Introduction

Deep cerebellar nuclear (DCN) neurons were among the first to be shown to generate rebound bursts following membrane hyperpolarizations, which are now incorporated into several models of cerebellar function. Rebound bursts are typically evoked in vitro using step membrane hyperpolarizations through direct current injection at the soma, although similar bursts have been reported following synaptic inhibition. We recently examined the properties and ionic basis for rebound burst discharge in DCN cells in vitro in relation to current-evoked membrane hyperpolarizations.4,8 T-type Ca²⁺ channels of the CaV3 family have long been known to participate in rebound bursts in DCN cells. These channels exhibit significant inactivation during the tonic spike activity found at rest in these cells, with membrane hyperpolarizations serving to make CaV3 channels available again for activation upon release from hyperpolarization. We found a striking correlation between the expression patterns of two CaV3 T-type Ca²⁺ channel isoforms and the generation of distinct phenotypes of rebound bursts in large diameter cells. One cell type that expresses CaV3.1 protein generates high frequency “Transient Bursts” of up to 450 Hz, while a second “Weak Burst” group expressing CaV3.3 channels responds to the same magnitude of membrane hyperpolarization with less than 140 Hz rebound frequency. We showed that the two burst phenotypes are shaped by a differential activation of inward T-type Ca²⁺ current and the degree of functional coupling between CaV3 channels and outward Ca²⁺-activated K⁺ channels. Altogether it is clear that specific patterns of ion channel expression and their interactions are key to generating the intrinsic membrane properties that underlie Transient and Weak Burst phenotypes, at least in relation to current-evoked membrane hyperpolarizations.

An important consideration is the extent to which these two phenotypes can be activated by the extensive GABAergic inhibitory synaptic input from Purkinje cells of cerebellar cortex. In this regard, Alvina et al.12 recently used a combination of recordings...
in the in vitro slice preparation and unit recordings in vivo to examine the ability for synaptic inhibitory inputs to evoke rebound bursts. They found that only a small percentage of DCN cells (15%) were able to generate a rebound burst in response to a 10-pulse, 100 Hz stimulus to Purkinje cell axons in vitro, with an even lower rate of occurrence detected in vivo. Based on their data the authors raised questions regarding the likelihood of DCN cell rebound contributing to cerebellar output. This is an important issue in cerebellar physiology and one that has not been substantially addressed. We therefore extended our previous analyses of Transient and Weak Burst cells to determine the probability for detecting rebound increases in firing rate in relation to inhibitory synaptic activation.

Results

Defining rebound bursts. Transient and Weak Burst phenotypes are readily identified in whole-cell recordings using direct current injection. In keeping with the study of Alvina et al., we relied extensively on extracellular recordings to avoid altering cytrosol contents to determine if these phenotypes could be distinguished following synaptic stimulation, but compared them to established properties of whole-cell recordings. There are two important considerations when examining rebound burst generation, in particular when using extracellular recordings and synaptic stimulation.

First, it is necessary to establish criteria that will objectively identify whether a cell has generated a rebound burst beyond the level of preceding tonic activity. Investigations to date have relied on relatively subjective measures, such as the presence of a transient burst component or a predefined increase in firing rate over the pre-stimulus baseline. To establish a statistical measure we defined rebound bursts according to an increase in post-stimulus firing rate exceeding twice the standard deviation of the tonic firing rate for 1 sec preceding the hyperpolarization. Because DCN cells fire tonically with great regularity, this test allows us to observe even small increases in firing rate over time. Using these criteria the increases in firing frequency were much longer (up to 3 secs) than the initial 100 ms we previously used to define burst phenotypes, but not significantly different between Transient and Weak Burst cells (Fig. 1A and Table 1). However, the maximum rebound frequency still occurred within -100 ms following hyperpolarizations and differed as predicted for Transient vs Weak Burst cells (Fig. 1A). A similar long time frame for rebound increases in frequency was reported by Usisari et al., who referred to this extended time in terms of the rate of spike accommodation. We chose to include the entire duration of the elevated period of firing as part of the rebound burst response.

