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Analysis of distinct short and prolonged components in rebound spiking of deep cerebellar nucleus neurons

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Abstract

Deep cerebellar nucleus (DCN) neurons show pronounced post-hyperpolarization rebound burst behavior, which may contribute significantly to responses to strong inhibitory inputs from cerebellar cortical Purkinje cells. Thus, rebound behavior could importantly shape the output from the cerebellum. We used whole cell recordings in brain slices to characterize DCN rebound properties and their dependence on hyperpolarization duration and depth. We found that DCN rebounds showed distinct fast and prolonged components, with different stimulus dependence and different underlying currents. The initial depolarization leading into rebound spiking was carried by HCN current, and variable expression of this current could lead to a control of rebound latency. The ensuing fast rebound burst was due to T-type calcium current, as previously described. It was highly variable between cells in strength, and could be expressed fully after short periods of hyperpolarization. In contrast, a subsequent prolonged rebound component required longer and deeper periods of hyperpolarization before it was fully established. We found using voltage-clamp and dynamic clamp analyses that a slowly inactivating persistent sodium current fit the conductance underlying this prolonged rebound component resulting in spike rate increases over several seconds. Overall, our results demonstrate that multiphasic DCN rebound properties could be elicited differentially by different levels of Purkinje cell activation, and thus create a rich repertoire of potential rebound dynamics in the cerebellar control of motor timing.

Keywords

rat; brain slice; dynamic clamp; inhibition; persistent ion channel

Introduction

All output from the cerebellar cortex is carried by Purkinje cell axons that provide GABA-A mediated inhibitory input predominantly to neurons in the DCN (Palkovits *et al.*, 1977; De Zeeuw & Berrebi, 1995). This arrangement raises the question of how inhibition at the level of the DCN can encode the results of cerebellar cortical processing. Work in brain slices has shown that DCN neurons are intrinsic pacemakers with a spontaneous baseline spiking frequency of 10-15 Hz in cell attached recordings (Uusisaari *et al.*, 2007), while they show faster average spike rates in awake rats that increase from 29 Hz at 12-19d age to 43 Hz at 20-26d (LeDoux *et al.*, 1998). Spontaneous spiking in vitro can be easily interrupted by small negative current injection or inhibitory input (Aizenman & Linden, 1999). When

patterns of stochastic inhibitory input trains are applied to DCN neurons in brain slices via dynamic clamping, single output spikes are triggered by brief pauses in inhibitory input (Gauck & Jaeger, 2000). In this continuous mode of input processing, DCN neuron spike rates are controlled by the balance of excitation and inhibition as well as by the occurrence of depolarizing fluctuations in input which increase in number for synchronization in inhibitory or excitatory input streams (Gauck & Jaeger, 2003).

A different mode of inhibitory input coding can be evoked in DCN neurons by strong bursts of inhibitory inputs. Following such strong inhibitory inputs DCN neurons react with a pronounced fast rebound burst (FRB) of spiking (Jahnsen, 1986a; Aizenman & Linden, 1999). This FRB is found in both GABAergic DCN neurons projecting to the inferior olive as well as in the glutamatergic cell class projecting to the red nucleus and to thalamus (Uusisaari *et al.*, 2007). Previous work using negative current injection pulses in DCN neurons in brain slices to mimic strong inhibitory input bursts has shown that the FRB has a significant underlying component of T-type calcium current. Recently, it was found that the FRB can be strong or weak in subtypes of DCN neurons containing either Ca(v)3.1 or Ca(v)3.3 subunits mediating T-type calcium current (Molineux *et al.*, 2006). In addition previous work has also indicated that hyperpolarization-activated cyclic nucleotide gated (HCN) channels can contribute to the early rebound component (Aizenman & Linden, 1999), but the significance of this contribution remains unexplored.

In addition to the FRB, early studies also showed a longer lasting prolonged rebound period (PRP) following hyperpolarizing stimuli (Jahnsen, 1986a; b; Llinas & Muhlethaler, 1988), possibly due to a persistent sodium plateau potential also identified by these authors. This PRP could last several seconds and contribute more spikes to the overall rebound than the initial FRB. However, the detailed properties of the PRP and the dependence on stimulus parameters have not been carefully examined to date.

In the present study we provide a detailed characterization of the dependence of FRB and PRP DCN rebounds on the depth and duration of preceding hyperpolarization, and provide evidence for an underlying persistent Na conductance in the case of PRP rebounds. This characterization is important to better understand what the stimulus conditions in vivo required to trigger rebounds would be, an issue about which a rigorous debate has recently ensued (Alvina *et al.*, 2008; Tadayonnejad *et al.*, 2009; Pedroarena, 2010).

Materials and Methods

Whole Cell Preparation

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) aged 14-19 days were anesthetized with halothane and decapitated using a small animal guillotine. The cerebellum was immediately removed and immersed in ice-cold ACSF containing (in mM): NaCl 124, KCl 3, KH₂PO₄ 1.2, NaHCO₃ 26, CaCl₂ 2, MgSO₄ 1.9, and glucose 20, bubbled with 95% oxygen + 5% CO₂ to maintain a pH of 7.4. Parasagittal slices were cut with a thickness of 300 μ m using a vibratome (Slicer HR2, Sigmund electronics) and placed upon a nylon mesh under ACSF where they incubated at 32° C for at least one hour prior to recording. All procedures used were approved by the Emory University IACUC and conform to the NIH Guide for the Care and Use of Laboratory Animals.

