Questioning the role of rebound firing in the cerebellum

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Karina Alviña[1,2], Joy Walter[1], Adam Kohn[1], Graham Ellis-Davies[3] and Kamran Khodakhah[1]

[1] Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, [2] Departmento de Ciencias Fisiologicas, Pontificia Universidad Catolica de Chile, Santiago, Chile, and [3] Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA 19102

Corresponding Author:

Kamran Khodakhah
Department of Neuroscience
Albert Einstein College of Medicine
1410 Pelham Parkway South
Kennedy Center, Room 506
Phone: (718) 430-3794
Fax: (718) 430-8821
E-mail: kkhodakh@aecom.yu.edu

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The centerpiece of recent theories on cerebellar function is rebound firing in neurons of the deep cerebellar nuclei (DCN). Despite the robustness of this phenomenon *in vitro*, *in vivo* studies have provided little evidence for its prevalence. Here we show that under physiological conditions, *in vitro* or *in vivo,* intact DCN neurons rarely show rebound firing, a finding that necessitates a critical re-evaluation of recent cerebellar models.

A vast amount of cortical and sensory information that converges onto the cerebellum is integrated by cerebellar Purkinje cells and subsequently conveyed to the neurons of the deep cerebellar nuclei (DCN) [1]. DCN neurons further process this information and generate the major output of the cerebellum, encoding the computational outcome of the cerebellar circuitry in their rate and temporal pattern of activity.

A stereotypic biophysical feature of DCN neurons is that they are capable of rebound depolarization [2-4]. Following a strong hyperpolarization their membrane potential briefly rebounds to a more depolarized level resulting in a transient increase in their firing rate; a phenomenon termed rebound firing [4,5]. Given the inhibitory GABAergic nature of Purkinje cell synapses onto DCN neurons, and primarily on the basis of this stereotypic biophysical property *in vitro*, rebound firing has been extensively incorporated into recent theories of cerebellar function [6-8]. Several functional roles, from timing to encoding information and mediating plasticity have been assigned to this phenomenon [6-10].

However, even though rebound firing is robust when examined using intracellular recordings *in vitro*, there is little direct evidence in support of its physiological prevalence *in vivo* [11-13]. We investigated this discrepancy.

DCN rebound depolarization is most likely mediated by low-threshold T-type calcium channels [3-5]. Factors that determine the extent of contribution of these channels to rebound firing are average membrane potential prior to hyperpolarization, and the level and duration of hyperpolarization [2,4,5] . Using acutely prepared rat cerebellar slices, we designed our experiments to replicate these factors as close to their physiological parameters as possible (see Supplemental data). We avoided intracellular recordings because they inevitably alter the membrane input resistance, and the cytosolic ionic composition. Therefore, we monitored the activity of DCN neurons extracellularly to preserve their baseline firing rate and the true reversal potential of their GABAergic inputs. Two sets of experiments were done to mimic strong hyperpolarizations that may occur under physiological conditions. First, with excitatory transmission blocked, GABAergic synaptic inputs were stimulated using a train of 10 electrical pulses @ 100 Hz (Figure 1a). The strength of the stimulation was adjusted such that it efficiently paused firing in the target cell (average pause duration=199.3±9.1 ms, n=39 cells). Using this paradigm we only observed rebound firing in 21% (8/39) of the cells examined. In the second set of experiments GABA was photoreleased on the dendrites and soma of the target DCN neuron (Figure 1b). This method allowed us to produce longer pauses (average 455.3±19.5 ms, n=52 cells). Nonetheless, only 14% (7/52) of the cells examined showed rebound

firing.

