Transient reversal of the sodium/calcium exchanger boosts presynaptic calcium and synaptic transmission at a cerebellar synapse

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Roome CJ, Power EM, Empson RM. Transient reversal of the sodium/calcium exchanger boosts presynaptic calcium and synaptic transmission at a cerebellar synapse. J Neurophysiol 109: 1669–1680, 2013. First published December 19, 2012; doi:10.1152/jn.00854.2012.—The sodium/calcium exchanger (NCX) is a widespread transporter that exchanges sodium and calcium ions across excitatory membranes. Normally, NCX mainly operates in its “forward” mode, harnessing the electrochemical gradient of sodium ions to expel calcium. During membrane depolarization or elevated internal sodium levels, NCX can instead switch the direction of net flux to expel sodium and allow calcium entry. Such “reverse”-mode NCX operation is frequently implicated during pathological or artificially extended periods of depolarization, not during normal activity. We have used fast calcium imaging, mathematical simulation, and whole cell electrophysiology to study the role of NCX at the parallel fiber-to-Purkinje neuron synapse in the mouse cerebellum. We show that nontraditional, reverse-mode NCX activity boosts the amplitude and duration of parallel fiber calcium transients during short bursts of high-frequency action potentials typical of their behavior in vivo. Simulations, supported by experimental manipulations, showed that accumulation of intracellular sodium drove NCX into reverse mode. This mechanism fueled additional calcium influx into the parallel fibers that promoted synaptic transmission to Purkinje neurons for up to 400 ms after the burst. Thus we provide the first functional demonstration of transient and fast NCX-mediated calcium entry at a major central synapse. This unexpected contribution from reverse-mode NCX appears critical for shaping presynaptic calcium dynamics and transiently boosting synaptic transmission, and is likely to optimize the accuracy of cerebellar information transfer.

DURING SYNAPTIC TRANSMISSION, the timing of the rise and fall of presynaptic Ca\(^{2+}\) is critical for neurotransmitter release and various forms of synaptic plasticity. Homeostatic mechanisms that actively control presynaptic internal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) dynamics are therefore fundamental for the fidelity and timing of synaptic transmission (Augustine 2001; Zucker and Regehr 2002). To this end, a variety of mechanisms at presynaptic terminals are known to influence both residual [Ca\(^{2+}\)]\(_i\) and activity within presynaptic terminals (Blaustein et al. 1996; Blaustein and Oborn 1975; Juhaszova et al. 2000; Regehr 1997). Previous work has suggested distinct roles for NCX at synaptic terminals. Physiological Ca\(^{2+}\) measurements show that NCX operates in its forward (Ca\(^{2+}\) exit) mode to recover residual Ca\(^{2+}\) levels and influence short-term synaptic plasticity (Jeon et al. 2003; Kim et al. 2005). In contrast, chemical depolarization of synaptosomes (Blaustein and Oborn 1975; Taglialetela et al. 1990) or prolonged tetanic stimulation at crayfish neuromuscular junctions (Zhong et al. 2001) revealed reverse-mode NCX-mediated Ca\(^{2+}\) entry, but the physiological relevance of the extra Ca\(^{2+}\) is unclear (Minami et al. 2007). To address these apparent inconsistencies and to further elucidate the physiological role for NCX during presynaptic Ca\(^{2+}\) dynamics, we have taken advantage of the well-characterized parallel fiber-to-Purkinje neuron (PF-PN) synapse, where the dynamics of presynaptic residual Ca\(^{2+}\) underlies its facilitatory behavior (Atluri and Regehr 1996). NCX is expressed at this synapse (Li et al. 2000), and a previous study reported NCX activity during PF activation (Regehr 1997). From a wider perspective, the modulation of short-term facilitation at the PF-PN synapse by NCX may be critical for reliable information transfer (Rotman et al. 2011) in the cerebellum.

To address the role of NCX at this important synapse, we combined fast presynaptic Ca\(^{2+}\) imaging during physiologically relevant stimulation patterns with pharmacology and whole cell electrophysiology. At the same time we informed our experimental interpretations with mathematical simulations of Na\(^{+}\) and Ca\(^{2+}\) fluxes within PFs. Uniquely, reverse-mode NCX drove Ca\(^{2+}\) entry into PFs during short bursts of high-frequency activity typical of their behavior in vivo (Chadderton et al. 2004). Mechanistically, the switch to reverse-mode NCX relied on the accumulation of Na\(^{+}\) during multiple presynaptic action potentials. Physiologically, reverse-mode NCX transiently boosted Ca\(^{2+}\) entry into the PF terminals and promoted PF-PN synaptic transmission. Together, these findings define NCX as an important, new, and fast presynaptic Ca\(^{2+}\) entry route.

METHODS

Ethical approval. All animal husbandry and procedures minimized animal suffering and were carried out using internationally recognized protocols approved by the University of Otago Animal Ethics Committee working to the New Zealand Animal Welfare Act (1999).

Slice preparation. Longitudinal cerebellar slices 300 \(\mu\)m thick were prepared from 3- to 4-wk-old C57 B6 male mice for imaging PFs and as sagittal slices for electrophysiology (Empson et al. 2007). Slices were maintained at 24°C (model TC324B; Harvard Apparatus) in a flow (3 ml/min) of artificial cerebrospinal fluid (aCSF), equilibrated with 95% O\(_2\) and 5% CO\(_2\), containing (in mM) 126 NaCl, 2.5

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To interleaved single (1×) and repetitive burst stimulation of PFs (a burst consisted of 5 stimuli delivered at 200 Hz, also called 5×) were recorded before application of pharmacological tools to manipulate NCX. In most (but not all) experiments, single 1× and burst 5× stimulation-evoked Ca2+ transients were obtained from the same slice. Application of pharmacological agents took place over a period of 5–10 min, after which the Ca²⁺ transients were again recorded in the continued presence of the pharmacological agent.