Second, it is necessary to establish criteria for setting stimulus intensity to compare between recording modes and single cells recorded in different slice preparations. Unless otherwise indicated, stimulus intensity was set to a level ~60–70% of that required to evoke a maximal IPSC during whole-cell recordings. Using this parameter we found equivalent IPSCs for Transient and Weak Burst cells, evoked with amplitudes of 221 ± 27.4 pA (n = 5) for Transient Burst cells and 213 ± 34.2 pA (n = 7) for Weak Burst cells. For unit recordings stimulus intensity was increased until no further increase in rebound frequency was observed, and then intensity reduced to ~60–70% of the maximum value. To compare this intensity to that used for whole-cell recordings we obtained a series of sequential on-cell followed by whole-cell recordings, and found that this intensity criteria evoked a comparable IPSC of 214 ± 27 pA (n = 12 combined Transient and Weak Burst recordings).

To further ensure that the activity of DCN cells was similar between on-cell recording and whole-cell recording modes we compared several baseline firing characteristics. We found that
Table 1  Rebound burst properties in whole-cell and on-cell recordings of large diameter DCN neurons

<table>
<thead>
<tr>
<th></th>
<th>Tonic Freq.</th>
<th>Transient Burst</th>
<th>Weak Burst</th>
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<td></td>
<td>(Hz)</td>
<td>Rebound Freq.</td>
<td>Rebound Duration (s)</td>
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<tr>
<td>On-cell (aCSF)</td>
<td>17.8 ± 3.02 (16)</td>
<td>73.8 ± 9.58</td>
<td>2.5 ± 0.32</td>
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<tr>
<td>On-cell (K-Gluc.)</td>
<td>18.4 ± 3.13 (5)</td>
<td>69.7 ± 8.83</td>
<td>2.2 ± 0.45</td>
</tr>
<tr>
<td>Whole-cell (K-Gluc.)</td>
<td>17.6 ± 2.16 (5)</td>
<td>58.6 ± 7.83</td>
<td>2.3 ± 0.47</td>
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Mean values of spike output under resting conditions and during rebound bursts following a 100 Hz, 10 stimuli train of Purkinje cell inputs (60–70% of maximal intensity). Rebound burst frequencies reflect the maximum frequency attained beyond baseline tonic firing rates and rebound duration the time required for spike firing to fall back within two standard deviations of the mean of baseline tonic firing. No significant differences were detected between on-cell or whole-cell recordings for tonic or rebound burst properties. Whole-cell recordings were made without applied current. aCSF, artificial cerebrospinal fluid, K-Gluc., potassium gluconate. n values are shown in parentheses.
Reliability of rebound bursts in deep cerebellum

As stimulus intensity was increased, the ability to evoke rebound bursts of higher frequency increased in both transient and weak burst cells. IPSP amplitude is correlated to FSL. It became apparent that the membrane potential during repetitive stimulation at 100 Hz for different stimulus intensities appeared to stabilize (presumed ECl) relatively early on during the stimulus train (Fig. 2A). This could provide the impression that the net inhibition evoked by the different intensities used here was fairly similar, or even that additional factors beyond GABA receptor activation might contribute to generating rebound responses. However, we confirmed that activating synaptic inputs (mossy fiber/climbing fiber) in the presence of picrotoxin to block Cl- channels (see Materials and Methods) produced no rebound increase in firing following a 10 pulse 100 Hz stimulus train (Fig. 2A). Thus the rebounds we observed, even at the highest stimulation intensities, are GABA_A receptor mediated, rather than through metabotropic receptors (i.e., mGluRs) or other synaptic inputs (i.e., aminergic) that might be activated from our stimulation sites.

