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ORIGINAL PAPER

Links Between Single-Trial Changes and Learning Rate in Eyelid Conditioning

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Abstract The discovery of single-trial learning effects, where the presence or absence (or the number) of climbing fiber inputs produces measureable changes in Purkinje cell response and in behavior, represents a major breakthrough in cerebellar learning. Among other things, these observations provide strong links between climbing fiber-mediated plasticity and cerebellar learning. They also demonstrate that cerebellar learning is stochastic, with each instantiation of a movement producing a small increment or decrement in gain. The sum of the small changes give rise to the macroscopic properties of cerebellar learning. We used a relatively large data set from another example of cerebellar-dependent learning, classical conditioning of eyelid responses, to attempt a behavioral replication and extension of single-trial learning effects. As a normal part of training, stimulus-alone trials provide instances where the climbing fiber response would be omitted, similar to non-climbing-fiber trials (gain down) during smooth pursuit training. The consequences of the stimulus-alone trial on the amplitude and timing of the conditioned response on the following paired trials were examined. We find that the amplitude of the conditioned response during the trial after a stimulus-alone trial (no climbing fiber input) was measurably smaller than the amplitude on the previous trials, and this single-trial effect on amplitude is larger for longer interstimulus intervals. The magnitude of the single-trial effect parallels the rate of extinction at different interstimulus intervals supporting the previously observed link between single-trial effects and learning.

Keywords Climbing fiber · LTD · LTP · Stochastic learning · Timing

Introduction

A strong understanding of cerebellar learning requires establishing causal links between plasticity at cerebellar synapses and changes in behavior mediated by the cerebellum. It is also important to determine how properties of plasticity interact with properties of circuits to confer to the cerebellum its processing and learning capabilities. A series of studies from the Lisberger lab [1, 2] provided a remarkable step toward these goals using smooth-pursuit eye movements. They demonstrated that the presence or absence of a climbing fiber input to a Purkinje cell produces measurable changes in both the Purkinje cell response and the pursuit response on the next trial. Because the direction of these changes is consistent with the climbing-fiber-induced plasticity at cerebellar synapses, these experiments represent as strong a correlation as can be established between plasticity at Purkinje cell synapses and cerebellar learning. These data also demonstrate that cerebellar learning is incremental and to a degree stochastic. Each movement will either involve a climbing fiber input or not, and thus the next movement should either be a little larger or smaller, respectively.

The fundamental importance of these findings highlights the value of replication using a different cerebellar-dependent form of learning such as eyelid conditioning. Particular properties of eyelid conditioning permit a relatively powerful yet simple to implement test of single-trial changes in cerebellar learning. Eyelid conditioning involves pairing a conditioned stimulus (CS) such as a tone with a reinforcing unconditioned

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stimulus (US), which in our experiments involves subdermal stimulation of the skin near the eye. In untrained animals, the US elicits a reflex response—the eyelids close. With paired presentations of CS and US, the tone CS acquires the ability to elicit a learned eyelid closure. As outlined in numerous reviews, what makes this learning useful is that the CS is conveyed to the cerebellum via activation of mossy fibers and the US by activation of climbing fiber inputs to the cerebellum [3, 4]. Output from the anterior interpositus deep nucleus drives the expression of conditioned responses (CRs) [5–7]. Eyelid conditioning therefore provides an experimentally tractable means to control cerebellar inputs and to infer cerebellar output via measurement of eyelid responses. In order to gain more control over the precise mossy fiber inputs to the cerebellum relative to a tone stimulus, it is possible to directly electrically stimulate mossy fibers as a substitute for the peripheral CS. This technique isolates learning mechanisms in the cerebellum from outside influences and produces learning that is indistinguishable from learning with a tone CS [8, 9].

Here, we make use of a common feature of eyelid conditioning training to implement a test of single-trial changes in behavior mediated by the cerebellum. Most training sessions involve occasional CS-alone trials to reveal the full time course of the CRs uncontaminated by the reflexive response to the US. When CS-alone trials are given repeatedly, extinction occurs, where response amplitude decreases until, with enough CS-alone training, the responses can fully disappear [10]. Previous studies have demonstrated that climbing fiber inputs to Purkinje cells are suppressed during the expression of a conditioned response (CR) on CS-alone trials [11, 12] and that indeed this suppression is the signal for extinction (or gain down) learning in the cerebellum [13]. This means that in well-trained animals, these occasional CS-alone trials represent instances where the CS was presented almost certainly in the absence of a climbing fiber input.