**Electrophysiology.** Whole cell recordings from Purkinje neuron (PN) soma under visual control were performed using glass electrodes (5–7 MΩ) containing (in mM) 140 KCl, 2 MgCl₂, 10 HEPES, 10 glucose, and 500 mM 3 M picrotoxin (Sigma-Aldrich) and prevented by 20 μM felodipine (all from Tocris Cookson). We conducted imaging and electrophysiology experiments, consisted of 5 stimuli at 200 Hz. Whole cell voltage-dependent Ca²⁺ currents from undifferentiated PC12 cells (kindly provided by Drs. Stephen Bunn and Istvan Abraham, University of Otago) 1–2 days after they were plated onto poly-L-lysine coverslips were obtained using the same recording amplifier and set up as described above. The extracellular solution contained (in mM) 140 NaCl, 10 TEACl, 10 CaCl₂, 10 HEPES, 10 glucose, and 26 sucrose, pH 7.3; the intracellular solution contained (in mM) 100 Cs-methanesulfonate, 10 TEACl, 10 EGTA, 7.5 NaCl, 3.5 MgCl₂, 10 lidocaine, 20 CsOH, 8 sucrose, 10 HEPES, 4 MgATP, and 0.4 Na,GTP, pH 7.2. Series resistances, cell leak, and capacitance were fully compensated. Inward currents were evoked by a 400-ms depolarization from −80 to +10 mV, and the steady-state current was expressed as a current density (pA/pF) using the capacitance measurement recorded directly from the Axopatch amplifier. Inward currents were similar to those previously reported (Garber et al. 1989); they were also voltage dependent and reversibly abolished by zero extracellular Ca²⁺ (n = 3) and 100 μM CdCl₂ (n = 3). Under these conditions the mean steady-state current changed from 3.4 ± 0.7 to 0.08 ± 0.3 pA/pF (P < 0.01, n = 6, paired t-test).

**Solutions and pharmacological treatments.** EPSCs were evoked in the presence of 50 μM picrotoxin (Sigma-Aldrich) and prevented by 20 μM (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoline-7-sulfonamide) (NBQX; Tocris Cookson). We conducted imaging and electrophysiology protocols in the presence of KB-R7943 (10–20 μM), SN-6 (10 μM), ω-agatoxin IVA (0.5 μM), cyclopiazonic acid (CPA; 10 μM), ouabain (80 mM), 20 μM NBQX, 10 μM felodipine (all from Tocris Cookson), or low extracellular Na⁺ (63 mM NaCl, 63 mM choline chloride; Sigma-Aldrich). In some experiments we applied serial applications of some of the two above treatments, e.g., ω-agatoxin IVA followed by KB-R7943. All pharmacological tools (less choline chloride and NBQX, both dissolved in water) were stored as 1,000× stock solutions in DMSO solvent. DMSO controls for both imaging and electrophysiology were negative.

**Theoretical simulations.** We generated a single-compartment model to simulate Ca²⁺ and Na⁺ fluxes across the PF presynaptic terminal membrane. The model incorporated a previous model of NCX flux from Weber et al. (2001) that used some parameters from PFs developed by Regehr (1997) together with a model for PF presynaptic Ca²⁺ dynamics modified from Erler et al. (2004) that included voltage-dependent Ca²⁺ entry, PMCA-mediated Ca²⁺ removal, and endogenous and exogenous Ca²⁺ buffers.

**Fig. 1.** Presynaptic parallel fiber (PF) Ca²⁺ transients during 1× and 5× burst stimulation protocols. A: a brightfield image (left) of the longitudinal cerebellar slice showing the position of the stimulating electrode (Stim) and the direction of PF stimulation (arrows) in the outer two-thirds of the molecular layer (ML), and the corresponding fluorescence (right) from Ca²⁺ dyes that has been actively transported from the loading site along a beam of PFs. The red rectangle shows the region of interest quantified in B. Scale bar, 0.5 mm. B: examples of normalized fluorescence intensity changes (ΔF/F) in the region of interest in response to a single (1×) stimulation (lower trace) and to a short burst of 5 stimulations (5×) at 200 Hz (5-ms separation). The bar graph (inset) shows the average amplitude of the peak normalized percent fluorescence for 1× and 5× stimulation, showing that the response to burst stimulation (5×) was approximately 5 times larger than the response to a single stimulus (1×). These fluorescence-based Ca²⁺ signals were therefore within the linear range and allowed accurate interpretation of changes in the amplitude of the fluorescence response. C: group mean recovery dynamics of the Ca²⁺ transients, determined from two-phase exponential decay function fits following the peak. t₁ and t₂ represent the first and second phases of recovery, respectively, for transients evoked by 1× or 5× stimulation. Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001, paired r-tests; all comparisons are between 1× and 5× values.
Description of the single-compartment model for Na\(^+\) and Ca\(^{2+}\) flux simulations. Because of the small volume of presynaptic terminals, spatial gradients in [Ca\(^{2+}\)]\(_i\) are assumed to dissipate very rapidly on the terminal on much shorter timescales than are relevant to the dynamics of residual presynaptic Ca\(^{2+}\) (Regehr 1997). Therefore, these simulations consider only average homogenous [Ca\(^{2+}\)]\(_i\) dynamics within a single compartment. The compartment had a surface-to-volume ratio (G) of 3/0.5 μm (Erler et al. 2004; Palay and Chan-Palay 1974) and boundaries that maintained a constant resting membrane potential (V\(_m\)) at ~70 mV. Under resting conditions the external Ca\(^{2+}\) and Na\(^+\) concentrations ([Ca\(^{2+}\)]\(_e\) and [Na\(^+\)]\(_e\)) were held constant.

Our experimental manipulations suggest little contribution of NCX (or PMCA; Empson et al. 2007) at rest, but within the simulation it was necessary to include additional Ca\(^{2+}\) and Na\(^+\) fluxes to exactly oppose Ca\(^{2+}\) and Na\(^+\) flux via PMCA and NCX at rest. Appropriate setting of the time course of these opposing ion fluxes enabled basal [Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\) to be held constant under “no-stimulus” conditions as a way to best match the experimental situation.

During “synaptic stimulation” in the model the membrane potential was briefly depolarized (to +20 mV for 1 ms) by using Gaussian functions to represent action potentials arriving at the presynaptic terminal. Membrane depolarizations triggered Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs), and since both [Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\) are known to accumulate in real Pf's during high-frequency stimulus trains (Regehr 1997), we also incremented the internal Na\(^+\) concentration (Δ[Na\(^+\)]\(_i\)) with each action potential to more realistically simulate internal Na\(^+\) (and Ca\(^{2+}\)) buildup during the 5× stimulus train. At the same time, the simulations also enabled us to determine how changes in [Na\(^+\)]\(_i\) and changes in membrane potential influenced the direction of net NCX flux during PF stimulation. The change in the free internal Ca\(^{2+}\) and Na\(^+\) concentrations for each time increment (Δt) was described by ordinary differential equations (Eqs. 1 and 2, respectively):

\[
\frac{\Delta [Ca^{2+}]_i}{\Delta t} = \frac{G}{zF} [J_{Ca_{in}} - J_{pmca} + J_{Ca_{leak}}] - \frac{1}{1 + T_{en} + T_{ex}} \tag{1}
\]

\[
\frac{\Delta [Na^+]_i}{\Delta t} = \frac{G}{zF} [J_{Na_{in}} - 3J_{ncx} + J_{Na_{leak}}], \tag{2}
\]