Figure 2. Rebound bursts and post-stimulus first spike latency are sensitive functions of stimulus intensity. (A) Representative traces of rebound spike responses in separate transient burst cells following a train of evoked IPSPs (10 pulses, 100 Hz, 20% and 100% intensity) in (i) on-cell recordings or (ii) whole-cell recordings, and (iii) in response to a current-evoked hyperpolarization in a whole-cell recording. Higher intensity synaptic activation, or an increase in hyperpolarization (red traces), evoke a higher rebound spike frequency and shorter post-stimulus FSL (dashed lines). (B) Plots of rebound frequency and the FSL/ISI ratio in extracellular unit recordings in response to increasing stimulus intensity of inhibitory input (10 pulses, 100 Hz; n = 16 transient, n = 12 weak burst cells). Dashed lines indicate a failure of approximately half of the units (in brackets) to exhibit a rebound burst at 20% of maximal intensity, and an associated sharp increase in FSL. Stimulus artifacts and spike amplitudes were truncated in (A). Burst frequencies are plotted as the increase above baseline tonic firing frequency.

A value of less than 1 thus indicates a decrease in FSL in relation to the original tonic firing rate.

We found that increasing the intensity of synaptic stimulation or membrane hyperpolarization produced a graded decrease in the latency before the cell resumed spiking. This property was apparent with synaptically evoked inhibition for both on-cell recordings (n = 28) and whole-cell recordings (Fig. 2A and B) (n = 19). We then tested this in whole-cell configuration and confirmed that weak hyperpolarizations gave a long FSL while stronger current pulses shortened the FSL (n = 40) (Fig. 2A). As a result, we find that a stimulus intensity chosen to maximize post-stimulus FSL represents the lowest intensity that will inhibit the cell sufficiently to stop firing during a stimulus train. For our weakest stimulus intensity (20% of maximum) approximately half of the cells failed to show a rebound increase in frequency, at which point FSL also sharply increased (Fig. 2B). Thus at 20% intensity the FSL ranged from 17–57 ms (Transient Burst 40.8 ± 5.8 ms; n = 11; Weak Burst 38.8 ± 5.17 ms; n = 8) and up to 59 ms for those cases that failed to generate a burst (Transient Burst 52.8 ± 3.8 ms; n = 5; Weak Burst 51.2 ± 5.06 ms; n = 4) (Fig. 2B). We note that even small increases in stimulus intensity to 40% or more of maximal intensity shortened FSL and consistently evoked rebound bursts in all cells (Fig. 2B).
established physiological evidence for multiple release sites on synaptic terminals of Purkinje cell axons and a widespread activation of GABA receptors that offsets synaptic depression during high frequency input. Although smaller amplitude IPSPs have been reported in DCN cells, they were attributed to potential activation of local recurrent inhibitory projections within the nucleus. Our stimulation site outside of the nucleus is expected to avoid this problem. We then more closely examined the relationship between stimulus intensity, IPSP amplitude, and FSL. We found that an increase in stimulus intensity was always associated with a measurable and similar change in IPSP amplitude for both Weak and Transient Burst cells, as shown for 19 different cases in Figure 3B. A plot of IPSP amplitude against the corresponding FSL/ISI ratio for each record further revealed a strong correlation, indicating that FSL is tightly linked to the amplitude of the initial IPSP in an afferent spike train (Fig. 3B). We can thus conclude that our stimulus intensities progressively increased the strength of synaptic inhibition in a manner that closely reflects a change in FSL and the probability of obtaining rebound bursts.

Discussion

The cellular properties that underlie rebound discharge and spike output in DCN cells have been examined in several studies using in vitro slice preparations. Some evidence exists for the generation of rebound-like bursts in vivo following periods of reduced firing of DCN cells (including presumed Purkinje cell-evoked inhibition) or rebound responses in motor control. Yet the potential for intrinsic membrane properties of the type assessed in vitro to generate rebound bursts in response to Purkinje cell inhibitory input has not been extensively studied.