Electrophysiology

Whole cell recordings from DCN neurons were obtained using either an Axoclamp 2B in bridge mode for current clamp experiments or a Multiclamp 700B for voltage clamp experiments (Axon Instruments). Recording electrodes were pulled from 1.5 mm glass pipettes and coated with Sylgard (#184, Dow-Corning, Midland, MI) to reduce their

capacitance. Electrodes were filled with intracellular solution containing (in mM): K-gluconate 140, HEPES 10, NaCl 6, MgCl₂ 2, EGTA 0.2, NaATP 4, NaGTP 0.4, spermine 0.05, and glutathione 5. Final electrode resistances ranged from 4 to 10 MΩ. Prior to whole-cell recording, pipette capacitance and electrode resistance was electronically neutralized and the pipette voltage offset was nullified. We did not subtract a junction potential from recorded voltages, which for our intracellular solution containing 140 mM K-gluconate is calculated with JPCalc (Barry, 1994) to be 10 mV. Therefore, all shown voltage traces are likely to be 10 mV more depolarized than the actual membrane potential. Recordings were rejected if the voltage drifted more than 5 mV or the spike overshoot amplitude was less than 10 mV. Initial recordings to characterize rebound properties were taken at 32° C, whereas voltage clamp and dynamic clamp recordings were obtained at room temperature (approx. 25° C) to improve voltage clamp quality and recording stability over multiple drug applications. Synaptic activity was blocked in all experiments using the broad-spectrum ionotropic glutamate receptor blocker kynurenic acid (1 mM) and the GABA-A receptor blocker picrotoxin (40 μM). Neurons were visualized using an Olympus 60x water immersion lens and DIC-IR optics. Images were captured by a video camera (Dage IR-1000, Dage-MTI, Michigan) mounted on a 4x phototube and displayed on a monitor. Glutamatergic projection neurons of the DCN were distinguished from GABAergic neurons based upon soma size and the presence of a fast after-hyperpolarization potential (Uusisaari *et al.*, 2007). The data pool consisted only of neurons that were greater than 15 μm in diameter and also displayed a noticeable fast after-hyperpolarization consistent with the properties of glutamatergic projection neurons. Nevertheless, some heterogeneity in the recorded pool is likely since multiple morphologically distinguishable cell types exist in the DCN (Chan-Palay, 1977), and previous work has shown heterogeneity in ion channel composition and rebound behavior even for large neurons in the same nucleus (Molineux *et al.*, 2008). Because the boundary of nuclear subregions is hard to verify in fresh slices and histological post-processing was not performed, we did not identify the nuclear region from which individual recordings were obtained. Data were acquired at a 10 kHz sampling rate using acquisition software that was developed in-house using LabView (National Instruments, Austin, TX) and subsequently were analyzed using custom written Matlab scripts and Matlab statistical routines (Mathworks, Natick, MA). A confidence limit of $P < 0.05$ was used to judge significance for all statistical tests used.

Dynamic Clamp application of persistent sodium current

The dynamic clamp technique (Robinson & Kawai, 1993; Sharp *et al.*, 1993) is a real-time application allowing one to add to a neuron any artificial membrane conductance at the site of recording, usually the soma. We implemented dynamic clamping in Real-Time LabView (National Instruments) with an update frequency of 10 kHz to monitor ongoing changes in membrane potential in computing driving forces and voltage dependent Hodgkin and Huxley gating variables. In particular we implemented a dynamic clamp model of persistent sodium current (NaP) to allow the addition or subtraction of this current to determine its effects on rebound spiking. Our specification of NaP is given by the following set of Hodgkin-Huxley equations:

$$I_{NaP} = \bar{g}_{NaP} m^3 h (E_{Na} - V_m)$$

with $dm / dt = (m_{\infty} - m) / \tau_m$ and $dh / dt = (h_{\infty} - h) / \tau_h$. The steady state values for m and h are

$$\text{written as } m_{\infty} = \frac{1}{1 + e^{-(V - V_{m1/2})/K_m}} \text{ and } h_{\infty} = \frac{1}{1 + e^{-(V - V_{h1/2})/K_h}} \text{ with } K_m = 4.1 \text{ mV } K_h = 5 \text{ mV.}$$

The time constants $\tau_h = 1$ sec and $\tau_m = 0.3$ milliseconds and half-activation values $V_{m1/2} = -55$ mV and $V_{h1/2} = -65$ mV were tuned to the values that allowed for the best match to the kinetics of the presumptive endogenous persistent sodium current. After these optimum kinetic values were found during the course of several early experiments, they were subsequently left unchanged and only $\bar{g}_{NaP}$ was varied as a free parameter during the course of individual experiments. Although we adjusted the kinetics of our NaP conductance to fit the observed time course of our prolonged rebound, the final properties were closely aligned to previously published properties of this conductance, including slow inactivation (Fleidervish & Gutnick, 1996).

Analysis of rebound spiking

Fast rebound bursts (FRB) and prolonged rebound periods (PRP) were separated and scored using customized Matlab scripts. The presence of an FRB in a given neuron was determined by analyzing the response to the largest negative current injection. FRBs were identified if the onset of a post-hyperpolarization response showed an instantaneous firing rate greater than 1.5 standard deviations faster than the average PRP firing rate for the cell. This allowed us to score FRBs that corresponded to weak bursters (Molineux *et al.*, 2008), which do not show a clear bimodality in spike rate in our hands but do show a distinct initial fast component in the rebound that does not occur in pure PRP responses. While the cutoff of 1.5 SD clearly presents a heuristic method, it corresponded well with a visual classification of distinct response components. The FRB rate was then calculated as the reciprocal of the first interspike interval within the burst. Using the value of the first interval only for burst rate calculation allowed us to compare FRB intensity for bursts with variable numbers of spikes. A diminishing number of burst spikes generally occurred when the level of negative current injection was reduced. The first interval in the FRB was typically the shortest burst interval thus yielding a maximal burst rate estimate. The feature of increasing burst interval duration is typical for rebounds riding on a T-type calcium low threshold spike (Jahnsen & Llinás, 1984).