where Δ[Ca\(^{2+}\)]\(_i\) and Δ[Na\(^+\)]\(_i\) are the changes in free internal ion concentrations, G is the single-compartment geometry factor (surface-to-volume ratio), and F is the Faraday constant. \(J_{Ca_{in}}\) and \(J_{Na_{in}}\) are Ca\(^{2+}\) and Na\(^+\) influx, respectively. \(J_{pmca}\) and \(J_{ncx}\) are NCX- and PMCA-mediated Ca\(^{2+}\) flux, respectively. Assuming the rapid buffer approximation, endogenous Ca\(^{2+}\) buffers and exogenous Ca\(^{2+}\) buffers (i.e., fluo-4 dextran) were included by means of a correction factor 1/(1 + T\(_{ex}\) + T\(_{en}\)), which depends only on Ca\(^{2+}\) concentration, buffer concentration (\(b\(_{en}\)\) and \(b\(_{ex}\)\)), and the buffer dissociation constant (\(K_{en/ex}\)) (Erler et al. 2004) (Eq. 3):

\[
T_{en/ex} = \frac{b_{en/ex}K_{en/ex}}{K_{en/ex} + [Ca^{2+}]_i} \tag{3}
\]

where \(K_{en/ex}\) and \(K_{en/ex}\) are the Ca\(^{2+}\) binding and unbinding rate constants, respectively.

Calcium influx through VDCCs. Calcium influx (\(J_{Ca_{in}}\)) through VDCCs is characterized by an ohmic voltage-current relationship through an open channel of conductance (\(g\(_c\)) and density (\(\rho\(_c\)) and \(g\(_c\)(\(V\(_{e}\) - \(V(t)\)), and a voltage-dependent opening probability, \(g\(_c\)(\(V\(_{e}\)\)) (Tsien 1983), shown below:

\[
J_{[Ca_{in}]}(V(t), \tilde{V}(t)) = \rho_c g_c(V(t)) \tilde{g}_c(\tilde{V}(t) - V(t)), \tag{4}
\]

where \(V\) is the membrane potential and \(\tilde{V}\) is the reversal potential as described by the Nernst equation:

\[
\tilde{V}(c) = \frac{RT}{zF} \ln \left( \frac{[Ca^{2+}]_i}{[Na^+]_i} \right) - \Delta V_{eff}, \tag{5}
\]

where \(R\), \(T\), \(z\), and \(F\) are universal constants (8.315 J/K/mol, 300 K, 2, and 96,485 C/mol, respectively), and \(\Delta V_{eff}\) corrects the exact reversal potential for the linear approximation (Erler et al. 2004).

The time dependence of the single-channel open probability is modeled by a single exponential approximation:

\[
\tilde{\tilde{g}}_c(V) = \frac{1}{\exp\left(\frac{(V_h - V) \frac{1}{k}}{\kappa}\right) + 1}, \tag{7}
\]

where \(V_h\) is the half-activation voltage and \(\kappa\) describes the steepness of the asymptotic opening probability.

PMCA Ca\(^{2+}\) efflux via PMCA was described by a simple Hill equation (Elwess et al. 1997; Erler et al. 2004):

\[
J_{pmca} = \tilde{I}_{pmca} \cdot \left( \frac{[Ca^{2+}]_i^n}{([Ca^{2+}]_i + K_{pmca})^n} - \rho_{pmca} \right), \tag{8}
\]

where \(\tilde{I}_{pmca}\) is the maximal Ca\(^{2+}\) current via PMCA, \(K_{pmca}\) is the Ca\(^{2+}\) efflux-dependent Hill constant, \(n\) is the Hill coefficient, and \(\rho_{pmca}\) is the PMCA protein density.

NCX Ca\(^{2+}\) and Na\(^+\) exchange. The NCX current (\(J_{ncx}\)) is described as the product of an electrochemical factor (\(\Delta E\), Eq. 12) and an allosteric factor (Allo, Eq. 11) (Weber et al. 2001) and was used to describe NCX-mediated Ca\(^{2+}\) and Na\(^+\) influx/efflux (Eqs. 9 and 10, respectively):

\[
\Delta E = \left( \frac{[Na^+]_i}{[Ca^{2+}]_i} + K_{mca}[Ca^{2+}]_i + K_{mna}[Na^+]_i \right) \left( 1 + \frac{[Ca^{2+}]_i}{K_{mca}} \right) + K_{mca}[Na^+]_i \left( 1 + \frac{[Na^+]_i}{K_{mna}} \right) + [Na^+]_i[Ca^{2+}]_i + \left( \mu_{na} \kappa \left( \frac{(\eta - 1)\nu}{R} \right) \right) \right) \tag{12}
\]

\[
\Delta E = \left( \frac{[Na^+]_i}{[Ca^{2+}]_i} + K_{mca}[Ca^{2+}]_i + K_{mna}[Na^+]_i \right) \left( 1 + \frac{[Ca^{2+}]_i}{K_{mca}} \right) + K_{mca}[Na^+]_i \left( 1 + \frac{[Na^+]_i}{K_{mna}} \right) + [Na^+]_i[Ca^{2+}]_i, \tag{11}
\]

\[
Allo = \left( \frac{[Ca^{2+}]_i^n}{([Ca^{2+}]_i + K_{pmca})^n} - \rho_{pmca} \right) \tag{9}
\]

\[
Sodium influx = 3J_{ncx} = -3\tilde{I}_{ncx} \cdot \Delta E \cdot (Allo) \cdot \rho_{ncx} \tag{10}
\]

\[
Calcium efflux = -J_{ncx} = -\tilde{I}_{ncx} \cdot \Delta E \cdot (Allo) \cdot \rho_{ncx}, \tag{11}
\]
where $I_{ncx}$ is the maximal Ca$^{2+}$ current via NCX, $K_{ncx}$ is the activation Ca$^{2+}$ concentration, and $p_{ncx}$ is the NCX protein density. $K_{	ext{diss}}(\text{internal})$ and $K_{	ext{diss}}(\text{external})$ are dissociation constants for internal ($i$) and external ($e$) Na$^+$ and Ca$^{2+}$, $v$ is the position of the energy barrier of NCX in the membrane electric field, and $K_{sam}$ is a factor controlling saturation of $I_{ncx}$ at negative potentials. See Weber et al. (2001) for a complete description of the NCX model.

A complete list of universal constants and parameters used in the simulations is given in Table 1. We implemented all simulations using MATLAB (code available on request).