The baseline firing rates of the 109 DCN neurons examined covered a range of 6-70 spikes per second, comparable to the range of baseline firing rates seen *in vivo* [12-14]. Within this range, there was no correlation between firing rate and rebound firing (Figure 1c). Similarly, elevating extracellular potassium concentration to 4 mM (10 cells), or omitting blockers of excitatory synaptic transmission (9 cells) did not increase the occurrence of rebound firing (Figure 1c). Moreover, there was no correlation between the pause duration and whether a cell showed rebound firing (Figure 1c). We also did not see rebound firing in any of the 8 adult (2-3 months old) DCN neurons examined suggesting that the low prevalence of rebound was not a function of developmental stage (Figure 1c). Collectively, only 18 out of the 109 cells (15%) dispersed throughout the three deep cerebellar nuclei showed rebound firing after a strong inhibitory input. Comparable data were obtained when rebound firing was examined in mice cerebellar slices (Supplemental data).

Despite the prominent pauses produced with our stimulation protocols, it is possible that the magnitude of the hyperpolarization was not large. We performed experiments to establish whether the synaptic stimulations used were strong enough to hyperpolarize the cells to their GABA reversal potential. We first recorded the activity of a DCN neuron extracellularly and determined whether a train of electrical stimulations resulted in rebound firing (Figure 2a). The cell was then whole-cell current-clamped and the extent to which its membrane hyperpolarized by the same train of stimuli was measured (Figure 2b). Lastly, the

reversal potential of GABAergic synaptic inputs was determined in voltage-clamp mode (Figure 2c). Only in one of the 8 cells examined with this extended protocol did we see rebound firing, even though the train of synaptic stimulations on average paused the extracellularly monitored activity for 188.9±9.2 ms (Figure 2a). Current-clamp recordings from the same cells showed that synaptic inputs hyperpolarized the membrane potential to an average of -69.2±1.1 mV, a level not statistically different from the imposed reversal potential of the GABAergic conductance (-71.1±0.8 mV, p=0.16 (Figure 2c). Based on the whole-cell data data, it is reasonable to conclude that during the extracellular recordings the GABAergic conductance was sufficiently large to hyperpolarize the intact cells to their true GABA reversal potential. In contrast to the single cell that showed rebound firing with synaptic stimulation, in all 8 cells prominent rebound firing was seen when their membrane potential was strongly hyperpolarized by current injection (Figure 2d). Thus, extended hyperpolarization of most DCN neurons to their GABA reversal potential is not sufficient to produce rebound firing although, in agreement with prior reports [2-5], we find that they all have the required conductances to generate rebound firing with stronger (physiologically irrelevant) hyperpolarizations.

The chloride reversal potential in a cell is determined, in part, by chloride transporters. Given recent evidence that phosphorylation [15] alters expression level of chloride transporters, we considered the possibility that the chloride reversal might have been different in DCN cells recorded in acutely prepared cerebellar slices compared to that *in vivo*. We thus examined the prevalence of

rebound firing in anaesthetized adult mice *in vivo*.

Interpreting data obtained from *in vivo* experiments using sensory stimuli to examine DCN activity is inherently ambiguous because an increase in the DCN firing rate is as likely to be caused by direct olivary and mossy fiber excitatory inputs as it is to be the consequence of rebound firing [11,13]. To avoid such an ambiguity, we took advantage of the somatotopic projection of Purkinje cells onto DCN neurons and monitored the activity of DCN neurons while directly activating the overlaying Purkinje cells that projected to them (Figure 3).

Stimulation of Purkinje cells by a train of electrical stimuli (10 @100 hz) effectively paused spontaneous activity of DCN neurons (Figure 3a), producing an average pause duration of 282.3±15.9 ms (n=20 cells, 5 animals). In this (Figure 3 b,c), and in fact most cells, the post-pause firing rate was comparable to the baseline firing rate (average baseline firing rate in all cells 26.5±4.1 spikes per second vs post-pause firing rate of 21.9±2.9 spikes per second; n=20). In 2 of the 20 cells examined the post-pause firing rate was marginally higher than the pre-pause firing rate (Figure 3d). Although one could consider these two cells to have rebound, the increases in their firing rate vs baseline were quite small (<20 spikes per second – in slices the post-pause firing rate of cells that showed rebound was >150 spikes per second) and their absolute post-pause firing rates were below 30 spikes per second. Figures 3e-f demonstrate the combined cumulative occurrence of pre- and post-pause interspike interval histogram and the post-pause interspike interval Z-score. Barring major differences in awake behaving animals, our data suggest that even *in vivo* strong inhibition of DCN

neurons by Purkinje cells has little effect on their post-pause firing rate.