We made use of the very large number of animals that have been trained in the delay eyelid conditioning paradigm to ask whether there is a detectable decrease in CR amplitude in the trial following the CS-alone trials. While this is a purely behavioral—and one-directional—attempt at replicating the Medina and Lisberger [1] findings, the large number of animals that have been trained this way provides strong statistical power to investigate the details of the behavioral effects of CS-alone trials on CRs. In addition, because different groups of animals were conditioned with either tone or electrical mossy fiber stimulation as a CS, comparing single-trial effects between these groups, we can either detect or exclude contributions of upstream CS processing from specifically cerebellar learning mechanisms. Moreover, for both CSs, animals were trained with a broad range of inter-stimulus intervals (ISIs), ranging from ISI 200 to ISI 1500, thus permitting investigation of the interaction between single-trial effects with ISI length. We have observed, for example, an almost 20-fold difference in the rate of extinction of CRs between the shortest and longest ISIs examined in the present analyses (Ohyama and Mauk, unpublished). Whereas, at the shortest ISI, responses may require several 100-trial sessions to extinguish, responses from animals trained at the longer ISIs can require as few as 10 trials to extinguish. Thus, we hoped to see whether there are ISI-dependent differences in the changes in CRs that immediately follow CS-alone trials.

Our results provide a clear replication of the main single-trial effect first shown by Medina and Lisberger and further demonstrate that the size of the changes as a consequence of single CS-alone trials scales with ISI duration and matches the different rates of extinction for those ISIs. The data also clearly demonstrate that quite similar results are observed independent of whether training involves a tone CS or stimulation of mossy fibers, and thus that any factors upstream of the cerebellum can be excluded. These findings support the implications of the original single-trial findings by showing that single trials with no climbing fiber input produce detectable decreases in responses on the next trial. The present data are also clearly consistent with the incremental and stochastic nature of cerebellar learning first revealed by Medina and Lisberger.

Methods

Subjects

Data were obtained from 160 male New Zealand albino rabbits (*Oryctolagus cuniculus*, Myrtle's Rabbitry, Thompsons Station, TN). The animals weighed between 2.5 and 3 kg at the time of surgery, were individually housed, were fed daily, and had free access to water. Treatment of animals and surgical procedures were in accordance with National Institutes of Health Guidelines and an institutionally approved animal welfare protocol.

Surgery

Regardless of experimental preparation, all rabbits received a similar surgery with respect to the method of behavioral data acquisition. Before surgery, a preanesthetic (40 mg/kg ketamine and 5 mg/kg acepromazine) was injected subdermally, and each rabbit was positioned in a stereotaxic restrainer such that lambda was 1.5 mm ventral to bregma. General anesthesia was maintained with isofluorene (2 % mixed in oxygen), and sterile procedures were used throughout the surgery. After a lidocane injection into the scalp, a midline incision was made and the skin and underlying tissue were retracted and held in place with hemostats. Three holes were drilled in the skull, which allowed insertion of stainless steel anchor screws. Anchor screws also functioned as ground screws in the mossy fiber stimulation animals. Rabbits prepared for mossy fiber

stimulation received a single or two laterally spaced (1 mm) stimulating electrodes (A-M Systems, Carlsborg, WA; tip exposed to obtain impedance of 100–200 kΩ) in the middle cerebellar peduncle ipsilateral to the trained eye (5.5 mm lateral, 16 mm ventral, and 3 mm anterior from lambda). The anchor/ground screws, mossy fiber stimulation electrodes, and a head bolt for the infrared emitter/detector were all secured to the skull with dental acrylic. Stainless steel loops terminating in gold pins were inserted into the caudal and rostral periorbital region of the left eye for delivery of the stimulation US. Rabbits were given postoperative analgesics and antibiotics for 2 days after surgery and were allowed to recover for a week before experiments began.

