments in cerebellar nuclear cells, with recordings made in high Na/Ca and low Na/Ca. Both solutions contained $0.3$ mM Cd²⁺ to reduce Ca currents; this concentration of Cd²⁺ has only a minor effect on Na current amplitude (Swensen and Bean 2003). Also, to minimize entry into slow inactivated states, the steady-state inactivation curve was assessed with 100-ms, rather than 200-ms, conditioning steps. The results obtained for cerebellar nuclear cells were consistent with the observations in Purkinje cells. Specifically, increasing the external Na concentration shifted the $V_{1/2}$ of inactivation by about $+8$ mV (Fig. 8A, $P < 0.001$, from $-58.6 \pm 1.8$ mV in low Na/Ca to $-50.7 \pm 1.6$ mV in high Na/Ca) with no significant effect on $k$ ($P = 0.11$, from $6.5 \pm 2$ mV in low Na/Ca to $5.9 \pm 2$ mV in high Na/Ca, $n = 4$). Additionally, as shown in Fig. 8B, with the shift to high Na solutions, the transient current amplitude at $+30$ mV increased $5.7 \pm 0.9$-fold, not significantly different from the GHK prediction of $4.6$-fold ($P = 0.3, n = 4$). In contrast, the resurgent current amplitude at $-30$ mV in high Na/Ca was $7.4 \pm 3.0$ times that in low Na/Ca, much larger than the GHK prediction of a $3.1$-fold increase (Fig. 8, B and C, $P < 0.01, n = 3$). Converting these current amplitudes to conductances gave a resurgent-to-transient conductance ratio in high Na/Ca to low Na/Ca of $241 \pm 47\%$ ($n = 3$). When the fourth cell was exposed to high Na/Ca, the steady-state current at positive potentials also increased, resulting in a tail current at $-30$ mV that obscured the rising phase of any resurgent current. Consistent with the other cells, however, the amplitude of the current elicited upon repolarization measured after 4 ms (the mean time of the resurgent current peak; Grieco et al. 2002) was 8.3 times the

![Diagram](https://example.com/diagram.png)
resurgent peak in low Na/Ca. The putative resurgent-to-transient conductance ratio in this cell was therefore 289%, similar to the other cells.

The disproportionate increase in resurgent current, along with the significant positive shift in the inactivation curve with high external Na⁺, is suggestive of a direct influence of external Na⁺ ions on the Na current. Since resurgent current apparently flows when an endogenous open channel blocker, which binds on depolarization, is expelled from the pore on repolarization, the preferential enhancement of resurgent current in physiological concentrations of Na⁺ is consistent with knock-off models of open-channel blockers by permeating ions (see DISCUSSION).

The result that many cerebellar cells have large resurgent Na currents in physiological ionic conditions is suggestive of a role for this component of current in cerebellar function. Observations made in isolated cells, however, cannot necessarily be directly extrapolated to the intact brain. A common concern raised about recordings from isolated cells is that channel properties might change during cell preparation, particularly as a consequence of enzymatic treatment. Although little evidence exists for the idea that Na current kinetics can change during enzymatic dissociation, certain channel types do appear especially susceptible to enzymatic cleavage, thereby reducing current densities (Akaike et al. 1988; Budde et al. 1994; Raman and Trussell 1992). In the worst case, the resurgent currents that we have measured may be a nonphysiological phenomenon induced by the process of cell isolation.

Several observations make this possibility unlikely. First, across isolated neurons, the presence or absence of resurgent current varies with cell class, not with the type of enzyme used for dissociation and not with duration of enzyme exposure (Do FIG. 7. Positive shift of steady-state inactivation curve in cerebellar nuclear and unipolar brush cells by quasi-physiological solutions. A and B: plots as in Fig. 6, but showing data for a cerebellar nuclear cell (left) and a unipolar brush cell (right). Note y-scale differences on the top and bottom traces. Junction-potential-corrected parameters of the fits for \( V_{1/2} \) and \( k \) for the cerebellar nuclear cell are \(-64.5 \) and \( 6.9 \) mV (low Na/Co) and \(-50 \) and \( 6.2 \) mV (high Na/Co), respectively, and for the unipolar brush cell, \(-59.5 \) and \( 5.0 \) mV (low Na/Ba) and \(-48 \) and \( 4.2 \) mV (high Na/Ba), respectively. C: summary data of \( V_{1/2} \) (left) and \( k \) (right) for 9 paired cells (CBN) and 9 unpaired cells (UBC). For clarity, only Na⁺ concentration is indicated. *P < 0.05. Values and statistics are given in Table 1.
Second, within isolated Purkinje cells, the presence or absence of resurgent current varies with expression of specific Na channel genes (Grieco and Raman 2004; Raman et al. 1997). Third, in any given membrane patch from an isolated Purkinje cell, the presence or absence of resurgent current varies with phosphorylation state of an intracellular, membrane-associated factor (Grieco and Raman 2004; Grieco et al. 2002).