Collectively, our findings demonstrate that rebound firing is not a common response to physiologic inhibitory inputs. It is possible that such inputs produce a small rebound depolarization that is not large enough to cause rebound firing but may still make DCN cells more responsive to subsequent excitatory inputs. Alternatively, it is plausible that only a small fraction of DCN neurons need to respond with rebound firing to be of functional significance. In either case our findings compel a critical reassessment of current cerebellar models that regard rebound firing as the stereotypic response of all DCN neurons to Purkinje cell input.

Figure 1. Rebound firing is not frequently observed in DCN neurons.

Whether DCN neurons respond by rebound firing (bottom traces) or not (top traces) was examined by extracellular recording using two stimulation paradigms: (a) electrical stimulation (10 pulses at 100 Hz) and (b) photorelease of GABA. (c) Fraction of cells showing rebound firing and 3-D scatter plot of whether a cell showed rebound firing as a function of its baseline firing rate, or stimulus-induced pause. Black symbols represent electrical stimulation, and the remaining colors correspond to GABA photorelease. The cells represented with red symbols were recorded in high extracellular potassium (10 cells), and those in green were obtained from adult rats (8 cells).

Figure 2. Extended hyperpolarization to GABA reversal potential infrequently results in rebound firing.

The response of DCN neurons to a train of inhibitory inputs was monitored first extracellularly (a) and then by current-clamp recording (b). The raw traces show the typical response (i.e. no rebound firing) in the same cell. Individual (circles) and average baseline firing rates and the duration of stimulus-induced pauses in all 8 cells examined are shown in the scattergram (a). The white symbol represents the one cell that showed rebound firing. (c) The reversal potential of GABAergic inputs determined by voltage-clamp recordings, and the maximum hyperpolarization achieved by the train of inhibitory synaptic inputs in current-clamp mode in the same cells. (d) Strong hyperpolarization beyond the GABA reversal potential by direct current injection in the same cell shown in

parts (a) and (b) produced prominent rebound firing (arrow).

Figure 3. Rebound firing is not a common response *in vivo*.

Single unit activity of DCN neurons was monitored *in vivo* and the Purkinje cells overlaying them were activated by a train of 10 electrical pulses @100 hz to produce prominent pauses in DCN firing. (a) Raw data trace, raster plot of 50 trials, and the corresponding PSTH of a typical cell. Despite producing long pauses, synaptic inhibition did not result in rebound firing (a,b). (c) The post-stimulus interspike intervals (ISIs) of the cell shown as Z score relative to the distribution of pre-pause ISIs. (d) Average post-pause firing rate vs pre-stimulus firing rate in 20 DCN neurons examined as above. (e) Cumulative post- (red) and pre-pause (black) ISIs for all 20 cells examined, and the Z score of their post-pause ISI (f).

