Neuronal Excitability

Climbing Fiber Regulation of Spontaneous Purkinje Cell Activity and Cerebellum-Dependent Blink Responses[1,2,3]

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Abstract

It has been known for a long time that GABAergic Purkinje cells in the cerebellar cortex, as well as their target neurons in the cerebellar nuclei, are spontaneously active. The cerebellar output will, therefore, depend on how input is integrated into this spontaneous activity. It has been shown that input from climbing fibers originating in the inferior olive controls the spontaneous activity in Purkinje cells. While blocking climbing fiber input to the Purkinje cells causes a dramatic increase in the firing rate, increased climbing fiber activity results in reduced Purkinje cell activity. However, the exact calibration of this regulation has not been examined systematically. Here we examine the relation between climbing fiber stimulation frequency and Purkinje cell activity in unanesthetized decerebrated ferrets. The results revealed a gradual suppression of Purkinje cell activity, starting at climbing fiber stimulation frequencies as low as 0.5 Hz. At 4 Hz, Purkinje cells were completely silenced. This effect lasted an average of 2 min after the stimulation rate was reduced to a lower level. We also examined the effect of sustained climbing fiber stimulation on overt behavior. Specifically, we analyzed conditioned blink responses, which are known to be dependent on the cerebellum, while stimulating the climbing fibers at different frequencies. In accordance with the neurophysiological data, the conditioned blink responses were suppressed at stimulation frequencies of $\geq$4 Hz.

Key words: climbing fibers; eyeblink conditioning; Purkinje cell; simple spikes; spontaneous background firing

Significance Statement

The cerebellum is vital for many important functions, including predicting sensory events, adjusting reflexes, allowing smooth movements, and acquiring associations between different stimuli. Purkinje cells, in the cerebellar cortex, have a spontaneous activity that is regulated by climbing fiber input from the inferior olive. Here we show that stimulating climbing fibers result in a frequency-dependent suppression of Purkinje cell activity. Moreover, such stimulation abolishes the expression of conditioned blink responses, which are known to rely on the cerebellum. These results demonstrate that cerebellar function is crucially dependent on normal levels of spontaneous activity in Purkinje cells.

Introduction

Purkinje cells of the cerebellar cortex receive input through two afferent pathways. The mossy/parallel fiber pathway carries input from a large number of nuclei in the brainstem and the spinal cord, while the climbing fiber pathway carries input from the inferior olive. Whereas activation of the mossy/parallel fibers elicits simple spikes in Purkinje cells, activating climbing fibers results in the generation of complex spikes (Eccles et al., 1967). Simple spikes consist of single Na^+-dependent action potentials. At rest, Purkinje cells fire at an average rate of ~44 Hz (Armstrong and Rawson, 1979) but can be modulated up to 200 Hz (Thach, 1967). Complex spikes, in comparison, occur at low rates, normally between 0.5 and 1.5 Hz, and rarely exceed 5 Hz (Armstrong and Rawson, 1979; Womack and Khodakhah, 2002; Bengtsson et al., 2004; Cerminara and Rawson, 2004). While input from mossy/ parallel fibers and climbing fibers can modulate their activity, Purkinje cells are tonically active (Woodward et al., 1974; Gähwiler, 1975; Llinás and Sugimori, 1980; Hounsgaard and Midtgaard, 1988; Häusser and Clark, 1997; Raman and Bean, 1999; Cerminara and Rawson, 2004). The direct effect of climbing fiber input to Purkinje cells is depolarization. However, increasing or decreasing the climbing fiber firing frequency beyond its normal range results in decreased or increased simple spike firing, respectively (Colin et al., 1980; Rawson and Tilokskulchai, 1981; Montarolo et al., 1982; Demer et al., 1985; Andersson and Hesslow, 1987b; Bengtsson et al., 2004; Cerminara and Rawson, 2004).

Several lines of evidence suggest that balanced simple and complex spike activity is essential for normal behavior (Llinás et al., 1975; Zbarska et al., 2008). Consequently, lesions of the inferior olive, as well as blocking the effect of GABA in the cerebellar nuclei, which results in the inhibition of the olive, abolishes cerebellum-dependent conditioned responses (Yeo et al., 1986; Parker et al., 2009). Balanced output from the inferior olive relies on

GABAergic input from the cerebellar nuclei, which in turn relies on activity in Purkinje cells (Andersson and Hesslow, 1987a,b; Lang et al., 1996; Bengtsson et al., 2004; Bengtsson and Hesslow, 2006, 2013; Bazzigaluppi et al., 2012; Chaumont et al., 2013; Najac and Raman, 2015). Although this interdependence between different parts of the cerebellar circuit is a fundamental principle of cerebellar function, the exact relationship between climbing fiber activity and simple spike activity has not yet been fully described. In this study, we provide a detailed analysis of the effect of climbing fiber activation at different frequencies on simple spike activity and the expression of cerebellum-dependent conditioned blink responses.