**Statistics.** For analysis we used pCLAMP 10 (Molecular Devices), Prism (GraphPad Software), and TableCurve2D (Jandel Scientific Software) all off-line. Akaike’s information criteria (AIC) in Prism confirmed that all Ca$^{2+}$ transient recoveries (in response to both 1× and 5× stimulation) recovered with a two-phase exponential function, returning a probability of >99.99% that a two-phase fit (compared with a one-phase fit) provided the best fit to the data. We used these fits to record half-lives of fast ($t_1$) and slow ($t_2$) phases of Ca$^{2+}$ recovery. In all cases, the amplitude components of the double-exponential fits ($A1$ and $A2$) were ~0.7 and 0.3, where $A1 + A2 = 1$. $A1$ and $A2$ were also not significantly altered by KB-R7934, SN-6, and Ca$^{2+}$-sensory stimulation (Chadderton et al. 2004) and also to sufficiently elevate PF presynaptic residual Ca$^{2+}$ to activate the low-affinity internal Ca$^{2+}$ activation site of NCX (Blautstein and Lederer 1999). The mean amplitude of Ca$^{2+}$ transients in response to high-frequency stimulation was about five times greater than after a single stimulation (Fig. 1B, 1×; $P < 0.001$, $t$-test, $n = 7$). The Ca$^{2+}$ transient evoked by 5× stimulation also recovered more slowly, as indicated by the longer fast ($t_1$,

### RESULTS

Beams of parallel fibers (PFs) previously loaded with the Ca$^{2+}$-sensitive dye fluo-4 dextran (Fig. 1A) responded to electrical stimulation with a transient rise and fall in cytosolic Ca$^{2+}$. We used two stimulation protocols: a single stimulus (1×) and a repetitive high-frequency (200 Hz) stimulus burst (5 stimuli at 200 Hz, 5×; Fig. 1B). We chose the latter to simulate afferent activity typically encountered in PFs during sensory stimulation (Chadderton et al. 2004) and also to sufficiently elevate PF presynaptic residual Ca$^{2+}$ to activate the low-affinity internal Ca$^{2+}$ activation site of NCX (Blautstein and Lederer 1999). The mean amplitude of Ca$^{2+}$ transients in response to high-frequency stimulation was about five times greater than after a single stimulation (Fig. 1B, 1×; $P < 0.001$, $t$-test, $n = 7$). The Ca$^{2+}$ transient evoked by 5× stimulation also recovered more slowly, as indicated by the longer fast ($t_1$,

### Table 1. Parameters used in the simulations of PF Na$^+$ and Ca$^{2+}$ movements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abbreviation</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faraday constant</td>
<td>$F$</td>
<td>96.485 C/mol</td>
<td></td>
</tr>
<tr>
<td>Gas constant</td>
<td>$R$</td>
<td>8.315 J·K$^{-1}$·mol$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>$T$</td>
<td>300 K</td>
<td></td>
</tr>
<tr>
<td>Geometry factor</td>
<td>$G$</td>
<td>(3/0.5) × 10$^{-6}$ m</td>
<td>Palay and Chan-Palay (1974)</td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>$V_m$</td>
<td>$-70 \pm 10^{-3}$ V</td>
<td>Koester and Sakmann (2000)</td>
</tr>
<tr>
<td>Extracellular Ca$^{2+}$ concentration</td>
<td>$[Ca^{2+}]_e$</td>
<td>0.05 × 10$^{-6}$ M</td>
<td>Regner (1997)</td>
</tr>
<tr>
<td>Resting Na$^+$ concentration</td>
<td>$[Na^+]_i$</td>
<td>10 × 10$^{-3}$ M</td>
<td>Regner (1997)</td>
</tr>
<tr>
<td>Extracellular Na$^+$ concentration</td>
<td>$[Na^+]_e$</td>
<td>150 × 10$^{-3}$ M</td>
<td>Regner (1997)</td>
</tr>
<tr>
<td>Change in $[Ca^{2+}]_i$, per action potential</td>
<td>$\Delta[Ca^{2+}]$</td>
<td>500 × 10$^{-6}$ M</td>
<td>Brenowitz and Regehr (2007)</td>
</tr>
<tr>
<td>Change in $[Na^+]_i$, per action potential</td>
<td>$\Delta[Na^+]$</td>
<td>80 × 10$^{-6}$ M</td>
<td>Regner (1997)</td>
</tr>
</tbody>
</table>

**Endogenous/exogenous buffers (based on calibrinin and fluo-4 dextran)**

- **Dissociation constant** | $K_{ncx}$ | 1.5 × 10$^{-6}$ M | Schwaller et al. (2002) |
- **Buffer concentration** | $B_m$ | 2.000 × 10$^{-6}$ M | Schwaller et al. (2002) |
- **Voltage-dependent Ca$^{2+}$ channels**
  - **Open pore conductivity** | $\tilde{g}_c$ | 14 × 10$^{-12}$ S | Fisher et al. (1990) |
  - **Half-activation voltage** | $V_0$ | $-4 \times 10^{-3}$ V | Fisher et al. (1990) |
  - **Steepness of opening probability** | $K_0$ | $6.3 \times 10^{-3}$ V | Fisher et al. (1990) |
  - **Channel time constant** | $\tau$ | $1 \times 10^{-3}$ s | Magee and Johnston (1995) |
- **Ca$^{2+}$ reversal potential** | $V_0$ | $47 \times 10^{-3}$ V | Fisher et al. (1990) |
- **VDCC density** | $\rho_v$ | $7 \times 10^9$ m$^{-2}$ | |

**PMCA density** | $\rho_{pmca}$ | 2.7 × 10$^{-13}$ [Ca$^{2+}$]$^{-1}$ | Elwess et al. (1997) |

**PMCA density** | $\rho_{pmca}$ | 3 × 10$^{-14}$ m$^{-2}$ | adjusted |

**Na$^+$/Ca$^{2+}$ exchanger**

- **Maximum activity rate** | $I_{ncx}$ | 2.7 × 10$^{-13}$ [Ca$^{2+}$]$^{-1}$ | Elwess et al. (1997) |

Parameters used in the simulations of parallel fiber (PF) Na$^+$ and Ca$^{2+}$ ion movements are listed with their abbreviations, values, and references providing their source. NCX, Na$^+$/Ca$^{2+}$ exchanger; PMCA, plasma membrane Ca$^{2+}$-ATPase; VDCC, voltage-dependent Ca$^{2+}$ channel.

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half-life recovery) and slow (t2, half-life recovery) components of two-phase exponential fits (Fig. 1C; \( P = 0.01 \) and \( P = 0.03 \), respectively, t-tests, \( n = 7 \)).