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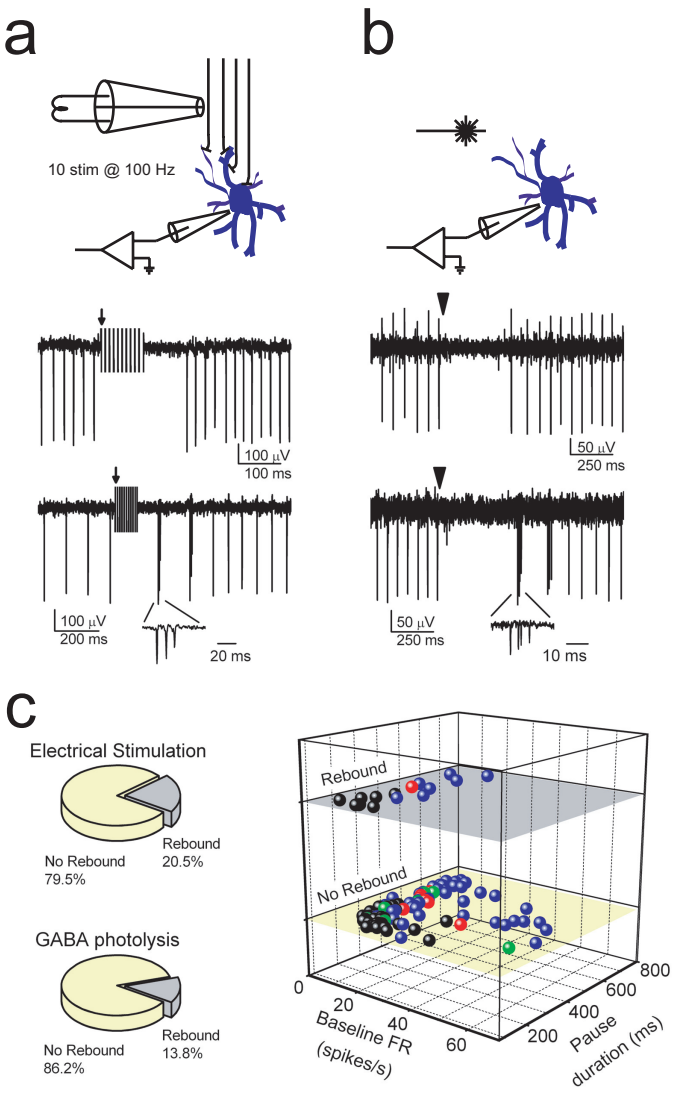
a
b
10 stim @ 100 Hz
100 µV
100 ms
100 µV
200 ms
20 ms
50 µV
250 ms
50 µV
250 ms
10 ms
c
Electrical Stimulation
No Rebound
79.5%
Rebound
20.5%
GABA photolysis
No Rebound
86.2%
Rebound
13.8%
Rebound
No Rebound
0
20
40
60
Baseline FR
(spikes/s)
200
400
600
800
Pause
duration (ms)

Figure 1

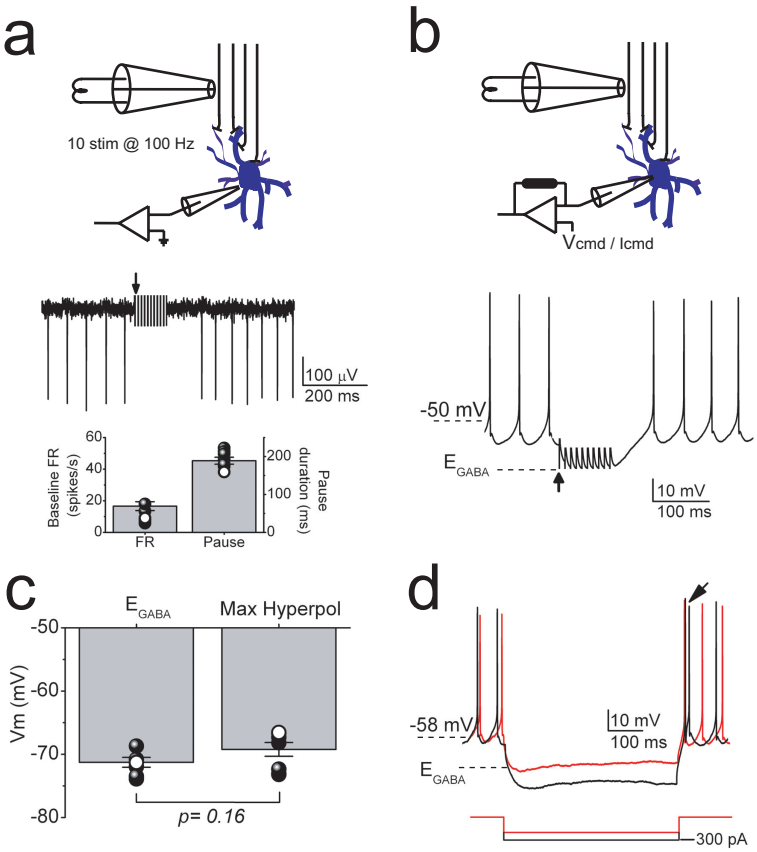
a
10 stim @ 100 Hz
100 µV
200 ms
Baseline FR (spikes/s)
Pause duration (ms)
FR
Pause
b
Vcmd / Icmd
-50 mV
E GABA
10 mV
100 ms
c
E GABA
Max Hyperpol
Vm (mV)
p= 0.16
d
-58 mV
E GABA
10 mV
100 ms
300 pA