Nevertheless, the concern remains. To address this issue directly, we made whole cell recordings from neurons in slices. Although the voltage control of voltage-gated currents is undoubtedly compromised in the slice preparation, we reasoned that resurgent Na current, if it existed, would be evident on repolarization, and that the modest amplitude and slow kinetics of this component of current would make it reasonably amenable to voltage clamp. Moreover, any resurgent-like current should be blocked by TTX. To minimize K and Ca currents, as well as synaptic currents, 5 mM TEA and 0.3 mM Cd were included in the bath. A K-based pipette solution was used to keep $E_K$ near $-90 \text{ mV}$ to avoid inward K tail currents in the range of potentials where resurgent current would be most likely to flow. The pipette solution included 5 mM TEA to decrease K current amplitudes.

Despite these procedures, the attempt to voltage-clamp Na currents in the slice was unsuccessful in many cells, particularly at temperatures above $30^\circ \text{C}$; notably, the voltage protocol (Fig. 9, top) often elicited escaping action potentials on the repolarization phase, superimposed on large inward currents.

**FIG. 8.** Changes in steady-state inactivation and resurgent current produced by increasing external Na concentration, with divalent cations held constant. A: steady-state availability curve from a representative cerebellar nuclear cell. Junction-potential-corrected parameters of the fits for $V_{1/2}$ and $k$ for the cerebellar nuclear cell are –57.3 and 6.5 mV (low Na/Ca) and –48.2 and 5.3 mV (high Na/Ca), respectively. B: transient and resurgent currents elicited by the voltage protocol shown in the same cell as in A. Inset: same traces at higher gain. The transient current at $+30 \text{ mV}$ increases 4.5-fold, and the resurgent current at $-30 \text{ mV}$ increases 8-fold. C: current-voltage relation for peak resurgent currents for the cell in A and B. Group data are given in the text.

and Bean 2003; Pan and Beam 1999; Raman and Bean 1997; the present data). Second, within isolated Purkinje cells, the presence or absence of resurgent current varies with expression of specific Na channel genes (Grieco and Raman 2004; Raman et al. 1997). Third, in any given membrane patch from an isolated Purkinje cell, the presence or absence of resurgent current varies with phosphorylation state of an intracellular, membrane-associated factor (Grieco and Raman 2004; Grieco et al. 2002).

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**FIG. 9.** Resurgent Na current in neurons in cerebellar slices. Top: voltage protocol. Top traces: superimposed traces of currents evoked from a Purkinje cell at 10-s intervals as TTX was washing into the bath solution. Bottom traces: same traces as above, but with the final trace of complete TTX blockade subtracted from each record. The repolarization-evoked current decreases in parallel with the transient current and retains kinetic features of resurgent Na current measured in isolated cells.
with resurgent kinetics is the facilitation of high-frequency firing, however, this behavior was rather suggestive than not of the presence of resurgent current. At temperatures below 30°C, we were able to obtain recordings in seven Purkinje neurons and two granule cells in which the clamp appeared adequate for our measurements.

Figure 9 (top traces) shows superimposed currents evoked repeatedly in a Purkinje cell at 27°C, as TTX was washing into the bathing solution. In addition to reducing and ultimately blocking the depolarization-evoked (transient) current, TTX blocked a significant component of current that was elicited upon repolarization in control conditions. A similar block of repolarization-evoked current was recorded in 7/7 Purkinje cells and 2/2 granule cells. Further evidence that the repolarization-evoked current was indeed the TTX-sensitive resurgent current of isolated cells, rather than clamp error, came from subtracting the trace obtained with maximal TTX block from each trace in the series obtained while TTX was washing into the bath (Fig. 9, bottom traces). Although the decay phases of the currents clearly deviate from the smooth exponentials obtained under ideal voltage-clamp conditions, the subtractions reveal currents that are reminiscent of the kinetics seen in isolated cells, including the slowly rising, slowly decaying current elicited on repolarization. Also, TTX block of all components of current was reversible. These data suggest that resurgent Na current is not an artifact of enzymatic treatment. Instead, it is more likely to be a physiological characteristic of a subset of Na channels in the nervous system.

**Discussion**

These experiments provide us with three primary pieces of information. First, the expression of resurgent Na current is widespread in neurons of the cerebellum. Second, the resurgent component of Na current is much larger in physiological concentrations of Na⁺ and divalent cations than it is in the ion-substituted solutions commonly used for biophysical studies. Third, the steady-state availability curve of Na channels in cerebellar neurons is also significantly affected by the ionic composition of the external saline.