Materials and Methods

Surgery

Eight male ferrets were initially anesthetized with a mixture of O_2 and air, with 1.5–2% isoflurane (Baxter Medical), which was subsequently replaced intravenously by propofol (10 mg/ml Diprivan; AstraZeneca). During anesthesia, a tracheotomy was performed, and the gas was led directly into a tracheal tube. The end-expiratory CO_2 concentration, arterial blood pressure, and rectal temperature were monitored and kept within physiological limits throughout the experiment. During the whole experiment, infusion was given intravenously [50 mg/ml glucose and isotonic acetate Ringer's solution (proportion, 1:1) with 0.004 mg/ml albumin fraction V (from bovine serum; Sigma-Aldrich), 2 mg/kg/h]. After fixation of the head in a stereotaxic frame, the skull was opened on the left side, and the caudal half of the left cerebral hemisphere, together with a substantial part of the thalamus on the left side, were removed by aspiration. The animals were decerebrated by sectioning the brainstem with a spatula 1–2 mm rostral to the superior colliculus. After decerebration, anesthesia was discontinued. With the cerebellum and colliculi exposed, a pool was constructed of cotton-reinforced agar and filled with warm high-density perfluorocarbon liquid (FC-40 Fluorinert; 3M). To ensure mechanical stability, animals were immobilized by curare, artificially ventilated, and kept hanging by the spine. A bilateral pneumothorax was performed to minimize chest movements and movements caused by changes in venous blood pressure. The dura covering the cerebellum was removed, and the surface was covered with agarose gel (15 mg/ml) to improve stability and prevent edema. This study was reviewed and approved by the local Swedish Ethical Committee.

Climbing fiber stimulation

The experimental setup, including stimulation and recording sites, is illustrated in Figure 1A. Climbing fibers were stimulated in the ipsilateral inferior cerebellar peduncle by lowering a tungsten electrode (diameter, 30 μm; uninsulated tip, 50 μm) 4.0–5.0 mm below the posterior cerebellar surface, at an angle of 45°, 4 mm lateral to the midline, and 4 mm rostral to the caudal border of the vermis. While tracking, single stimulus pulses were applied and the evoked field potentials were recorded in the C3 zone of the hemispheric lobule VI, which was identified
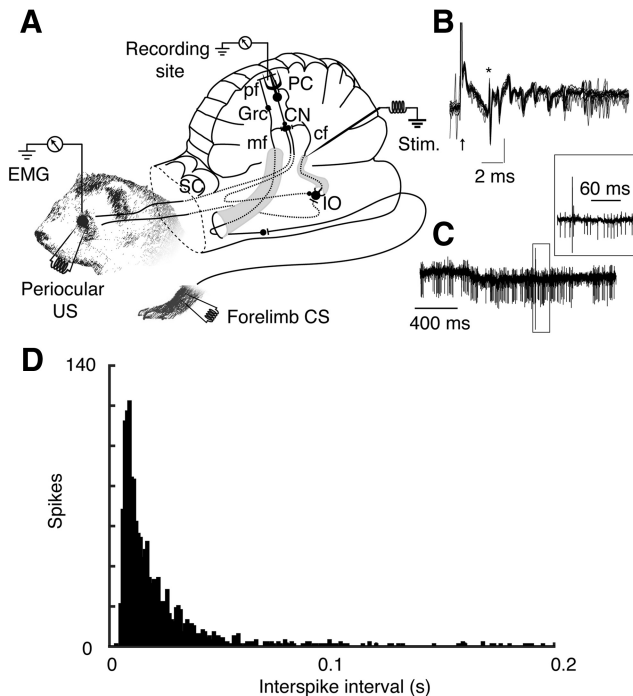
Figure 1. Experimental setup, cerebellar circuit diagram, and single unit records. **A**, Schematic illustration of the experimental setup with recording and stimulation sites. mf, Mossy fiber; cf, climbing fiber; pf, parallel fiber; PC, Purkinje cell; GrC, granule cell; CN, cerebellar nuclei; IO, inferior olive; SC, superior colliculus. **B**, Complex spikes evoked by climbing fiber stimulation. Thin black traces represent ten superimposed complex spikes. Thick trace is the average waveform. The asterisk indicates the onset of the complex spike and the black arrow indicates the time of the stimulation. **C**, Extracellular recording of a representative Purkinje cell. Bottom trace shows an unfiltered recording and the inset shows, in greater detail, the complex spike evoked by climbing fiber stimulation. **D**, Archetypal interspike interval distribution before the start of the climbing fiber stimulation.

by previously established criteria (Hesslow, 1994a; Hesslow and Ivarsson, 1994). The effectiveness of all stimulation sites and stimulus intensities were verified again and adjusted, if necessary, when recording the activity of single Purkinje cells.

Purkinje cell recording
Five extracellular single-unit records of Purkinje cells were obtained using microelectrodes with pulled and ground tips, 30–40 μm metal core diameter (Thomas Recording). When a Purkinje cell with the appropriate input characteristics was found, we began stimulating climbing fibers at 0.5 Hz after which the frequency was increased by 0.5 Hz every 5 min up to 4 Hz. Above this frequency, the stimulation was increased by 1 Hz every 5 min. In one experiment, we included 60 s periods between every switch in stimulation frequency during which no stimulation was given. This was done to determine whether the effect on background activity lasted beyond the stimulation. To optimize the recording stability, animals were curarized when we searched for or recorded the activity of Purkinje cells.

Analysis of Purkinje cell activity
Voltage changes related to neural activity were passed unfiltered through an amplifier and sampled at 40 kHz using a Power 1401 A/D converter unit (CED). Action potentials were isolated on-line with Spike2 software (version 7.11; CED Electronics Design). All recordings were subsequently reanalyzed off-line. Identification of complex spikes followed a series of inclusion criteria: the presence of spikelets after the large initial spike (Fig. 1B), a short latency inhibition ~10 ms after the complex spike (Fig. 1C), as well as archetypal interspike interval distributions (Fig. 1D). As the firing rate of Purkinje cells is highly variable between different cells, firing rates were normalized relative to a baseline segment recorded before the start of the stimulation protocol. Population responses were calculated by averaging over Purkinje cells for the same session. Unless otherwise stated, we report activity as the mean $\pm$ SEM.