Figure 2

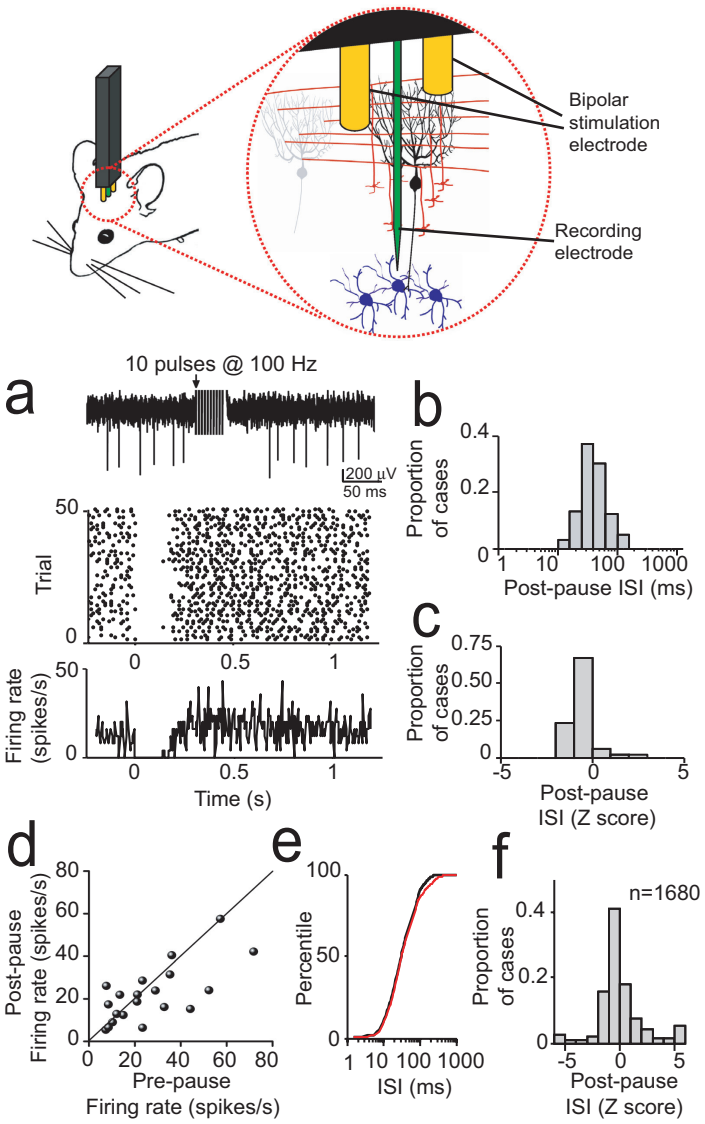
Bipolar
stimulation
electrode
Recording
electrode
a
10 pulses @ 100 Hz
200 µV
50 ms
Trial
Firing rate
(spikes/s)
Time (s)
b
Proportion
of cases
Post-pause ISI (ms)
c
Post-pause
ISI (Z score)
d
Post-pause
Firing rate (spikes/s)
Pre-pause
Firing rate (spikes/s)
e
Percentile
ISI (ms)
f
n=1680
Post-pause
ISI (Z score)

Figure 3