**External ions and channel block**

Divalent cations have long been known to affect Na channels (Armstrong and Cota 1991; Hille et al. 1975; Nilius 1988), both by affecting surface potential and by direct blocking interactions. Our experiments are consistent with work showing that transient as well as resurgent currents at moderately negative potentials are highly sensitive to the species and concentration of divalent cations (Swensen and Bean 2003). The effects of divalent cations cannot, however, entirely account for the increase in resurgent current and shift in availability curves in quasi-physiological solutions. First, relative to the 2 mM Ca²⁺ and 2 mM Mg²⁺ in the modified Tyrode’s solution, the 2 mM Co²⁺ in the low Na solution will shift the surface potential by about +3 mV, whereas 2 mM Ba²⁺ will shift it by about ~3 mV (Hille et al. 1975). Based on data from Hille et al. (1975), to obtain large enough changes in surface potential to account for the observed shifts in the availability curves, the low Na solutions would have to contain ~1 mM Ba²⁺ or ~300 μM Co²⁺. Second, unipolar brush cells were studied in low Na with 0.3 mM Cd²⁺ as the Ca channel blocker rather than 2 mM Co²⁺; 0.3 mM Cd²⁺ is not expected to block Na currents significantly (Swensen and Bean 2003). Nevertheless, unipolar brush cells showed qualitatively the same changes as Purkinje and cerebellar nuclear cells, suggesting that factors other than divalent ions affected the Na currents. Finally, even with divalents held constant, switching Purkinje cells and cerebellar nuclear cells from low to high Na indicated that resurgent current increased more than predicted by the GHK equation and also produced a positive shift of the availability curve.

This preferential increase in resurgent current by raising external Na is consistent with several previous studies of open-channel blockers. In Shaker K channels, the N-terminus “ball” (Hoshi et al. 1990; Zagotta et al. 1990) acts as an open-channel blocker, inactivating the channel at positive potentials. During recovery at negative potentials, K channels reopen as the ball is expelled from the pore, allowing a brief passage of current before the channel deactivates (Demo and Yellen 1991), much like Na channels that carry resurgent current. Increasing the concentration of external K accelerates recovery from N-type (ball-and-chain) inactivation, probably as a result of permeant ions facilitating unbinding of the pore blocker (Baukrowitz and Yellen 1995; Demo and Yellen 1991; Gómez-Lagunas and Armstrong 1994). In contrast to Shaker K channels, in Na channels, recovery from fast inactivation does not appear to proceed through open states (Kuo and Bean 1994), and the fast inactivation gate does not behave like an open channel blocker (Tang et al. 1996). Nevertheless, in Na channels from squid axon and from cardiac cells, raising the concentration of external Na facilitates the expulsion of exogenously applied intracellular blockers, such as strychnine, tetra-alkylammonium ions, and the KIFMK inactivation peptide (Eaholtz et al. 1994; O’Leary et al. 1996; Shapiro 1977; Tang et al. 1994).

Our observation that raising external Na increases resurgent current to a greater extent than predicted by the changes in permeant ion concentration, driving force, and divalent cations is therefore consistent with the idea that permeating Na ions may displace an endogenous, voltage-dependent, open-channel blocker from a binding site within the pore.

**Fast and slow inactivation**

Increases in external Na⁺ concentration may affect steady-state availability curves by limiting fast and/or slow inactivation. Evidence for an interaction between external Na and the fast inactivation gate has been obtained in recordings from CA1 pyramidal cells, in which high external Na accelerated recovery from fast inactivation (Kuo and Liao 2000). In those experiments, raising external Na from 50 to 150 mM produced a ~4-mV positive shift in the steady-state inactivation curve, similar to our data from Purkinje cells. Not all preparations show a dependence of inactivation on permeating ions; however, in squid axons and cardiac cells, for instance, fast inactivation is relatively insensitive to changes in the concentration of external Na (Armstrong and Bezanilla 1974; Townsend and Horn 1997)

An additional property that may be affected by external Na⁺ is slow inactivation. In many Na and K channels, prolonged or repeated depolarizations can lead to slow (C-type) inactivation, which is independent of fast inactivation (Cannon 1996) and
which appears to result from a constriction of the external mouth of the channel (Yellen et al. 1994). In Shaker K channels, entry into slow inactivated states can be reduced by manipulations that promote binding of K ions in the external mouth of the pore, such that a constant outward K flux is maintained (Baukrowitz and Yellen 1995; Gómez-Lagunas and Armstrong 1994). Similarly, in cardiac Na channels, high concentrations of external Na (or other alkali cations) slow the onset of slow inactivation as well as accelerate recovery from it (Townsend and Horn 1997).