Eyeblink conditioning
Three ferrets were trained in a standard delay conditioning paradigm, where the conditional stimulus (CS) was a 320 ms, 50 Hz train of 1 ms electrical pulses, applied subcutaneously through a pair of needle electrodes inserted ~5 mm apart through the skin of the left forelimb (intensity range, 1.2–2 mA). Prior to training, we verified that the stimulation did not elicit any eye muscle activity. The unconditional stimulus consisted of three 1 ms pulses at 50 Hz, with a stimulation intensity of 3 mA, applied bilaterally to the periocular skin, 300 ms after the onset of the conditional stimulus. We used a pseudo-random 16 $\pm$ 1 s intertrial interval. The stimuli used in this study are known to be effective for eyeblink conditioning in this preparation (Hesslow and Ivarsson, 1996; Ivarsson et al., 1997). Conditioning started approximately 1 h after completion of the surgery and continued until the animal emitted conditioned eyeblink responses (ECRs) on at least 7 of 10 consecutive CS-alone trials. During training, the animals were paralyzed through curare. This paralysis was interrupted every 30 min to monitor the behavioral response.

Behavioral analysis
To examine the acquisition of conditioned blink responses we recorded electromyographic (EMG) activity bilaterally through pairs of stainless steel electrodes. The signal was amplified, high-pass filtered at 5 kHz, and digitized at 40 kHz with a Power 1401 data acquisition analog-to-digital (A/D) converter unit (CED). Data analysis was performed using Spike2 version 7 software (CED) and custom routines developed in Matlab (MathWorks). Eyeblink responses from each valid trial were examined off-line. The recorded EMG was rectified, and the mean power of the signal was obtained calculating the root mean square in a 5 ms time window. In general, ECRs could easily be discriminated by visual inspection of the raw signal. Muscle activity between 50 and 298 ms after CS onset, with at least twice the amplitude of spontaneous muscle activity 0-100 ms before CS, was considered to be an ECR. The onset latency was defined as the time when activity exceeded and stayed above this threshold for at least 50 ms. Responses with a latency of <50 ms

were classified as alpha responses and were excluded from the valid ECRs trials. Means of ECR incidence (i.e., the ratio between trials that elicited an ECR and the total number of trials within a block) and baseline activity were calculated and plotted for blocks of 10 consecutive trials. Onset latency, peak latency, and response amplitude were determined only for those trials with an ECR. All group data are presented as the mean ± SEM.

Results

Purkinje cell recordings

We recorded the activity of Purkinje cells in the blink-controlling C3 zone of the cerebellar cortex, identified by characteristic field potentials evoked by periocular stimulation (Bengtsson et al., 2004; Jirenhed et al., 2007; Jirenhed and Hesslow, 2015). Five Purkinje cells with periocular and climbing fiber input were recorded from five different animals. The recordings were stable, with a good signal-to-noise ratio (Fig. 1C). The Purkinje cell firing rate before the start of the stimulation was between 8.85 and 33.7 Hz (mean, 20.2 ± 9.37 Hz). Climbing fibers were stimulated in a stepwise incremental fashion starting at 0.5 Hz and increasing 0.5-1 Hz every 5 min. The exact frequencies used are shown in Figure 2A. A complete experimental session lasted for 65 min (78 min when the 60 s interval between switches in frequency was introduced). At all frequencies, the stimulation reliably elicited a complex spike with the expected 2-3 ms latency (Fig. 1B). Sometimes, the stimulation also elicited a second complex spike that was indirectly evoked by the climbing fiber reflex (Eccles et al., 1966). However, in these experiments, the stimulation strength was adjusted to minimize the occurrence of such reflexive climbing fiber responses.

Effect of climbing fiber stimulation on Purkinje cell firing rate

A repeated measures ANOVA with a Greenhouse–Geisser correction showed that increasing the frequency of the climbing fiber stimulation had a reliable inhibitory effect on Purkinje cell firing ($F_{(1.713,6850)} = 21.737, p = 0.0012$; Table 1). An example of the effect of sustained climbing fiber activation over an entire experimental session is illustrated in Figure 2A. Whereas stimulation with intensities below the threshold for eliciting complex spikes did not induce any suppression at any frequency tested, above threshold stimulation in the range 0.5-10 Hz, suppressed simple spike activity in a graded manner (Fig. 2B–E). Post hoc testing, using Bonferroni correction, revealed that stimulation at 4 Hz consistently resulted in a strong suppression of Purkinje cell activity ($84.04 ± 5\%, p = 0.0067$), and stimulation at 5 Hz all but silenced the Purkinje cells ($96.6 ± 1.5\%, p = 0.00002$). As illustrated in Figure 3, increasing the climbing fiber stimulation frequency, and thus decreasing Purkinje cell activity, did not affect the interspike interval distribution substantially. This inhibitory effect is consistent with results reported in previous investigations in which a complete suppression was induced at stimulation frequencies of 4–5 Hz (Rawson and Tilokskulchai, 1981; Demer et al., 1985). In one Purkinje cell, a 76.3% reduction in simple spike firing was observed already at 1 Hz, and increasing the stimulation frequency to

1.5 Hz all but silenced the cell (97.19% suppression over the 5 min session).

In the last stimulation session, we switched back to a stimulation frequency of 0.5 Hz. This resulted in a recovery of the Purkinje cell activity, yet the suppression persisted for an average of 118.6 ± 42.56 s. The average firing rate in the last 0.5 Hz stimulation session was 59.3 ± 12.5% ($p = 0.812$), although the Purkinje cell that exhibited almost complete suppression at 1.5 Hz remained suppressed throughout this session (Fig. 2B, Exp 4).