The magnitude of the PRP was quantified as the maximum firing rate of the rebound discharge after skipping the preceding temporal components of the rebound dominated by T-type Ca and HCN currents (Muri & Knopfel, 1994; Aizenman *et al.*, 1998; Molineux *et al.*, 2006). Through visual inspection of rebound behavior of recorded neurons we determined that 300 ms after the offset of negative current injection early rebound components had terminated (also see Figure 1). The firing frequency of the PRP was determined as the instantaneous firing frequency at 300 ms post-stimulus time. This frequency was obtained from the first 10 spikes following this time point as the intercept of a linear least squares fit of firing frequency (see Figure 1). We avoided using a single ISI to score PRP frequency at 300 ms as this measure was quite variable. Finally, the spontaneous pre-stimulus firing rate was averaged and subtracted from the calculated value PRP firing frequency to give a measure of the net increase in the PRP frequency over the spontaneous rate. It should be noted that by tying the PRP frequency estimates to a the 300 ms post-stimulus time point we excluded possible higher PRP frequencies occurring earlier but may be overlapping with FRB components.

Results

Multiple components of DCN neuron rebound behavior

Recordings of 67 cells were obtained from 41 rats. All neurons showed some form of fast and/or prolonged rebound components following negative current injection pulses. Different temporal rebound patterns were generated depending on the strength of each component (Fig. 1). The initial fast rebound burst (FRB) of spikes occurred at a variable latency (Fig. 1

insets) and was followed by a brief pause in a subpopulation of cases (42%). All neurons also showed a prolonged period of increased spike rate (PRP) following a hyperpolarizing stimulus of sufficient amplitude and duration (Fig. 1).

Different rebound properties show distinct relations to stimulus amplitude and duration

The duration and amplitude of hyperpolarization necessary to express rebound behavior are important criteria to better understand how inhibitory input conditions in vivo might trigger rebounds. Therefore we characterized the dependence of each rebound component on stimulus amplitude and duration in 18 neurons (Figs. 2-3). The rate of the initial fast rebound burst (FRB) was calculated as the inverse of the first burst interval as described in the methods section. FRBs were typically 2 to 8 spikes in length and their frequency can be seen to rapidly saturate as stimulus duration increased beyond 250 milliseconds and stimulus magnitude increased beyond 150 pA (Figs 2A, 3A, $n = 10$ cells, $P < 0.010$ for all group comparisons, student's two-tailed t-test). Some cells showed clear FRBs only at stimulus intensities above 250 pA (e.g. bottom panel green line Fig. 2A), which was primarily due to their low effective input resistance and small voltage responses with current injection (Supp. Fig. 1). The onset latency of the rebound was also significantly dependent on stimulus strength and duration (Figs. 2C, 2B, $P < 0.010$ for all group comparisons, student's two-tailed t-test). This latency was calculated as the time between the offset of the hyperpolarizing pulse and the first spike of the rebound. In comparison to FRB frequency, rebound latency had a more graded dependency on stimulus amplitude and duration and showed incremental decreases for input durations up to ~1s and input amplitudes up to ~400 pA. This finding suggests that the mechanism underlying rebound latency is not just tied to the strength of the following FRB because the latter saturated at much lower stimulus amplitudes and durations than the decrease in onset latency.

The magnitude of the prolonged rebound period (PRP) also significantly increased with both pulse duration and pulse magnitude (Figs. 2B, 3C, $P < 0.010$ for all group comparisons, student's two-tailed t-test). It increased gradually until it saturated for stimuli at ~1s duration and ~400 pA amplitude (Fig. 2B). It therefore showed a similar stimulus dependence as the onset latency, however, as we will show below, the ionic bases of latency and PRP differ.

The expression of each rebound property was quite variable between neurons but the dependence on stimulus amplitude and duration was consistent for each component regardless of its strength (Fig. 2). A potential confounding factor in the analysis shown is that neurons with different input resistances would yield different voltage deflections for the same stimulus amplitude. In fact, for one group of neurons the FRB frequency at -200 pA current injection for 1 s showed a near linear dependence on effective input resistance (Supp. Fig. 2). In this group FRB frequency was lower for high-input resistance neurons. Therefore, this relationship was the opposite from what would be expected if a deeper level of hyperpolarization reached during a negative current injection (high input resistance) resulted in a stronger FRB. A second group of neurons showed a much weaker FRB and was restricted to neurons with an intermediate input resistance (Supp. Fig. 2). This group most likely corresponds to the weak bursters previously identified (Molineux *et al.*, 2008). The PRP frequencies showed no clear relationship with effective input resistance, suggesting that they were primarily determined by a highly variable amplitude of underlying rebound conductance. For a given neuron the PRP frequency elicited with a fixed amplitude stimulus was quite stable (Supp. Fig. 3), and neurons with strong or weak PRPs did or did not exhibit an FRB. Further, a scatter plot of FRB and PRP frequencies against each other at a fixed stimulus size did not reveal any clear relationship (Supp. Fig. 4). Thus, each neuron could be tuned to respond with a specific rebound pattern to a strong hyperpolarizing stimulus due to the presence of separate FRB and PRP rebound conductances.

HCN current modulated rebound latency, but was not required for FRB or PRP rebound components

The HCN conductance is a plausible candidate for a current underlying rebound spiking in DCN neurons as HCN activation during hyperpolarization results in a depolarizing transient at subthreshold potentials following the offset of hyperpolarizing current pulses (Aizenman & Linden, 1999). The presence of HCN current in DCN neurons was readily apparent from the disappearance of a “sag” potential during hyperpolarization following HCN block with cesium or ZD7288 (Fig. 4A). HCN current activates at hyperpolarized potentials with time constants of tens to several hundred ms in different neurons, and deactivates with similar time constants following the offset of a hyperpolarizing stimulus (Pape, 1996). Thus remaining HCN activation could potentially provide a depolarization sufficient to drive a rebound spiking. However, block of the HCN current revealed that HCN block did not remove FRB or PRP rebound components ($n = 10$ cells, examples in Fig. 4A).