We confirmed the presynaptic origin of the 1× and 5× PF transients, since they were abolished by zero extracellular \([Ca^{2+}]_o\) (\( n = 4 \)) and unaffected by the postsynaptic glutamate receptor antagonist 20 μM NBQX (\( n = 3 \)). CPA (10 μM; \( n = 4 \)) did not influence 1× and 5× Ca\(^{2+}\) transients, suggesting little contribution from intracellular Ca\(^{2+}\) stores.

**Inhibition of reverse-mode NCX reduced the amplitude and accelerated the recovery of 5× PF Ca\(^{2+}\) transients.** To test the contribution of the NCX to PF Ca\(^{2+}\) dynamics, we recorded Ca\(^{2+}\) transients evoked by both 1× and 5× stimulation protocols in the presence of two selective pharmacological inhibitors of NCX. We used KB-R7943 (Iwamoto et al. 1996) and SN-6 (Niu et al. 2007), which both exhibit a preference to inhibitors of NCX. We used KB-R7943 (Iwamoto et al. 1996) and SN-6 (Niu et al. 2007), which both exhibit a preference to inhibitors of NCX.

Application of 20 μM KB-R7943 reduced the amplitude of the 5× Ca\(^{2+}\) transient by around 40% (Fig. 2, A and B; \( P = 0.0009 \), paired t-test, \( n = 9 \)) and also accelerated the early phase (t1) of its recovery (Fig. 2C; \( P < 0.0015 \), paired t-test, \( n = 9 \)). The slower recovery phase (t2) was not affected (Fig. 2D; \( P = 0.8 \), paired t-test, \( n = 9 \)). In six of these experiments we tested KB-R7943 action on 1× stimulation Ca\(^{2+}\) transients, but they remained unaffected (see Fig. 2). (Neither their amplitude, %ΔF/F, or recovery rates, t1 and t2, were altered: \( P = 0.91 \), \( P = 0.95 \), and \( P = 0.13 \), respectively, paired t-tests, \( n = 6 \)). KB-R7943 did not influence basal fluorescence (4,370 ± 696 vs. 4,033 ± 521; \( P = 0.26 \), paired t-test, \( n = 9 \)).

A lower concentration of KB-R7943 (10 μM) also reduced the amplitude of the 5× Ca\(^{2+}\) transient, from 5.00 ± 0.2 to 3.5 ± 0.3%ΔF/F, and t1 changed from 121.3 ± 6.3 to 111 ± 3.8 ms (both \( P < 0.05 \), paired t-test, \( n = 3 \)). The second reverse-mode inhibitor, 10 μM SN-6, also reduced the peak amplitude of the 5× Ca\(^{2+}\) transient (4.5 ± 0.4 to 3.5 ± 0.3%ΔF/F; \( P = 0.03 \), paired t-test, \( n = 5 \)) and also reduced t1 (144.5 ± 25 to 129.4 ± 22.9 ms; \( P = 0.03 \), paired t-test, \( n = 5 \)) with no effect on t2 (715.4 ± 174 to 589 ± 133 ms; \( P = 0.34 \), paired t-test, \( n = 5 \)). The amplitude and recovery dynamics of 1× stimulation-evoked Ca\(^{2+}\) transients were not influenced by SN-6 (1.4 ± 0.2 vs. 1.1 ± 0.16%ΔF/F; \( P = 0.21 \), paired t-test, \( n = 3 \); t1 unchanged from 74 ± 19 to 67 ± 20.1 ms, \( P = 0.62 \), paired t-test, \( n = 3 \); t2 unchanged from 236.3 ± 62.4 to 227.7 ± 54.1 ms, \( P = 0.57 \), paired t-test, \( n = 3 \)).

These results indicate that reverse-mode NCX contributes to early Ca\(^{2+}\) influx into PFS only following a high-frequency presynaptic burst and not after a single action potential or under basal conditions. However, thus far our interpretation...
relies on the specificity of the inhibitors. SN-6 has few off-target ion channel actions (Niu et al. 2007), but KB-R7943 influences other targets, including L-type voltage-gated Ca\(^{2+}\) channels (Birinyi et al. 2005). Although L-type Ca\(^{2+}\) channels are not present in PF terminals, other voltage-gated Ca\(^{2+}\) channels are, so the potential remained for our observed influx through voltage-gated Ca\(^{2+}\) channels. First, in the presence of 0.5 \(\text{M} \) reverse high-frequency stimulation. We therefore conducted additional experiments with KB-R7943 to determine if P/Q type currents might be recruited. A strong resemblance to HVA currents in cerebellar granule cells (Neal et al. 2010; Zhang et al. 2007) and their kinetics bear strong resemblance to HVA currents in cerebellar granule cells (Pearson et al. 1995). Importantly, 20 \(\mu\text{M} \) KB-R7943 did not significantly influence the amplitude of HVA Ca\(^{2+}\) currents in PC12 cells. Felodipine (10 \(\mu\text{M}\)) an L-type Ca\(^{2+}\) channel blocker, reduced the HVA current in these cells from 6.4 \(\pm\) 1.4 to 5.1 \(\pm\) 1.2 pA/pF (similar to Garber et al. 1989), but in the same cells KB-R7943 did not alter the inward current further: it remained at 5.0 \(\pm\) 1.3 pA/pF \((n = 6, P > 0.05, \text{repeated-measures 1-way ANOVA, Bonferroni multiple comparison})\). It is therefore unlikely that KB-R7943 blocked P/Q-, R-, and N-type channel-mediated Ca\(^{2+}\) entry into the PF terminals in response to stimulation (Mintz et al. 1995; Myoga and Regehr 2011). Together, these controls and the similarity of action of KB-R7943 and SN-6 all strongly support reverse-mode NCX-mediated Ca\(^{2+}\) entry into PFs during short bursts of high-frequency stimulation.

Na\(^{+}\) accumulation in PFs provided the necessary conditions for NCX reversal and Ca\(^{2+}\) entry. We therefore sought to identify the basis for the reverse-mode NCX activity. Since we cannot directly measure NCX flux from the tiny presynaptic PF terminals, we instead modeled its activity. Using known parameters for PFs, we estimated presynaptic Ca\(^{2+}\) dynamics using a model adapted from Erler and applied this together with a model previously used to simulate NCX flux in the heart (Weber et al. 2001). The model allowed us to identify and modify factors that influence NCX flux directionality (Fig. 3).