Contribution of post-complex spike pauses to the simple spike suppression

Complex spikes are usually followed by a characteristic pause in Purkinje cell activity that can last from 10 ms to a couple of hundred milliseconds (Latham and Paul, 1971; Simpson et al., 1996). Could the suppression of Purkinje cell activity reflect the combined effect of all post-complex spike pauses? To quantify the contribution from the post-complex spike pauses to the simple spike suppression, we calculated the average duration of the post-complex spike pause (population mean, 0.038 ± 0.021 s). Then we estimated the firing rate that should have been observed only if these post-complex spike pauses contributed to the suppression. The firing rate variation relative to the baseline was then recalculated. The estimated contribution of the post-complex spike pauses to the total suppression would account for at most 1.9% (range, 1.0–3.5%) at the lowest stimulation frequency used (0.5 Hz), and 34% (range, 18–63), at the highest stimulation frequency used (10 Hz).

Effects of sustained climbing fiber discharge on overt behavior

After stable conditioning had been achieved, curarization was discontinued, and the animals were tested with the climbing fiber stimulation protocol. This protocol consisted of seven experimental sessions separated by 5 min resting periods in which no stimulation was applied. Each session was composed of four blocks of 10 trials. The first block, which consisted of 10 paired trials, served as a control condition. Following this were two blocks, each consisting of 10 CS-alone trials. During these CS-alone trials, climbing fibers in the cerebellar peduncle were stimulated at a fixed frequency. Between sessions, we increased the frequency of the climbing fiber stimulation in a stepwise manner going from 1 to 4.5 Hz, in 0.5 Hz increments. To avoid the presence of stimulation artifacts in the electromyographic trace, climbing fiber stimulation was stopped for 1 s, starting 200 ms before the CS onset. A final block without climbing fiber stimulation served as a second control condition.

All animals developed ECRs at a normal rate, reaching a stable level of conditioning within 3–5 h (~500–700 trials). At this point, the CS elicited ECRs on 86 ± 5.7% of the trials. Figure 4 illustrates a climbing fiber field potential recorded on the cerebellar cortex (Fig. 4A) and an overt ECR recorded with EMG electrodes (Fig. 4B). The mean onset latency of the ECRs was 163 ± 39 ms, and the mean peak latency was 236 ± 44 ms after CS onset. The acquisition process was similar to that observed in previ-
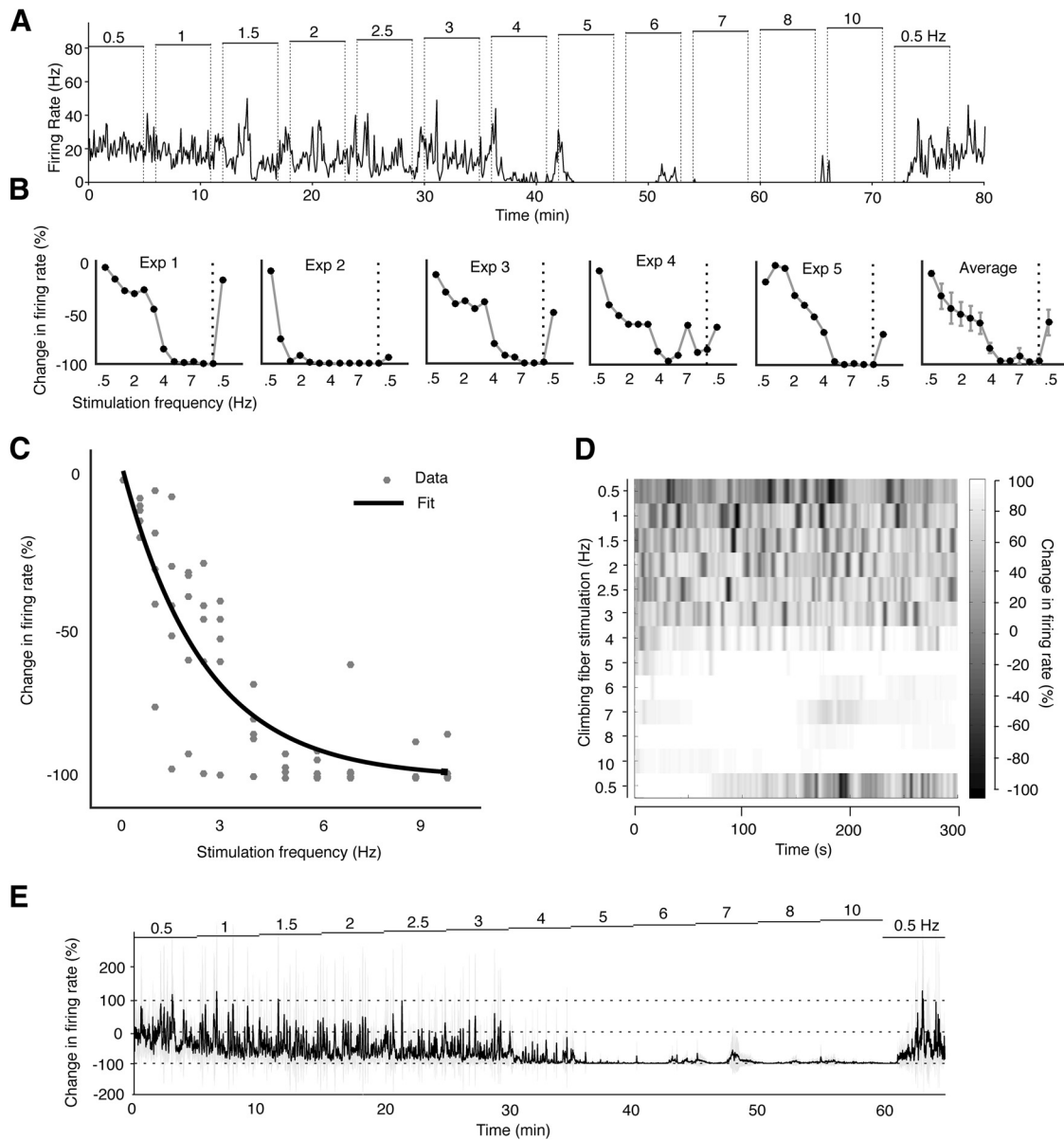
Figure 2. Stimulating climbing fibers suppresses Purkinje cell activity in a frequency dependent manner. **A**, Time course of the simple spike suppression in a Purkinje cell during a complete experimental session. Climbing fibers were stimulated with electrical pulses (duration, 0.1 ms; intensity, 240 μA), for 5 min at incremental frequencies, with 60 s breaks in between each switch in frequency. The firing rate was estimated through convoluting the spike train with a Gaussian kernel (sigma, 2 s). **B**, Change in Purkinje cell activity as a function of the climbing fiber stimulation frequency in each of the five cells, as well as the average change (right). **C**, Scatterplot illustrating the relation between the climbing fiber stimulation frequency on the x-axis and changes in Purkinje cell activity on the y-axis. The data fits an exponential curve (black line) described by a coefficient of 0.397. The best fit was obtained through the Matlab Curve fitting toolbox (MathWorks). **D**, Average raster plot of simple spike firing rate changes over time. The color of the shadings indicates simple spike activity changes relative to the pre-stimulation baseline (baseline = 0%). Lighter areas indicate inhibition of simple spike firing (i.e., 100% equals a complete suppression) and darker areas indicate increased activity. **E**, Time course of the simple spike activity changes over the entire stimulation session averaged for the five experiments. Each data point corresponds to the firing rate change over a 100 ms window. Light gray shadings represent a 95% confidence interval.