While HCN block did not remove FRB and PRP rebound components, it did have a noticeable effect upon the latency to rebound firing following stimulus offset (Fig. 4A). This effect is quantified in Fig. 4B1, showing a significant increase in rebound delay after HCN block ($P < 0.01$ for each of 6 neurons analyzed, student's two-tailed t-test). The average increase in delay was from 33 to 334 ms, indicating that the inward current associated with HCN at hyperpolarized potentials was a major factor in controlling rebound latency. In fact, when the amount of sag (Fig. 4B2) is taken as an indicator of HCN current amplitude, we found a significant linear correlation (using Matlab `corrcoef` function) between HCN expression and rebound latency leading to faster rebound onsets in the presence of increased HCN conductance (Fig. 4C, $r^2 = 0.77$, $P = 0.0020$, $n = 9$ cells). In addition, blocking HCN could lead to unstable firing behavior following stimulus offset that led to variable post-stimulus behaviors including repeated fast bursts (Fig. 4A2). This reduction of stability suggests that HCN was partly activated at rest and because the reversal potential of HCN current is close to the average membrane potential in DCN neuron during pacemaking, HCN activation would lead both to the reduction of burst-induced hyperpolarization (which can trigger repeated bursting) and decreased rebound intensity due to shunting of rebound currents with a reversal potential positive to HCN.

Calcium current or SK current block induced bistability and enhanced prolonged rebound spiking

Slowly inactivating plateau currents can be due to calcium channel current, as for example is present in the dendrites of cerebellar Purkinje cells (Llinas & Sugimori, 1980). We applied the non-specific calcium channel blocker cadmium ($125 \mu\text{M}$) to examine the possible dependence of the PRP in DCN neurons on calcium conductances. Instead of blocking PRP, however, cadmium consistently led to an enhanced PRP frequency in DCN neurons (Fig. 5A, $n = 6$ cells). In 3/6 cells examined cadmium caused bursting or bistable firing dynamics even in the absence of stimulus pulses (Fig. 5B-C). A likely explanation of the enhanced PRP and spontaneous bistability in the presence of cadmium is the loss of activation of the calcium-gated SK potassium current. Block of SK current with apamin removes a slow AHP from DCN neurons and induces bursting (Jahnsen, 1986b; Aizenman & Linden, 1999; Raman *et al.*, 2000). When we compare the effects of apamin (10 nM) with cadmium a similar induction of periods of high frequency firing (Fig. 5B) and bistable membrane potential distribution (Fig. 5C) was found. Apamin bursts tended to terminate earlier than cadmium induced plateau firing (Fig. 5B), however, suggesting that the offset of apamin elicited bursting may be due to the activation of other calcium dependent outward currents which are not present after block with cadmium. Overall, these findings indicate that calcium current presents an important regulator of DCN neuron excitability but is not responsible for the PRP following hyperpolarizing stimuli.

The prolonged rebound was sodium conductance dependent and reflected the kinetics of persistent sodium current

Early work on rebound depolarization in DCN neurons suggested that a persistent sodium current may underlie the prolonged rebound component (Llinas & Muhlethaler, 1988). This hypothesis has not been specifically addressed in subsequent studies on DCN rebound behavior, however. Therefore we placed a particular focus in our present study on this least explored component of DCN rebounds. First, we determined that the application of TTX (1 μ M) in addition to cesium (5 mM) not only abolished PRP spiking but also blocked any underlying prolonged depolarization following stimulus offset while a brief depolarization corresponding to the FRB remained (Fig. 6A, $n = 6$ cells). We then attempted to use riluzole (20 μ M), which has been shown to specifically block persistent sodium current in cortical neurons (Urbani & Belluzzi, 2000). However, in our hands the effect of riluzole in DCN neurons was not specific to persistent sodium current as it blocked both fast and slow components of sodium channel activation similarly to small doses of TTX.

In the absence of an available specific NaP blocker we used a voltage clamp protocol and TTX subtraction to look for the presence of a prolonged rebound current based on sodium conductance. In order to achieve a natural activation profile reflecting rebound properties of NaP conductance in voltage clamp we used previously recorded current-clamp traces from the same neurons as voltage-clamp command waveforms (Fig. 6B). When we subtracted post-TTX from pre-TTX current traces using these voltage-clamp waveforms we indeed found a slowly inactivating sodium current component following the offset of hyperpolarization (Fig. 6C). The trajectory of this current matched the period of increased spiking frequency of the PRP in the control current clamp trace of the same neurons (Fig. 6B,C). Note that large spike currents were truncated in this figure in order to highlight the smaller persistent current between spikes. Voltage clamping was carried out in the presence of apamin to reduce the effects of space-clamp errors in the voltage clamp currents. This was necessary as we found that without the use of Apamin, the endogenous action potential current activated SK conductance to a greater degree than the action potential waveforms applied in voltage clamp mode in the presence of TTX. This difference led to a mismatch of apamin sensitive current in the pre and post-TTX subtracted trials, obscuring I_{NaP} . To obtain an estimate of the level of persistent Na current in each neuron during the PRP we averaged the TTX subtracted inward current in the first second following the offset of hyperpolarization (Fig. 6D). This revealed average persistent Na current amplitudes between 14 and 36 pA. Note that these values are smaller than the maximal NaP current directly following current injection offset. Overall, these voltage-clamp data indicate that NaP current activation fits the profile required for supporting the PRP in our current clamp data. To address this question directly, we resorted to applying simulated NaP current via dynamic clamp to assess whether the measured current was sufficient to account for the observed spike rate increases.