![Fig. 3. Simulations of NCX flux direction in response to 5× PF presynaptic action potentials.](http://jn.physiology.org/)

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**Fig. 3.** Simulations of NCX flux direction in response to 5× PF presynaptic action potentials. A: simulation of the direction and magnitude of the NCX flux using the parameters in Table 1: forward, forward-mode flux (above zero); reverse, reverse-mode flux (below zero; shaded). During the burst of presynaptic action potentials, note the very fast transient reversal of the flux during each presynaptic action potential, marked with an arrow, consistent with a negative reversal potential for the electrogenic NCX exchanger (Blaustein 1988). After the burst, NCX remained in its reverse mode (flux is less than zero) for up to 400 ms after the burst. Removal of intracellular Na\(^{+}\) ion ([Na\(^{+}\)]) accumulation changed the flux considerably, as shown in B, so that forward mode was more dominant both during and after the burst, although transient reversal of NCX during each action potential remained (see asterisks). Lowering external Na\(^{+}\) concentration ([Na\(^{+}\)]) in the presence of Na\(^{+}\) accumulation, as shown in C, enhanced the amplitude and duration of the reverse-mode NCX flux both during the burst and also for more than 500 ms after the burst. Traces in D show the impact of the altered NCX flux for Ca\(^{2+}\) dynamics in the PFs. The parameters used to generate the Ca\(^{2+}\) transients are shown in detail in Table 1. The 5× stimulation protocol generated a Ca\(^{2+}\) transient with a peak and recovery (black trace). Removal of NCX from the model reduced the peak of the Ca\(^{2+}\) transient while accelerating the recovery from peak Ca\(^{2+}\), just as we had seen experimentally (gray trace). Furthermore, removal of NCX did not greatly influence single, 1× Ca\(^{2+}\) transients (dashed traces).

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The model incorporated an allosteric factor (Ca\(^{2+}\)- and Na\(^{+}\)-binding sites) and also an electrochemical factor based on changes in Na\(^{+}\) and Ca\(^{2+}\) concentrations across the terminal membrane during presynaptic action potentials (see Eqs. 9–12 in METHODS). Since [Na\(^{+}\)]\(_e\) accumulates in real PFs during high-frequency stimulation (Regehr 1997), we continuously incremented presynaptic ∆[Na\(^{+}\)]\(_e\), and (Δ[Ca\(^{2+}\)]\(_e\)) with each action potential during the 5× train to more accurately simulate buildup of ions in the PF. The simulation reported a sustained reversal of NCX flux (line below zero in Fig. 3A) that lasted ~400 ms after the burst and so closely approximated our experimental findings. When we neglected to accumulate [Na\(^{+}\)]\(_e\) in the model, NCX failed to switch into reverse mode but instead sustained forward-mode activity both during and after the burst (see Fig. 3B). These results supported the idea that activation of reverse-mode NCX in PFs required accumulated elevation of [Na\(^{+}\)]\(_e\), in a manner similar to a mechanism thought to operate in cardiac muscle (Leblanc and Hume 1990). To further test our model, we predicted that lowered [Na\(^{+}\)]\(_e\) and accumulated [Na\(^{+}\)]\(_i\) would favor reverse-mode NCX, and, as shown in Fig. 3C, these conditions further increased both the amplitude and duration of the after-burst reverse-mode NCX flux (below zero).

These NCX flux simulations indicated that bursts of high-frequency action potentials in real PFs allowed [Na\(^{+}\)]\(_e\) to accumulate to a level sufficient to activate reverse-mode NCX, as also indicated by our pharmacological experiments. However, since our experimental evidence relied on interpretation of fast Ca\(^{2+}\) signals, we incorporated the same NCX simulation parameters into a model of presynaptic Ca\(^{2+}\) (Fig. 3D). High-frequency bursts of presynaptic action potentials in the model evoked a large rise in Ca\(^{2+}\) with a fast recovery (Fig. 3D, black line). When NCX was removed, the model reported a reduction in the amplitude of peak Ca\(^{2+}\) and an accelerated Ca\(^{2+}\) recovery, consistent with our experimental observations with NCX inhibitors shown in Fig. 2.

Manipulation of Na\(^{+}\) gradients to favor reverse-mode NCX also boosted 5× PF Ca\(^{2+}\) transient. If, as the simulations suggested, internal Na\(^{+}\) accumulation is critical for NCX reversal, experimental manipulation of Na\(^{+}\) gradients should also modify PF Ca\(^{2+}\) transients. First, we lowered [Na\(^{+}\)]\(_i\) to favor NCX reverse mode (Fig. 3A, lower trace). When NCX is in reverse mode, lowering [Na\(^{+}\)]\(_i\) will favor NCX-mediated transport of Na\(^{+}\) out of the terminal, resulting in increased Ca\(^{2+}\) influx and a slowing of the early phase of recovery of the Ca\(^{2+}\) transient. As shown in Fig. 4, A and B, low external [Na\(^{+}\)]\(_e\) increased the peak of the 5× Ca\(^{2+}\) transient (P < 0.05, 1-way ANOVA, n = 6), an effect reversed by KB-R7943 (P < 0.001, 1-way ANOVA, n = 6). If was also significantly slowed by low [Na\(^{+}\)]\(_e\) (Fig. 4C; P < 0.05, 1-way ANOVA, n = 6) without influencing t2 (Fig. 4D; P = 0.97, 1-way ANOVA, n = 6). As shown in Fig. 4, B–D, low [Na\(^{+}\)]\(_i\) did not significantly alter the peak amplitude or t1 and t2 recovery half-lives of Ca\(^{2+}\) transients evoked by 1× stimulation (P = 0.84, P = 0.95, and P = 0.15, respectively, paired t-tests, n = 7). Low [Na\(^{+}\)]\(_e\) also did not influence basal fluorescence (mean values were unchanged from 3,373 ± 522 to 3,480 ± 443; P = 0.4, paired t-test, n = 7), indicating that NCX is inactive when PFs are at rest. Lowering [Na\(^{+}\)]\(_i\) will reduce the driving force for Na\(^{+}\) entry, which could broaden the PF presynaptic action potential (Hodgkin and Katz 1949) and thereby directly cause increased Ca\(^{2+}\) entry. As an additional approach, we therefore used a very low concentration of ouabain (80 nM) as an alternative way to enhance NCX reverse-mode flux. Ouabain (80 nM) inhibits the high-affinity α\(_e\)-isoform of the Na\(^{+}\)-K\(^{+}\)-ATPase (NKA) and will raise [Na\(^{+}\)]\(_i\), to favor reverse-mode NCX (Zhang et al. 2005), and it is unlikely to cause as significant a shift in driving force as halving [Na\(^{+}\)]\(_i\). As shown in Fig. 4, E and F, ouabain increased the peak of the 5× stimulation-induced Ca\(^{2+}\) transient (P < 0.05, 1-way ANOVA, n = 6). If was also significantly slowed by ouabain (Fig. 4G; P < 0.05, 1-way ANOVA, n = 6) without influencing t2 (Fig. 4H; P = 0.56, 1-way ANOVA, n = 6). Ouabain effects were also reversed by KB-R7943 (Fig. 4, E and F; P < 0.001, 1-way ANOVA, n = 6). Importantly, ouabain did not alter the peak amplitude or t1 and t2 of Ca\(^{2+}\) transients evoked by 1× stimulation (Fig. 4, F–H; P = 0.21, P = 0.35, and P = 0.9, respectively, paired t-tests, n = 3) and did not alter basal fluorescence (mean values were unchanged from 3,373 ± 522 to 3,480 ± 443; P = 0.4, paired t-test, n = 7).