ous studies (Hesslow et al., 1999; Wetmore et al., 2014). Mirroring the effect on Purkinje cell activity, climbing fiber stimulation suppressed ECRs in a frequency-dependent manner (Figs. 4C-D, 5). The rate of ECRs in the paired trials preceding the stimulation did not differ between sessions (mean ECRs, 81.6 ± 5%), and it was compara-

ble to the ECR frequency observed at the end of acqui-sition, indicating that no extinction occurred during or between the experimental sessions. Stimulating the climbing fibers at frequencies from 1 to 2.5 Hz did not result in a substantial reduction of ECRs. However, stim-ulation at ≥3 Hz induced a strong suppression of ECRs.

Table 1: Statistical analysis

	Data structure	Type of test	Power
a.	Normal	Repeated-measures ANOVA	0.0012
	4 Hz stimulation	*Post hoc* (Bonferroni corrected)	0.0067
	5 Hz stimulation	*Post hoc* (Bonferroni corrected)	0.00002
	0.5 Hz stimulation (final session)	*Post hoc* (Bonferroni corrected)	0.812

For the highest stimulation intensities tested (4–4.5 Hz), ECR expression was completely blocked in the second experimental block (Figs. 4C-D, 5). For all the stimulation frequencies tested, the rate of ECRs in the fourth block, when climbing fiber stimulation was discontinued, returned to the control level (mean, 76 ± 6.7%).

Consistent with the fact that Purkinje cells inhibit the cerebellar nuclei, climbing fiber stimulation, which induces a suppression of Purkinje cell activity, resulted in increased EMG activity at the higher stimulation frequencies (Fig. 5). On a few occasions, we also observed muscle tremor during climbing fiber stimulation at frequencies above the ones reported in this study. However, due to the risk of tissue damage around the stimulation electrode, such stimulation frequencies were not tested further.

Discussion

Results summary
Our results show that stimulating climbing fibers at 0.5–3 Hz induces a frequency-dependent, graded suppression of Purkinje cell activity. Climbing fiber stimulation at ≥4 Hz completely silences Purkinje cells and causes an abolition of conditioned blink responses, which are known to depend on the cerebellum. Given that these results were obtained in decerebrated, unanesthetized ferrets, we can exclude the possibility that the results were due to or influenced by anesthesia that has previously been shown to affect the cerebellum (Schonewille et al., 2006; Bengtsson and Jörntell, 2007).