Dynamic clamp application of artificial persistent Na current could cancel or enhance endogenous PRP spiking

Dynamic clamping is a specialized stimulation technique that allows for the real-time injection of time and voltage dependent currents that mimic the biophysical properties of ion channels or synapses (Robinson & Kawai, 1993; Sharp *et al.*, 1993). In the present study, we used this tool to apply artificial NaP current to DCN neurons to assess the likely role of NaP kinetics to shape rebound profiles. The kinetics of our artificial NaP current was modeled using the Hodgkin-Huxley formalism (see Methods for details) and voltage-dependent terms were updated with the recorded V_m at a refresh rate of 10 KHz. The kinetics of our model were closely aligned to established kinetics of NaP (Fleiderovich & Gutnick, 1996), however, we treated NaP as a separate conductance from fast inactivating Na current although in

reality NaP is likely to be a special gating mode of the same channel population (Alzheimer *et al.*, 1993; Brown *et al.*, 1994). Activation and inactivation half-voltages and time constants were optimized so that the rebound profiles resulting from applied NaP in current clamp matched that of the endogenous PRP. In particular, we tuned these parameters so that application of negative NaP conductance (inverted applied current) would cancel the endogenous PRP spiking. The best values found were $V_{m1/2} = -55$ mV, $V_{h1/2} = -65$ mV, $\tau_h = 1$ s, $\tau_m = 0.3$ ms. As we did not correct for junction potentials in our recordings, 10 mV should be subtracted from the half-voltages to compare with values in the literature (see Discussion). A representative neuron with added or subtracted artificial NaP is shown in Fig. 7A. The dynamic clamp current almost completely removed the PRP spiking of the control trace (Fig. 7A, middle) when an inverse current with a conductance $\bar{g}_{NaP} - 0.83$ nS was applied. Conversely, the PRP spiking was increased while retaining its time course when a $\bar{g}_{NaP} = +0.3$ nS was added. Thus, application of positive and negative artificial somatic I_{NaP} via dynamic clamp demonstrated that the kinetics of our artificial NaP conductance were suitable to mimic the current underlying the endogenous PRP spiking. The matched conductance parameters therefore represent an estimate of the kinetics of the endogenous PRP conductance.

To determine how the PRP frequency related to the amount of NaP conductance present, we varied applied $\bar{g}_{NaP}$ over a range of values for a set of neurons ($n = 6$) and calculated the resulting PRP spike frequencies as described above (Fig. 2). For each neuron, the PRP frequency scaled approximately linearly with $\bar{g}_{NaP}$ (Fig. 7B) and in most cases the PRP could be completely removed by applying an inverted I_{NaP} . The amount of $\bar{g}_{NaP}$ necessary to remove PRP spiking and return the neuron close to baseline pacemaking was calculated from the intercept at 0 PRP frequency of a linear fit for the PRP - $\bar{g}_{NaP}$ relationship (Fig. 7B). The values of $\bar{g}_{NaP}$ canceling endogenous PRPs ranged from -0.6 to -2.3 nS (Fig. 7C).

To serve as a valid prediction of endogenous PRP rebound conductance, the effects of applied $\bar{g}_{NaP}$ should also replicate the relationships found between stimulus amplitude and duration vs. PRP frequency (Figs. 2,3). Increases in PRP frequency with stimulus duration and amplitude reflect the increased deinactivation of the rebound conductance during the stimulus and the subsequent proportion of available channels for activation at stimulus offset. Because the PRP frequency varied nearly linearly with the amount of NaP conductance present (Fig. 7B) we could test the validity of artificial $\bar{g}_{NaP}$ inactivation and activation kinetics by examining the proportion of deinactivated channels of our simulated NaP conductance following different stimulus conditions. To perform this analysis, we computed the fraction of open NaP conductance ($m^3 \cdot h$) for different stimulus conditions by using the voltage trajectory of recorded neurons as input into the HH equations. An example of how the voltage trajectories of one neuron resulted in increasing p_{open} g_{NaP} with increasing durations of preceding hyperpolarizing current injection is shown in Fig. 8A,B. In a second step, the computed fractional conductance at 300 ms after stimulus offset was taken to estimate the expected effect of NaP on PRP frequency for each neuron. This analysis demonstrated a good match between fractional conductance as a function of stimulus amplitude and duration (Fig. 8C) and stimulus dependence of PRP frequency (Fig. 2B). One interesting aspect of this analysis was that it revealed a strong NaP activation immediately after stimulus offset (Fig. 8B). This effect is based on the fast activation properties of NaP (Baker & Bostock, 1997), and leads to the prediction that the FRB component of DCN rebounds contains a contribution from I_{NaP} as well as T-type calcium current. However, as the NaP conductance is relatively small, the FRB is likely dominated by the calcium current and evoked calcium-dependent potassium current.

Discussion

In order to better understand under which conditions DCN neurons may exhibit rebound firing we parameterized the time course and strength of rebound as a function of the preceding duration and depth of hyperpolarization. We found that DCN rebounds contain two distinct components that exhibit different time courses and a voltage-dependent activation profile. Notably, a short fast rebound we termed FRB required less time and depth of preceding hyperpolarization than a subsequent longer lasting prolonged rebound period we termed PRP. We find that the FRB and PRP depend on the de-inactivation profile of T-type Ca and persistent Na current, respectively, and could be expressed independently of each other in different cells. Further, HCN current activation made an important contribution to the onset timing of rebound activity but not rebound profile. Our findings give a new level of detail to the well established presence of strong rebound behavior in DCN neurons (Jahnsen, 1986a; Llinas & Muhlethaler, 1988; Aizenman & Linden, 1999). While the dependence of the FRB component on T-type (Cav3.1 and Cav3.2) channels has been well established (Llinas & Muhlethaler, 1988; Aizenman & Linden, 1999; Molineux *et al.*, 2006), the PRP has previously received much less attention and only a brief mention of possible persistent Na current dependence has been made (Jahnsen, 1986a). Nevertheless, we find that the PRP adds many more spikes to a DCN neuron baseline activity than the FRB does, and its prolonged time course could have important effects on driving muscle activity beyond the duration of a stimulus.