Identifying synaptically evoked rebound bursts. The present study demonstrates that rebound bursts are readily evoked in large diameter DCN cells following activation of Purkinje cell axonal inputs with a 10 pulse, 100 Hz stimulus train in vitro. Furthermore, we were able to discriminate the activity of two phenotypes of rebound inherent to DCN cells using a statistical definition of rebound bursts. These differences were apparent not only in whole-cell recordings, but also in on-cell recordings of unit activity in which cytosolic contents are not disrupted. Indeed, data recorded in on-cell or whole-cell modes were not statistically different in any respect. We could thus use rebound spike frequency in on-cell recordings to reliably identify the output of a Transient or Weak Burst phenotype in the Interpositus nucleus. These results are also important in demonstrating that DCN cells maintained in vitro under our conditions establish a resting potential and Cl- reversal potential that is sufficient to allow synaptic inhibition to shift the membrane potential past burst threshold. Since this work was conducted in a slice preparation, the results pertain only to the
Purkinje cell axons stimulated dorsal to the DCN nucleus, with no involvement of Purkinje cell activity or cerebellar cortical neurons, as they are disconnected in the preparation of slices. The rebound response evoked under these conditions was entirely dependent on picrotoxin-sensitive (Cl−-mediated) GABAergic inhibition, and not the result of activating any glutamatergic or other modulatory inputs. Previous work has also shown that rebound bursts can be recorded beyond the P12–P18 period tested here as current-evoked rebounds have been recorded in animals up to six months of age. The generation of rebound bursts is thus not confined to periods of earlier stages of development. The one key difference with previous work is that the maximal frequency of synaptically evoked rebound bursts we record is lower than that reported using large current injections (as reported in ref. 12). This is not entirely unexpected given the relative magnitude of membrane hyperpolarizations applied in either case, and we found that the differences in burst frequency between Transient and Weak burst cells were still apparent following synaptic activation. The data presented here thus establish that several facets of rebound burst discharge established in previous studies employing current injection are reproduced with inhibitory synaptic activation.

**Probability of evoking rebound bursts.** An important issue in cerebellar physiology is the extent to which physiological levels of synaptic input can evoke rebound bursts in DCN cells. A recent study reported a very low probability when using a stimulus intensity set to evoke a long post-stimulus pause (FSL) in spike firing. We found that the probability of evoking rebound bursts, and the associated FSL after the stimulus train, was highly sensitive to the intensity of stimulation and the amplitude of the evoked IPSP. We were able to obtain consistent rebound responses in all cells using stimuli set to ∼40% of maximum, corresponding to an IPSC of 183 ± 24.2 pA (n = 11). Importantly, a previous estimate of the IPSC amplitude associated with presumed single axon input to DCN cells when recorded under similar conditions was between 50–100 pA. Our threshold for reliably evoking bursts at 40% stimulus intensity should then correspond to the synchronous activation of only a small number of Purkinje cell inputs. This is likely a conservative estimate, but it indicates that our stimulation paradigms are well within the range expected to activate a reasonable number of the hundreds of Purkinje cell axon projections onto DCN cells from cerebellar cortex. Thus we find that rebound bursts are evoked in vitro with high probability using physiological levels of inhibitory activation.

**Inhibitory synaptic activation of rebound bursts.** It was previously reported that delivering inhibitory stimulus trains produced a frequency-dependent depression of the IPSP that resulted in different tonic levels of membrane hyperpolarization. We also found this for different stimulus intensities at 100 Hz, but with an apparent common stabilization of membrane potential at the presumed ECl (Fig. 2A). Despite this, rebound discharge intensity continued to increase with stimulus intensity. One explanation for this is an increased activation of inhibitory inputs terminating in dendritic locations of DCN cells, where a substantial number of synaptic terminals are attributed to Purkinje cell input. Imaging has also shown that step commands delivered at the soma invoke a significant portion of LVA-mediated Ca2+ current in proximal dendrites. Indeed, membrane hyperpolarizations at the soma were instead found to be associated with a decrease in internal Ca2+ levels during and shortly after the end of a hyperpolarizing pulse. Each of these results suggest that much of the Ca2+-mediated rebound depolarization is actually generated at dendritic locations. In this respect, Aizenman and Linden found that synaptic inhibition was more effective than somatic current injection at recruiting a rebound burst even when the resulting somatic hyperpolarizations were identical, emphasizing the potential importance of dendritic inhibitory inputs. It has been shown that DCN cells are electrically compact enough for step hyperpolarizations at the soma to control the state of inactivation of LVA Ca2+ channels involved in rebound generation. However, membrane filtering during electronic conduction of the synaptic voltage response to the soma may decrease the ability to detect differences in the amplitude of IPSPs generated in dendrites. It was further shown that release of GABA at multiple sites from Purkinje cell terminals leads to rapid spillover and potential activation of GABA receptors up to 700 nm away. Spillover and diffusion will extend the region of membrane brought under the hyperpolarizing influence of GABA receptor activation and thus the extent of T-type Ca2+ channel recovery from inactivation. The relatively constant hyperpolarization we observe during repetitive stimulation is then predicted from the characteristics of GABA release from Purkinje cell presynaptic terminals. Each of these factors will influence the ability for an inhibitory synaptic input to generate rebound discharge regardless of the apparent state of hyperpolarization recorded at the soma. We thus expect that increasing stimulus intensity acts to synchronously activate more Purkinje cell axons (including those that terminate at dendritic sites), promoting a larger local IPSP and recovery of Ca2+ channels from inactivation, thereby establishing the conditions required for the generation of a rebound burst.