Relationship between climbing fiber stimulation and Purkinje cell activity
Prior studies have explored how olivary input shapes Purkinje cell activity. These studies have provided ample evidence, from several species, that lesioning or cooling the inferior olive increases Purkinje cell firing, whereas stimulating the climbing fibers suppresses or silences the Purkinje cells (Colin et al., 1980; Rawson and Tilokskulchai, 1981; Montarolo et al., 1982; Demer et al., 1985; Savio and Tempia, 1985; Cerminara and Rawson, 2004). However, these reports mainly show that higher stimulation frequencies induce stronger suppression of Purkinje cell activity and then report the threshold for completely silencing Purkinje cells. Moreover, the reported estimates have ranged between 2 Hz (Colin et al., 1980) and 8–10 Hz (Rawson and Tilokskulchai, 1981). Possible reasons for this variance are that the data come from different species and that different types of anesthesia were used. Our study thus adds to the existing body of evidence by providing a detailed description of the gradual suppression of Purkinje cell activity induced by small incremental increases in the frequency of climbing fiber stimulation, in an unanesthetized animal. Overall, the relation between climbing fiber stimulation and simple spike
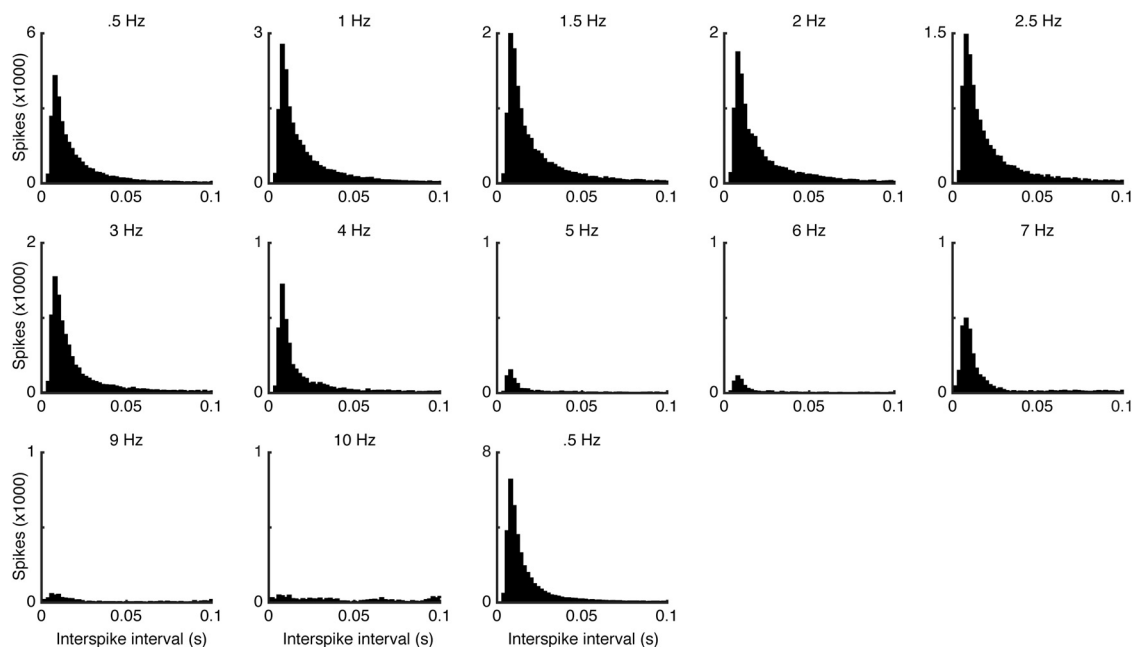

Figure 3. Interspike interval distributions for each climbing fiber stimulation frequency (bin size, 2 ms).
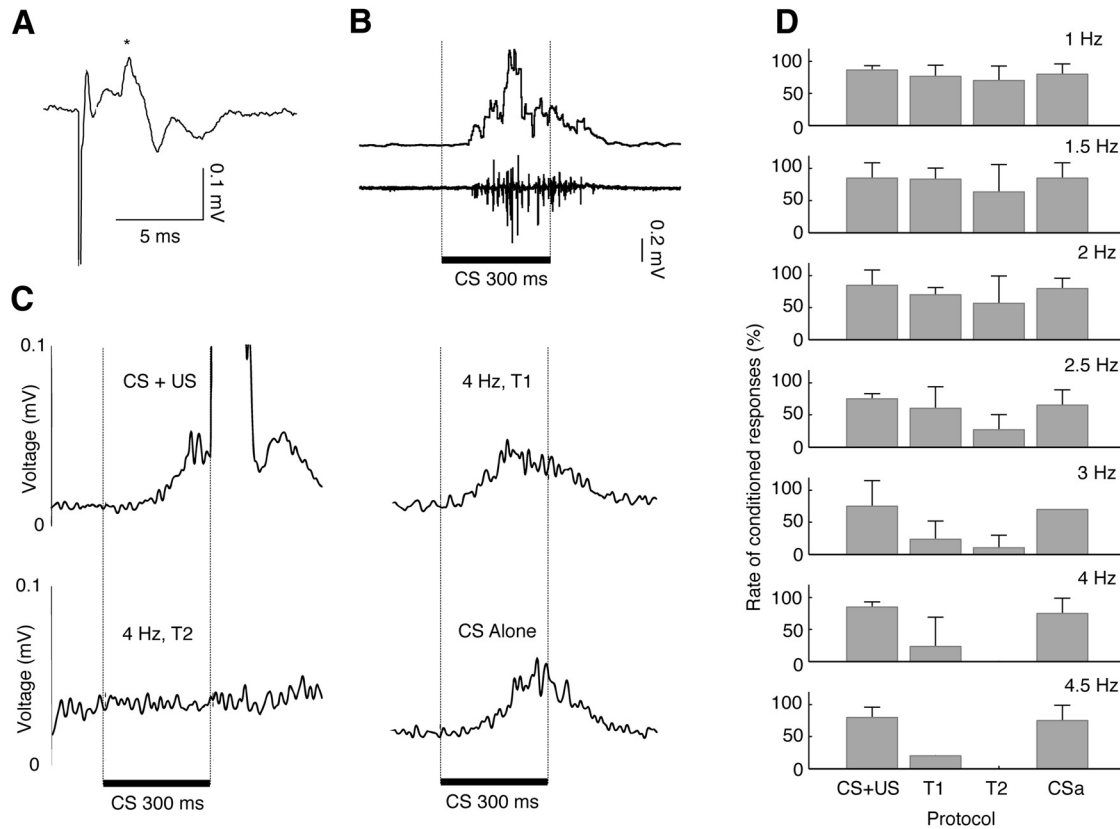
A