Reverse-mode NCX-mediated Ca\(^{2+}\) entry promoted synaptic transmission for a few hundred milliseconds after 5× PF stimulation. Thus far our findings indicated that a short window of high-frequency action potentials switched NCX into reverse mode as [Na\(^{+}\)]\(_i\) accumulated within the PF terminal. The outcome was a boost to NCX-mediated Ca\(^{2+}\) influx during a time window that included the peak of the Ca\(^{2+}\) transient and a few hundred milliseconds thereafter. To determine if this additional presynaptic Ca\(^{2+}\) was relevant for the behavior of the synapse, we took advantage of the fact that the size of the PF-PN synaptic response and the extent to which it exhibits facilitatory behavior of the synapse. As shown in Fig. 5A, we compared PPR just before the 5× burst stimulus and at 200-ms intervals thereafter. These times corresponded to the early phase of decay of the 5× Ca\(^{2+}\) transient where KB-R7943 was exerting its effect (see presynaptic Ca\(^{2+}\) transients in Figs. 2B and 5A). At 200 ms after the burst, when presynaptic Ca\(^{2+}\) was still high, PPR was reduced (Fig. 5, A and C) alongside an increase in the amplitude of the first of the pair of EPSCs (P = 0.0002, 1-way ANOVA; mean values changed from 267 ± 50 pA before 5× stimulus to 367 ± 61 pA at the 200-ms time point after the stimulus; P < 0.05, from the 1-way ANOVA Bonferroni multiple comparison at that time point, n = 5). The result supported the idea that sustained presynaptic Ca\(^{2+}\) 200 ms after the burst was sufficient to enhance glutamate release. Six hundred milliseconds later, both PPR and the first EPSC (and presynaptic Ca\(^{2+}\)) returned to prestimulus levels. (EPSC mean values were returned to 236 ± 49 pA, similar to values before 5× stimulation, P > 0.05, 1-way ANOVA, n = 5).

Since KB-R7943 reduced the boost to Ca\(^{2+}\) influx provided by reverse-mode NCX, we predicted that KB-R7943 should abolish the reduction of PPR and the increased size of the first EPSC at just after the burst. As shown in Fig. 5C, this was the case; in the presence of KB-R7943, PPR remained the same before and after the burst (P = 0.016, 2-way ANOVA, n = 6). As shown in the example cell in Fig. 5B, KB-R7943 also abolished the increased amplitude of the first EPSC recorded 200 ms after the burst; in fact, the amplitude of EPSCs

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remained constant at all time points after the burst \( (P > 0.05, 1\text{-way ANOVA, } n = 6) \). Critically, KB-R7943 did not alter PPR or EPSC amplitudes evoked by the \( 1\times \) stimulus independently from the \( 5\times \) stimulus. This result also provided additional further evidence to eliminate off-target effects of KB-R7943 at voltage-gated \( \text{Ca}^{2+} \) channels during presynaptic \( \text{Ca}^{2+} \) entry (see also above). PPR remained unchanged by KB-R7943 (from 1.91 ± 0.12 to 1.86 ± 0.07; \( P = 0.51 \), paired \( t \)-test, \( n = 5 \)), consistent with no change in the amplitudes of first or second EPSCs \( (P > 0.54 \) and \( P > 0.14 \), respectively, paired \( t \)-tests, \( n = 5 \)). (Mean values of the 1st EPSC remained unchanged from 248.2 ± 56 to 248.5 ± 52 pA, and those of the
2nd EPSC from 389 ± 72 to 422 ± 74 pA.) Thus, all together, our results support a functional role for a reverse-mode NCX-mediated boost to Ca\(^{2+}\) influx that enhanced synaptic transmission during a 400-ms time window following a high-frequency burst of PF action potentials.

DISCUSSION

We have defined a new functional role for NCX at the cerebellar PF-PN synapse. Although NCX is generally thought to help clear Ca\(^{2+}\) from synaptic terminals under physiological conditions, we report here that the transporter transiently switches into its reverse, Ca\(^{2+}\) influx, mode during the type of high-frequency bursts typical of PFs in vivo (Chaderton et al. 2004). Functionally, this extra Ca\(^{2+}\) transiently boosts PF-PN synaptic transmission immediately after the burst.

Several experimental findings support these conclusions. Two different pharmacological inhibitors of reverse-mode NCX (KB-R7943 and SN-6) reduced the amplitude of the burst-evoked PF Ca\(^{2+}\) transient. This suggests that NCX normally operates in reverse mode during bursts of PF afferent activity and contributes to Ca\(^{2+}\) influx into the terminals rather than clearance. The timing of the NCX-driven boost to the Ca\(^{2+}\) transient occurred at its peak and for a few hundred milliseconds thereafter, as evidenced by the fact that both NCX inhibitors reduced the peak amplitude of the Ca\(^{2+}\) transient and also accelerated the initial, fast phase of recovery. Pharmacological manipulation of NCX did not influence the Ca\(^{2+}\) transients evoked by a single PF stimulation (1×), suggesting that NCX was not functional under these conditions. Indeed, a recent report showed that NCX only operates in forward (Ca\(^{2+}\) clearance) mode under 1× stimulation conditions upon removal of PMCA, a major calcium Ca\(^{2+}\) mechanism in PFs (Roome et al. 2013).