Although a synaptic activation of rebound burst with inhibitory inputs in DCN neurons has been demonstrated (Llinas & Muhlethaler, 1988; Aizenman & Linden, 1999), a recent study questioned whether physiological inhibitory input *in vivo* is strong enough to elicit rebounds in most DCN neurons (Alvina *et al.*, 2008). This issue is under debate, as other authors found that strong inhibitory input *in vivo* creates a sufficient condition for rebound firing (Tadayonnejad *et al.*, 2009; Pedroarena, 2010) and others have found a role for rebound firing in eyeblink conditioning (Hesslow, 1994). While we did not directly address the question of synaptic activation of DCN rebounds, through using a systematic set of hyperpolarizing current injections of different amplitudes and durations we could delimit the conditions expected to result in rebound behavior. Importantly, we noted that rebounds are not all-or-none, but that inhibition of different intensity can result in incrementally stronger rebounds. In particular, for the FRB we found that a hyperpolarization level below -70 mV and a deinactivation period of approximately 150 ms at this level are needed to achieve full rebound strength, which is in good agreement with values previously described for T-type current driven rebounds in thalamic neurons (Jahnsen & Llinás, 1984). This voltage dependent regulation of FRB strength is superposed on the expression of weak FRB behavior via Cav3.2 channels or a strong FRB behavior via Cav3.1 channels (Molineux *et al.*, 2006; Molineux *et al.*, 2008). In addition we found that the FRB onset time after the stimulus was highly correlated with the amount of HCN current present in each neuron as judged by the sag amplitude during hyperpolarization, and HCN block led to a highly significant increase in rebound delay. This finding indicates that HCN current does not directly drive rebound spiking, but has an important influence on rebound timing and its activation during hyperpolarization is necessary to allow rebound events to occur with short delays after strong inhibitory events.

Important role of persistent Na current in prolonged rebound activity in DCN neurons

We found that the stimulus dependence of the PRP is different than that of the FRB in that it has a longer deinactivation period of about 1 second and also requires more hyperpolarized potentials for full deinactivation. Therefore the stimulus conditions *in vivo* that may trigger a strong PRP rebound component require deeper and longer-lasting hyperpolarization than those triggering a strong FRB. Previous work has described a slowly inactivating Na current

in DCN neurons (Gardette *et al.*, 1985), which seemed the best candidate to us to generate the depolarization underlying PRP spiking. From voltage clamp experiments in other neurons persistent Na current (NaP) has been shown to activate with sub-millisecond speed (Baker & Bostock, 1997) at potentials about 15 mV more negative than the fast Na current (i.e. -70 to -60 mV) and inactivate slowly with a time constant of 2 s (at 32° C) in some cell types (Fleidervish & Gutnick, 1996) though not others (Kay *et al.*, 1998). Removal of inactivation –if present– starts to occur at -70 mV with a time constant of 2.3 s and speeds up to a time constant of 1 s at -90 mV (Fleidervish & Gutnick, 1996). These values are fully compatible with the stimulus dependency and activation range we used for the conditions triggering PRP spiking in DCN neurons. Our own voltage clamp experiments also revealed a TTX sensitive persistent component activating and inactivating with the described properties of NaP in other neurons. Taken together, this evidence indicates that NaP is likely to be the major factor in triggering our observed slow PRPs. With dynamic clamp application of an artificial NaP conductance we could indeed verify that the kinetics of this conductance were well suited to explain the time course and amplitude of observed PRPs.

Rebound spiking in DCN neurons may be more complex than described by a simple 2 stage model of T-type and NaP activation during FRB and PRP components. First, our data indicate that NaP activation already occurs early in the rebound period and thus is likely to contribute to the FRB as well. Though easily overshadowed by a strong Ca(v)3.1 driven low-threshold calcium spike and ensuing calcium dependent potassium current, the FRB of DCN neurons that show only a weak T-type current carried by Ca(v)3.3 channels (Molineux *et al.*, 2006; Molineux *et al.*, 2008) may be significantly influenced by NaP activation as well, especially following prolonged periods of hyperpolarization. In addition, rebound spiking driven by electrical stimulation of inhibitory inputs (Aizenman & Linden, 1999) could include metabotropic glutamate receptor activation. Burst stimulation of excitatory inputs even during block of ionotropic receptors or in voltage clamp can lead to substantial inward currents through mGluR group 1 dependent activation of non-specific cation channels (Batchelor *et al.*, 1994; Guerineau *et al.*, 1995). The modulation of such slow currents through synaptic activity could play a significant role in modulating or strengthening rebounds in vivo and should be more closely examined in the DCN in future studies.

Heterogeneity of rebound activity in DCN

Our analysis was restricted to neurons with soma sizes $> 15 \mu\text{m}$ and the presence of a fast AHP, properties which previously have been associated with the subtype of glutamatergic projection neurons in the DCN (Aizenman *et al.*, 2003; Uusisaari *et al.*, 2007). However, previous work comparing large with small (Czubayko *et al.*, 2001; Aizenman *et al.*, 2003) or identified GABA $^{-}$ with GABA $^{+}$ (Uusisaari *et al.*, 2007) neurons have found robust expression of rebounds in all subtypes of DCN neurons. In fact, GABA $^{+}$ neurons showed a trend for longer rebound activity (Uusisaari *et al.*, 2007), suggesting that NaP currents in this cell type may be expressed more robustly. The FRB rebound component has been found to be weak or strong within two subtypes of large glutamatergic projection neurons based on the differential expression of Ca(v)3.3 or Ca(v)3.1 channels (Molineux *et al.*, 2006; Molineux *et al.*, 2008), whereas all GABA $^{+}$ neurons showed fast Ca(v)3.1 dependent bursts. Within our own data pool of putative glutamatergic projection neurons we also found strong and weak FRB components, and in addition a highly variable level of PRP component as well. The strength of the FRB and PRP rebound components was not correlated, and thus different neurons can show a wide variety of rebound patterns for the same stimulus. It will be important to determine in future work whether this variability in rebound properties is the result of excitability plasticity that could adapt the rebound of individual neurons to specific learning histories. Recent work has indicated that plasticity rules in DCN neurons are quite

complex, and include the enhancement of synaptic inward currents depending on a period of strong inhibition followed by rebound activity (Pugh & Raman, 2006; Pugh & Raman, 2008). If similar stimulus conditions also modulated intrinsic rebound currents, the variable rebound activity found could indeed be the result of different activation histories of recorded neurons.