B

C

D

Figure 4. A, Average of 10 consecutive field potentials recorded on the cerebellar cortex following direct stimulation of climbing fibers. Asterisk indicates the climbing fiber response. **B**, EMG from the orbicularis oculi muscle recording on a single CS-alone trial in a trained animal. The top trace shows a rectified and smoothed trace (smoothing window 10 ms). The bottom trace shows the raw signal. **C**, Rectified and smoothed EMG on paired trials without climbing fiber stimulation (top left), CS-alone trials with climbing fiber stimulation at 4 Hz (top right and bottom left), and on CS-alone trials without climbing fiber stimulation (bottom right). **D**, Effect of sustained climbing fiber stimulation at 1–4.5 Hz on the rate of conditioned eyeblink responses (ECR). Each bar plot shows the percentage of ECRs (+ SEM), in blocks of 10 trials, for each stimulation frequency.

activity conforms to observations made in previous studies. For instance, Demer et al., (1985) found that stimulating at ≥4 Hz silences most, but not all, Purkinje cells. It is probably no coincidence that the inferior olive fires only at frequencies >5 Hz under artificial circumstances (Colin et al., 1980; Ekerot et al., 1987). In one cell, we did not see complete suppression even when stimulating climbing fibers at 10 Hz. This could indicate that we did not saturate the underlying mechanism responsible for the suppression. Alternatively, it means that some cells are not completely silenced by high-frequency climbing fiber stimulation, which would also be consistent with observations made by Demer et al., (1985).

How do climbing fibers suppress Purkinje cell activity? So far, two potentially complementary mechanisms have been proposed. First, climbing fiber stimulation can suppress Purkinje cells through activating inhibitory interneurons in the cerebellar cortex, which then inhibit Purkinje cells (Bloedel and Roberts, 1971; Schwarz and Welsh, 2001; Badura et al., 2013). In line with this alternative, optogenetic stimulation of the inferior olive activates basket cells, which are powerful inhibitors of Purkinje cells (Mathews et al., 2012). One argument against this mech-

anism is that subthreshold stimulation of climbing fibers, which does not elicit complex spikes but probably activates interneurons near the recorded Purkinje cell, did not affect Purkinje cell activity. Other researchers have proposed that climbing fiber activity inhibits Purkinje cells directly (Rawson and Tilokskulchai, 1981), possibly through activation of small-conductance, calcium-activated potassium channels, which are found in Purkinje cells (Hosy et al., 2011). Activation of these on the Purkinje cells causes a suppression of the neuronal activity lasting up to a couple of hundred milliseconds. The fact that Purkinje cells are silenced completely when climbing fibers are stimulated at 4 Hz may indicate that these channels mediate the inhibition. It is also important to remember that the cerebellum consists of different zones (Voogd, 1969; Oscarsson and Iggo, 1973; Armstrong, 1974; Ekerot and Larson, 1979; Voogd and Glickstein, 1998), which operate according to somewhat distinct molecular mechanisms (Zhou et al., 2014; Cerminara et al., 2015). Thus, the climbing fiber inhibition of Purkinje cell activity might not act uniformly across the cerebellum. Indeed, these zonal differences could potentially explain the variance in the suppression caused by climbing fiber stimulation, which has been observed in different studies.
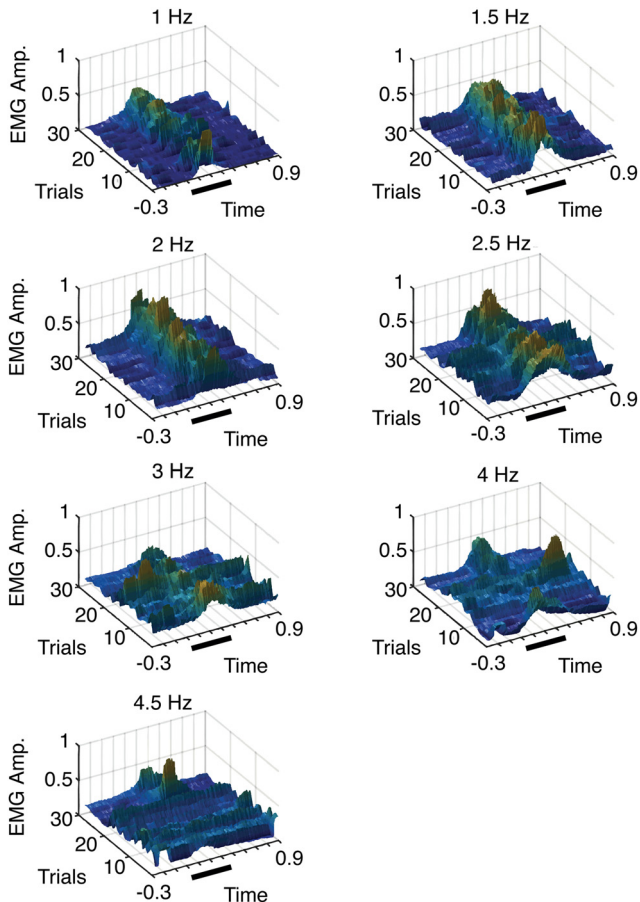
Figure 5. 3D surface plots illustrating orbicularis oculi EMG in one animal in the last three test blocks for each stimulation frequency. Data were rectified and binned over a 50 ms window and normalized to the highest value over the seven recording sessions.