The shifts in the availability curves that we have measured are consistent with an ability of high external Na⁺ to destabilize binding of the fast inactivation gate and/or to prevent slow inactivation, thereby increasing channel availability at moderately negative potentials. Notably, however, in cerebellar nuclear cells, the shift in inactivation was seen even with relatively short (100-ms) conditioning steps. Possibly, cerebellar nuclear cells enter “slow” inactivated states quite rapidly, and it is entry into these states that is minimized by high Na⁺; alternatively, the observed shift in the steady-state inactivation curve may result largely from destabilization of fast inactivation. In either case, this increase in availability may itself contribute to the increase in resurgent current amplitude. Relief of slow inactivation may provide more channels that could open, block, and unblock with repolarization, and relief of fast inactivation may make it less likely for stable binding of the fast inactivation gate to curtail the current that flows as channels become unblocked.

Maximization of availability and high-frequency firing

The net result of destabilization of open-channel block, fast inactivation, and/or slow inactivation at negative potentials is that physiological ionic concentrations limit the accumulation of channels in nonconductive states, thereby increasing channel availability. In this context, it is interesting that binding of the fast inactivation gate is also limited at positive potentials by the endogenous open-channel block (Grieco and Raman 2004; Raman and Bean 2001). The effect of the rapid unblock on repolarization, apparently promoted by physiological ion concentrations, is twofold: First, the resurgent current that flows following an action potential may provide some depolarizing drive for a subsequent spike (Do and Bean 2003). Second, the unblocked channels deactivate into “available” states, rather than remaining refractory (Raman and Bean 1997). The cycle of opening, blocking, and unblocking thereby minimizes the refractory period, a feature that may be suitable for enabling high-frequency firing (Khaliq et al. 2003; Raman and Bean 2001).

In fact, Purkinje cells, cerebellar nuclear cells, unipolar brush cells, and granule cells all produce action potentials at high rates. Purkinje neurons are not only spontaneously active at frequencies near 50 Hz, but fire at frequencies >100 Hz during cerebellar behaviors (e.g., Gilbert and Thach 1977; Kitazawa et al. 1998; Thach 1968). Cerebellar nuclear neurons tend to fire at lower rates than Purkinje cells, but they are also spontaneously active and can maintain firing frequencies of >50 Hz for prolonged periods (Aizemman and Linden 1999; Jahnse 1986; Ledoux et al. 1998; Thach 1968). Although the electrical activity of unipolar brush cells has been studied less extensively, it is known that their excitatory postsynaptic currents have an unusually long component that results from entrapment of glutamate in the synaptic cleft (Rossi et al. 1995). The resulting excitatory postsynaptic potentials can be large and long, evoking high-frequency action potentials superimposed on the synaptic depolarization (Rossi et al. 1995).

Finally, although granule cells do not appear to be spontaneously active, they fire at frequencies exceeding 100 Hz in response to excitatory stimuli (Chadderton et al. 2004; D’Angelo et al. 1998; Hartmann and Bower 2001; Mitchell and Silver 2003).

The extent to which rapid firing depends on the expression of Na channels with resurgent kinetics may vary across these cerebellar neurons. Conversely, the efficacy of resurgent current in promoting firing will depend strongly on the other ion channels expressed in the particular cells. Nevertheless, it is striking that the four classes of cerebellar neurons that we have tested share the property of producing resurgent current, particularly since this property initially seemed to be an unusual, if not unique, specialization of Purkinje cells. The idea that Purkinje cells were exceptional seemed plausible in part because resurgent current is absent from several neurons that express the same Na channel α subunits as Purkinje cells (Maurice et al. 2001; Pan and Beam 1999; Raman and Bean 1997), and in part because voltage-gated Na channels in expression systems lack a resurgent component (Smith et al. 1998). Recently, however, resurgent current was identified in neurons of the subthalamic nuclei (Do and Bean 2003), and the present results reveal that the current is a typical, rather than an unusual, feature of cerebellar neurons. The list of neurons with resurgent kinetics appears likely to grow. The widespread occurrence of this once-novel component of Na current suggests that the expression of a blocking factor that confers resurgent kinetics to Na channels may be a commonly utilized mechanism by neurons whose signaling involves sustained periods of high-frequency firing.

ACKNOWLEDGMENTS

Experimental contributions: Purkinje cells, cerebellar nuclear cells: F. S. Afshari and T. M. Grieco (laboratory of I. M. Raman); unipolar brush cells: K. Ptak (laboratories of D. R. McCrimmon and N. T. Slater); granule cells: Z. M. Khaliq (laboratory of I. M. Raman); slice experiments: Z. M. Khaliq, T. M. Grieco, and I. M. Raman. We are grateful to J. Pugh for dissociating cerebellar nuclear cells for low Na/Ca experiments.

GRANTS

This work was supported by the Harvard University Enrichment Program to F. S. Afshari, the Klingenstein Foundation to I. M. Raman, and National Institutes of Health Grants NS-39395 to I. M. Raman, NS-09904 to E. Mugnaini and N. T. Slater, and HL-072415 to D. R. McCrimmon.

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