Functional significance for rebound activity in DCN

The role of rebound properties in DCN neurons in the control of cerebellar output in behaving animals remains unclear at this point. Recent evidence in anesthetized animals suggests that rebounds are not readily elicited with electrical stimulation of inhibitory inputs (Alvina *et al.*, 2008). However, only 10 pulses at 100 Hz of input stimulation were applied in this study, and the resulting 0.1 s duration of inhibition is shorter than the time required for full rebound conductance deactivation shown by our results. In addition, the depth of hyperpolarization elicited with inhibitory inputs is limited by the reversal potential of chloride, which in DCN neurons may be around -75 mV for sharp intracellular recordings in whole brains (Llinas & Muhlethaler, 1988). Nevertheless, a recent study showed that strong inhibitory synaptic stimulation could result in robust rebounds (Tadayonnejad *et al.*, 2009) and previous experimental evidence suggests the involvement of DCN rebound firing in the control of eyeblink conditioning (Hesslow, 1994). It is also quite possible that the reversal potential of chloride *in vivo* is modulated and not uniform across neurons, which would allow a subpopulation of neurons to exhibit stronger rebounds. While rebound-specific excitability plasticity has not been described to date, synaptic plasticity rules strengthening excitatory input in the DCN have been found to be triggered by periods of hyperpolarization that can cause rebounds (Pugh & Raman, 2006; Pugh & Raman, 2008). Rebounds based on previous plasticity events could occur in specific situations when DCN neurons have acquired particular behavioral contingencies such as delayed eyeblink conditioning (Wetmore *et al.*, 2008). Rebound spiking could also be triggered by specific sensory stimuli, as for example in anesthetized animals DCN neurons frequently show periods of excitation following pronounced inhibitory periods after air-puffs to the face (Rowland & Jaeger, 2005). However, clearly not all excitatory responses of DCN neurons during behavior are due to rebounds. Strong increases in DCN activity in monkeys executing reaching movement for example occur in the absence of preceding inhibition (Gibson *et al.*, 1996). Thus, rebounds probably represent a specialized response property of DCN neurons in specific situations that yet need to be clarified with physiological recordings in behaving animals. It is also quite possible that rebounds occur only in a few trials of repeated behaviors and the commonly used trial-averaged display of neuron activity in studies of DCN activity needs to be augmented by single trial analysis for rebound activity. A specific role for PRP vs. FRB components of rebounds *in vivo* is clearly also not established at this time, though it might be speculated that multiple independent rebound components would allow DCN neurons to act as adjustable temporal pattern generators (Houk, 1987; Rowland & Jaeger, 2008) that could be involved in controlling phasic and tonic components of motor control, such as reach-grasp combinations (Gibson *et al.*, 1996).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Rebound spiking of three different neurons in response to -200 pA current injection illustrates the observed range of rebound characteristics. The insets above each rebound pointed to by arrows show the initial 300 ms of each rebound at an expanded time scale. In neuron 1, a distinct fast rebound burst (FRB) was followed by a prolonged rebound Period (PRP), in which spiking was still significantly elevated above baseline. Neuron 2 showed only a weak FRB but a similar PRP, while neuron 3 showed a strong initial FRB followed by a pause and a secondary fast burst before a less pronounced PRP ensued. The left axis denotes mV of membrane potential, while the right axis denotes instantaneous firing frequency shown by the grey diamond symbols denoting $1 / \text{ISI}$ in Hz for each ISI beyond 300 ms post-stimulus. As further described in Methods, the PRP frequency was quantified by fitting a linear regression line to the frequency given by the first 10 ISIs occurring 300 ms post-stimulus. The solid line between the diamonds (between 2.3 and 2.6 s on the time axis) denotes this regression and the intercept at 300 ms post stimulus (2.3 s on the time axis) is denoted by a grey star. The PRP frequency increase was calculated from this intercept by subtracting the pre-stimulus spontaneous firing rate.

Figure 2.

Dependence of FRB and PRP rebound components on stimulus duration and amplitude. A) Data are shown for neurons that showed an FRB component for at least the highest stimulation amplitude (11 of 18 neurons). Each line represents the rebound properties of a single neuron. Upper Panel: In most neurons, the FRB reached a maximal frequency when the duration of the preceding hyperpolarizing stimulus (-200 pA amplitude) exceeded 250 ms. Lower Panel: FRB frequency also depended on the amplitude of the stimulus and typically reached a maximum when the preceding stimulus exceeded -150 pA (1s stimulus duration). FRB frequency was calculated using the first ISI of the rebound if an FRB was detected (See Methods). B) The influence of stimulus amplitude and duration for the PRP is shown for all 18 cells. Note the more gradual effect of stimulus duration and amplitude on the PRP than the FRB, suggesting a different underlying voltage dependence of the PRP rebound conductance. C) The latency to first spike following the offset of a hyperpolarizing pulse ($n = 16$ cells) showed a strong increase at short stimulus durations and graded dependence on stimulus amplitudes. Identical colors in different subpanels correspond to the same cells.

Figure 3.

Significance in differences for rebound properties for short vs. long and small vs. large stimuli. Double asterisks denote a P -value of < 0.01 . For determining the effect of stimulus duration the stimulus amplitude was held at -200 pA, and for determining the effect of stimulus amplitude the duration was held at 1 s. A) Fast burst frequency increased by 800% between stimulus durations of 60 ms and 1.5 s for neurons with a full FRB response at -200 pA input. An increase in stimulus amplitude from -50 to -355 pA lead to an FRB increase by 270% for stimuli of 1 s duration. B) Rebound latency was 4 times longer for short stimulus durations and 2 times longer for small stimulus amplitudes. C) The PRP rate increased by an average of 680% as the stimulus duration was increased from 60 ms to 1.5 s and by 250% as hyperpolarizing stimulus magnitude increased from 50 pA to 355 pA.