In summary, the available data indicates that rebound bursts can invariably be recruited in DCN cells in vitro with moderate levels of inhibition, and to exhibit many of the salient properties of rebound discharge delineated in previous studies. Although rebound increases in DCN cell firing rate have been reported in vivo, the correspondence of these to intrinsic rebound burst mechanisms has not been fully established. Thus the factors that recruit rebound bursts in DCN cells under in vivo conditions remain to be identified.

**Materials and Methods.**

In vitro slice preparation. All tests were conducted on P12–P18 male Sprague-Dawley rats (Charles River Canada) maintained according to the Canadian Council for Animal Care. Parasagittal cerebellar slices of 250 µm thickness were maintained for recordings at 32–34°C (as in ref. 8). Recordings focused on large diameter cells in the Interpositus nucleus identified visually through differential interference contrast optics and infrared illumination. External aCSF contained (in mM): 125 NaCl, 3.25 KCl, 1.5 CaCl2, 1.5 MgCl2, 25 NaHCO3, and 25 D-glucose, pH 7.4.

**Recordings.** The internal electrolyte for whole-cell recordings was (in mM): 130 K-gluconate, 0.1 EGTA, 10 HEPES, 7 NaCl, 0.3 MgCl2, with 5 di-tris-creatine phosphate, 2 Tris-ATP and 0.5
Reliability of rebound bursts in deep cerebellum

Na-GTP, pH 7.3 with KOH. Using these ion concentrations we established an $E_{Cl}$ of -75.7 mV at 34°C, a value close to that for the peak of the IPSP in previous microelectrode recordings that produce minimal disruption of the Cl− gradient. To reference whole-cell membrane potentials with the K-glucuronate based electrolyte, we subtracted a junction potential of -10.6 mV. Unless otherwise indicated, whole-cell recordings were adjusted through current injection (<50 pA) to a nominal value of -61 mV according to the trough of AHPs during tonic firing. On-cell recordings were performed by forming a high resistance seal with the membrane through negative pressure using a HEPES-buffered aCSF (in mM): 150 NaCl, 3.25 KCl, 1.5 CaCl2, 1.5 MgCl2, 10 HEPES, and 25 D-glucose, pH 7.4 (n = 28), or a K-glucuronate based internal solution prior to moving to the whole-cell recording configuration (n = 12). Unless specifically noted FSL values do not include the duration of the 100 Hz stimulus train.

Stimulation. Purkinje cell axons were activated using a metal bipolar concentric stimulating electrode (Frederick Haer, MA) that was positioned dorsal to the DCN and outside of the Interpositus nucleus to avoid activating any axon collaterals to the nucleus. All recordings were made from cells positioned near the edge of the nucleus. Inhibitory synaptic responses were measured in the presence of 25 µM dl-2-amino-5-phosphonopentanoic acid (DL-AP5) and 10 µM 6,7-dinitroquinoxaline-2,3-dione (DNQX) to block glutamatergic responses. A combination of mossy and/or climbing fiber excitatory inputs were evoked in the presence of picrotoxin (50 µM) to block GABAA receptors by placing the stimulating electrode ventral to the DCN and outside of the Interpositus.

Average values are shown as mean ± SEM and statistical tests conducted by Students’ t-test.

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References