Together our experimental results indicated that short, high-frequency bursts of PF action potentials rapidly and selectively switch NCX into reverse (Ca\(^{2+}\) entry) mode. Several factors will influence the switch, the most significant being membrane depolarization beyond the reversal potential of NCX, and Na\(^{+}\) and Ca\(^{2+}\) binding to the internal reverse-mode activation sites of the NCX (Blaustein and Lederer 1999). The relative contributions of these factors are normally determined by directly measuring NCX flux, but this is technically impossible from the very small PF terminal. We therefore developed a simulation of NCX flux based on a previous model but with PF parameters. Critically, the simulation only reported reverse-mode NCX activity after a high-frequency burst of action potentials when presynaptic [Na\(^{+}\)], accumulated, as occurs in real PFs during high-frequency trains of presynaptic action potentials (Regehr 1997). Presumably, under these conditions, [Na\(^{+}\)]i rose sufficiently to activate the internal reverse-mode Na\(^{+}\) binding site (Blaustein and Lederer 1999). To experimentally test the importance of [Na\(^{+}\)]i accumulation highlighted by our modeling approach, we used two experimental strategies to manipulate Na\(^{+}\) gradients. We lowered [Na\(^{+}\)]i to favor Na\(^{+}\) exit (and Ca\(^{2+}\) entry) if [Na\(^{+}\)]i is accumulating by reverse-mode NCX. In separate experiments we also used very low concentrations of ouabain to inhibit the Na\(^{+}\)-K\(^{+}\)-ATPase as an alternative way to favor [Na\(^{+}\)]i accumulation. Both manipulations increased the amplitude of the 5× Ca\(^{2+}\) transients and slowed their initial recovery in a KB-R7943-sensitive manner.
These experimental results therefore endorsed the findings from the simulation and helped confirm the idea that Na⁺ accumulation in PFs augments NCX reverse-mode Ca²⁺ entry during, and immediately after, the high-frequency burst.

Whereas elevated [Na⁺], is critical to switch NCX into reverse mode, so too is internal, nontransported and activating Ca²⁺ (DiPolo 1979). In the PF terminal, action-potential-evoked residual Ca²⁺ levels are estimated at 600–900 nM (Brenowitz and Regehr 2007), close to the Kᵢ of the internal Ca²⁺-binding reverse-mode activation site (DiPolo 1979). Activation of forward-mode NCX by Ca²⁺ is typically thought to require somewhat higher levels of Ca²⁺, so it is possible that during synthetically evoked Ca²⁺ transients, forward- and reverse-mode NCX operate simultaneously (Yu and Choi 1997) but that certain conditions favor one over the other. Our findings indicate that depolarization, Na⁺ accumulation, and residual Ca²⁺ all combine during short bursts of presynaptic action potentials in PFs (and perhaps other small-volume neuronal compartments) to favor reverse-mode NCX. The mechanism shares some similarity with the (still controversial) contribution of reverse-mode NCX during the cardiac action potential (Leblanc and Hume 1990). In cardiac myocytes, the dyadic (or “fuzzy”) space between the plasma membrane and the sarcoplasmic reticulum membrane allows rapid accumulation of [Na⁺]. This is thought to drive transient, reverse-mode NCX-mediated Ca²⁺ entry and associated depolarization that enhances individual cardiac action potentials (Larbig et al. 2010). A similarly diffusion-restricted space exists in the PF presynaptic terminal (estimated as 0.8 μm long × 0.25 μm wide; volume 0.2 μm³; Palay and Chan-Palay 1974) and could provide the anatomic basis for accumulation of [Na⁺], that triggers reverse mode NCX, particularly during bursts of action potentials.

To identify how the reverse-mode NCX Ca²⁺ influx contributed to synaptic function, we used electrophysiological recordings from the Purkinje neuron to probe the behavior of the PF-PN synapse. We took advantage of the fact that paired-pulse facilitation at this synapse indirectly reports residual Ca²⁺ levels (Atluri and Regehr 1996) and applied paired stimulations immediately after the high-frequency burst, just as NCX was driving extra Ca²⁺ into the terminal. At this early time after the burst, coincident with NCX-mediated presynaptic Ca²⁺ influx, we observed enhanced PF-evoked EPSCs and reduced facilitation, both consistent with increased Ca²⁺-dependent glutamate release and PF residual Ca²⁺. Since both changes were prevented by KB-R7943, the results provide convincing evidence that NCX normally boosts Ca²⁺ influx sufficiently to enhance synaptic transmission during a time window of ~400 ms after the burst. Thus this is the first demonstration of a physiological and functional demonstration of reverse-mode NCX-mediated Ca²⁺ entry at a central synapse. A previous study identified how reverse-mode NCX-mediated presynaptic Ca²⁺ entry accompanied long periods (20 s) of tetanic stimulation at the crayfish neuromuscular junction (Zhong et al. 2001), but this extra Ca²⁺ did not influence long-term facilitation at this synapse (Minami et al. 2007). Another study showed that exposure of hippocampal neurons to veratridine for several seconds as a way to artificially raise [Na⁺], also enhanced transmitter release via reverse-mode NCX (Bouron and Reuter 1996). From a pathologic perspective, prolonged reverse-mode NCX-mediated Ca²⁺ influx into presynaptic terminals is undesirable. Indeed, nonphysiological [Na⁺], overload of sympathetic nerves drives norepinephrine release through reverse-mode NCX (Torok et al. 2008), and reverse-mode NCX drives excessive glutamate release in a preclinical model of multiple sclerosis (Rossi et al. 2010). The same could apply in models of severe ischemia where NCX reverse-mode inhibitors may exert their neuroprotective effects by curtailing excessive glutamate release (Iwamoto and Kita 2006; Matsuda et al. 2001). However, there are some conflicting opinions about the direction of NCX flux in pathological conditions (Cross et al. 2010). For example, milder ischemic episodes (Jeon et al. 2008; Tanaka et al. 2002) activate forward-mode NCX to expel Ca²⁺, and ischemic preconditioning elevates NCX expression and ameliorates stroke-induced brain damage (Pignataro et al. 2012). Therefore, Ca²⁺ clearance by forward-mode NCX remains an important neuroprotection target.

In addition to the important Ca²⁺ recovery roles for forward-mode NCX, our results support a transient and physiological switch of NCX direction that boosts Ca²⁺ entry during brief bursts of PF activity. We know that P/Q-, N-, and R-type voltage-gated Ca²⁺ channels all drive Ca²⁺-dependent glutamate release at the PF synapse (Mintz et al. 1995; Myoga and Regehr 2011), so why the need for additional Ca²⁺ entry via reverse-mode NCX? Perhaps during multiple high-frequency action potentials, rapid inactivation of voltage-gated channels limits Ca²⁺ entry and reduces the efficiency of glutamate release. If so, NCX reversal, triggered by fast accumulation of action potential-evoked Na⁺ influx, provides a plausible mechanism for activity-dependent amplification of Ca²⁺ entry into the PFs. As we have shown, this extra Ca²⁺ promotes and sustains synaptic transmission after the burst. It is tempting to speculate that the poor motor performance of NCX3 knockout mice (Sokolow et al. 2004) might be explained by critically weakened transmission of granule cell-encoded sensory input to PNs during this time window.

In summary, we have identified reverse-mode NCX as a new route for fast presynaptic Ca²⁺ entry at the cerebellar PF-PN synapse. By boosting Ca²⁺ entry following high-frequency afferent input, NCX generates a time window of enhanced glutamate release that may optimize physiologically relevant, frequency-encoded sensory information transfer.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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