The finding that even relatively low stimulation frequencies have effects on Purkinje cell activity highlights the importance of the regulatory effect of climbing fiber input. In addition to the suppression of simple spike firing, we sometimes also observed a suppression of the spontaneous complex spike activity. This was not further investigated here, and we can only speculate on the mechanisms behind the observation. Thus, as the simple spike activity drops, there should be an increase in the spontaneous activity in the nuclear cells. Presumably, this is true for the projection cells as well as the inhibitory olivary projecting cells (De Zeeuw and Berrebi, 1995). Consequently, the activity in the inferior olive, as well as the complex spike frequency in Purkinje cells, should be reduced (Andersson and Hesslow, 1987a,b). Importantly, the Purkinje cell firing returned to normal levels when high-frequency stimulation was stopped, suggesting that the sustained stimulation did not cause any pathological change to the Purkinje cells. It has been hypothesized that there is an optimal range of background activity within which the parallel fiber input is integrated (Cerminara and Rawson, 2004). This would ensure that the signal-to-noise ratio is kept within a range that is optimal both for synaptic

plasticity and for Purkinje cell control of activity in the cerebellar nuclei and, hence, the output from the cerebellum (Bengtsson and Hesslow, 2013).

While the inferior olive regulates Purkinje cell activity, the activity of the olive itself is influenced by activation history (Hansel and Linden, 2000) and by GABAergic input from the cerebellar nuclei (Hesslow, 1986; Andersson et al., 1988; De Zeeuw et al., 1988; Nelson et al., 1989; Lang et al., 1996). As stated above, activation of the climbing fibers is bound to have subsequent effects all through the cerebello-olivary circuit (Bengtsson et al., 2004; Bengtsson and Hesslow, 2006). Moreover, direct stimulation of climbing fibers, as in our case, is bound to activate many climbing fibers, resulting in synchronous activation of many Purkinje cells (Lang et al., 1999). This synchronous activation will activate or inhibit downstream targets like the inhibitory interneurons (Szapiro and Barbour, 2007) and the cerebellar nuclei (Llinás and Mühlethaler, 1988; Bengtsson et al., 2011; Person and Raman, 2012). As the level of synchrony was not measured here, we cannot exclude that the activation may have had exaggerated circuitry effects (Bengtsson et al., 2011). There is also ample evidence that the inferior olive, which fires in bursts of 1-6 spikes (Maruta et al., 2007; Mathy et al., 2009), can be regulated in a graded manner (Najafi and Medina, 2013; Rasmussen and Hesslow, 2014), and that the number of spikes in an olivary burst can influence subsequent learning (Rasmussen et al., 2013, 2015; Yang and Lisberger, 2014). In other words, even a small increase in olivary activity can potentially cause changes to all parts of the cerebellar network, including the olive itself.

Behavior

When extended to behavior, sustained climbing fiber stimulation in the intertrial period has a strong impact on the expression of ECRs. Stimulation at frequencies that silenced Purkinje cell activity also caused an abolition of ECRs. Given that there is a tight link between Purkinje cell activity and eyelid movements (Hesslow, 1994b; Heiney et al., 2014; Halverson et al., 2015), the present results suggest that high-frequency climbing fiber stimulation abolishes ECRs by silencing Purkinje cells, which leads to increased activity in the cerebellar nuclei and thus cerebellar output. In other words, high-frequency climbing fiber stimulation disrupts the entire cerebellar network and, in extension, cerebellum-dependent behavior. The fast recovery of ECRs when stimulation was stopped is consistent with the behavioral effect of injecting harmaline in the olive. Such injections influence NMDA receptors (Du and Harvey, 1997), leading to high levels of climbing fiber activity, which in turn prevent motor learning, including the acquisition of ECRs (Türker and Miles, 1984; Welsh, 1998). Our results are also in agreement with other pharmacological studies reporting an abolition of ECRs after manipulation of the inhibition of the cerebellar nuclei (Yeo et al., 1986; Parker et al., 2009). Suppression of the cerebellar nuclei through the application of a GABA agonist prevents conditioned responses because the nuclei cannot trigger motor responses. If instead activity in the cerebellar nuclei is increased through the application of a GABA antagonist or, as in this study, through climbing

fiber stimulation, conditioned responses are abolished because of sustained activation of eyelid muscles, which prevents further modulation of the blink response (Aksenov et al., 2004; Parker et al., 2009).

Conclusion

The fact that high-frequency climbing fiber stimulation disrupts Purkinje cell activity as well as cerebellum-dependent blink responses emphasizes the importance of considering the cerebellar network as a whole. Cerebellar function relies on the interaction among all parts of the cerebellar network, and disrupting any part of the network can affect other parts of the network in ways that can be difficult to anticipate. These effects are particularly important to consider when using anesthesia or when lesioning parts of the cerebellum, because such interventions can cast the entire system off balance.

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