Figure 4.

HCN current controls latency to rebound spiking. A) Two representative cells are shown before and after HCN block. In neuron 1 (A1) HCN was blocked with cesium (5 mM), in neuron 2 (A2) with ZD7288 (60 μ M). The stimulus current was reduced after HCN block to yield the same membrane potential at the end of the stimulus. This allowed us to examine the delay to rebound offset from the same preceding potential before and after HCN block. Following HCN block, the latency to rebound was strongly increased and a continued burst pattern developed in 2 of 6 cells (example in A2). Note that both the FRB and PRP rebound components remained present when bursting did not occur (A1). B1) For six cells, bar plots compare the rebound latency in control (dark) versus HCN block (light). Neuron 1 corresponds to the same cell shown in A2 and was blocked using ZD7288 while all other cells were blocked using cesium. B2) The same 6 cells showed a pronounced voltage sag during hyperpolarizing stimuli (dark area of bars) in control trials (sag period indicated by dashed lines in A1,A2) that was mostly abolished after HCN block (light area of bars). C) A linear relationship between sag amplitude and rebound latency prior to HCN block shows a significant negative linear correlation ($r^2 = 0.77$, $P=0.0020$, $n = 9$ cells, including the 6 shown in B).

Figure 5.

Calcium Current is not responsible for PRP but controls stability through SK channel activation. A) The nonspecific calcium channel blocker cadmium (125 μ M) resulted in a dramatic increase in PRP frequency indicating that the plateau current underlying it is not calcium dependent ($n = 6$ cells). B) Both apamin and cadmium result in similar loss of firing stability and lead to periods of high frequency firing, even in the absence of current pulse stimulation. C) The probability distribution of membrane potential (after digital removal of action potentials) was unimodal before cadmium or apamin application, but bimodal afterwards. This bimodality indicates the presence of separate up- and down states in the presence of these blockers. The y-axis is normalized so that the area under each probability density curve is 1.

Figure 6.

The PRP is sodium current dependent and reflects the time course of persistent sodium current inactivation. A) In the presence of cesium and TTX a small depolarizing transient with the timing of the FRB remains (Fig. 6A, arrow), but no prolonged rebound depolarization that could trigger the PRP is present ($n = 6$ cells). B) Three current clamp traces are shown from separate cells subjected to a -500 pA current injection pulse. Each trace was subsequently used as a command waveform in voltage clamp mode in the presence of apamin (100 nM) and subsequently in the presence of apamin (100 nM) and TTX (1 μ M). Subtracting post-TTX traces from pre-TTX ones allowed us to investigate the dynamics of sodium current activation with natural rebound spike patterns. C) An example

of TTX sensitive current is shown for the neuron corresponding to the top trace in panel in panel B (starred * trace). A long lasting inward current followed the offset of the hyperpolarized period of the command potential (time course of command potential is depicted by starred * trace in panel B). The voltage-clamp current shown is an average of 10 TTX-subtracted trials. Action potential currents are truncated. Red boxes denote the averaged current during each interspike interval. D) TTX sensitive current was averaged during the first second of the rebound period for each of the neurons shown in B. Star (*) denotes the same neuron as in panel C. Standard error bars denote between-trial variability (n = 10 trials).

Figure 7.

Dynamic clamp application of a persistent sodium current (I_{NaP}) to the soma replicated the endogenous current underlying the PRP. **A**) I_{NaP} conductance was subtracted ($\bar{g}_{NaP}$ value of -0.83 nS, top trace) or added ($\bar{g}_{NaP}$ values of 0.3 nS, bottom trace) with dynamic clamping during current clamp recording of a neuron expressing a typical PRP (middle trace, no applied I_{NaP}). A negative value of $\bar{g}_{NaP}$ means that the additive inverse of the current was injected into the cell to cancel an endogenous I_{NaP} component. In each trace, a 1 s hyperpolarizing current pulse of -300 pA was used to trigger deinactivation of endogenous and applied I_{NaP} . **B**) The PRP frequency (calculated as in Fig 1, see Methods) was positively correlated with the magnitude of applied $\bar{g}_{NaP}$. Each line represents a different neuron ($n = 6$) and error bars depict SEM between 12 repeated trials. Thin blue lines present linear fits to the relation between PRP frequency and applied $\bar{g}_{NaP}$ for each neuron. **C**) Predicted endogenous $\bar{g}_{NaP}$ is plotted as a bar graph for each cell from the data in **B**. Intercepts with zero as the point where the PRP caused by endogenous current was exactly canceled by applied negative $\bar{g}_{NaP}$ were calculated from linear fits for each neuron depicted in **B**.

Figure 8.

The dependence of endogenous PRP frequency on stimulus duration and amplitude is well matched with the kinetics of artificial I_{NaP} . A) Representative traces showing increased PRP frequency of one neuron with increasing stimulus duration. B) Simulation of artificial I_{NaP} time course during the traces shown in A. These traces were calculated by forcing the kinetics of our simulated I_{NaP} to follow the recorded voltage trace and using a $\bar{g}_{NaP}$ value approximating the endogenous value as determined from the intercept in Fig. 7B for this neuron. Asterisks denote the 300 ms post-stimulus time at which PRP frequency was scored (see Methods). C) The fraction of open NaP conductance at the offset of a hyperpolarizing current pulse was calculated from our artificial NaP kinetics for each cell shown in Fig. 2 as a function of stimulus duration and amplitude. Fractional NaP conductance was determined from simulations using the voltage traces of the original recordings as a voltage input to our artificial NaP conductance. The thicker black line denotes the average. A good match between NaP fractional conductance and PRP frequency shown in Fig. 2 was found.