Conditioning

Animals were trained in custom-designed, well-ventilated, and sound-attenuating chambers measuring 90×60×60 cm (length, width, height). Each chamber was equipped with a speaker that was connected to an audio source module (model V85-05, Coulborn Instruments, Allentown, PA) or Windows-based PC used to generate tones. Electrical leads from a stimulus isolator (model 2100, A-M Systems, Carlsborg, WA) were attached to the periorbital electrodes to deliver pulses of electrical stimulation used as the US. For rabbits trained with mossy fiber stimulation as a CS, separate stimulators were used to time the delivery of constant current pulses initiated by custom software through stimulus isolators (model 2300, A-M Systems, Carlsborg, WA), which were connected with gold pins to the electrodes implanted in the middle cerebellar peduncle. To measure eyelid position, an infrared emitter/detector was attached directly to the head stage of each rabbit to record movements of the left external eyelid. These detectors provide a linear readout of eyelid position by measuring the amount of infrared light reflected back to the detector, which increases as the eye closes [14]. At the start of each daily session, the detector was calibrated by delivering the US to elicit maximum eye closure. The amplification of the signal was adjusted to match an assumed maximum eye closure of 6 mm.

Stimulus presentation was controlled by custom-designed software for all experiments. Rabbits were given daily eyelid conditioning sessions comprised of 12 blocks of 9 trials (1 CS-alone trial and 8 paired trials per block, 108 trials total). In some cases, the CS-alone trial occurred at the beginning of each block while other training paradigms presented the CS-alone trial at the end of each block. Training trials involving presentation of a tone CS were typically a 1- or 9.5-kHz, 85-dB sinusoidal tone with a rise and fall time of 5 ms to avoid audible clicks from the speaker. The US was a 50-ms train of constant current pulses (50 Hz, 1-ms pulse width, 2–3 mA) delivered through the periorbital electrodes. Rabbits were trained at four different ISIs using the tone CS (128 animals), 200, 250, 500, and 1000.

Rabbits receiving daily eyelid conditioning sessions with mossy fiber stimulation as the CS (32 animals) consisted of passing cathodal current stimulation (100 Hz, 100-μs pulse width, 100 μA) through electrodes implanted in the middle cerebellar peduncle. Rabbits were trained with four different ISIs using mossy fiber stimulation as the CS, 250, 500, 750, and 1500. Both tones and mossy fiber stimulation as CSs lasted for 50 ms longer than the ISI to allow the CS and US to co-terminate. Trials using both tones and mossy fiber stimulation were separated by a mean intertrial interval of 30± 10 s.

Data Analysis

For each trial, 2500 ms of eyelid position (200 ms pre-CS, 2300 ms post-CS) was collected at 1 kHz and at 12-bit resolution. Data were stored to a computer disk and analyzed off-line using custom-written scripts in MATLAB. Eyelid position measured 200 ms before each trial established a baseline for detecting eyelid movement during each trial. Trials were excluded from the analysis if a movement greater than 0.3 mm occurred during the 200-ms baseline period before CS onset. A CR was defined as an eyelid closure of at least 0.3 mm with an onset between 30 ms after CS onset and the onset of the US. Amplitude of the CR was defined as the value of eyelid position at the time of US onset (or where it would had been for CS-alone trials). Error bars in all figures indicate SEM. CR percentage or likelihood, used in the analysis, refers to the fraction of trials where the response satisfied the CR criterion (number of CR trials divided by the total number of valid trials). In the present analysis, we used amplitude and CR likelihood measures to investigate the single CS-alone trial effect. For all analysis except acquisition results shown in Fig. 1, we included only sessions with at least 70 % CRs, corresponding typically to sessions 3–5 from Fig. 1

Results

All data were taken from animals trained for other purposes, such as acquiring CRs prior to a lesion or reversible inactivation experiment. All of these manipulations occurred after the data presented here. It is also the case that none of the animals had received prior training experience at a different ISI. The analyses make use of the fact that with a larger than usual number of subjects per group, it is possible to detect small but significant differences in the amplitudes of CRs in the trials that follow the CS-alone test trials that are presented as part of our normal training protocols. Figure 1a shows a prototypical acquisition curve for eyelid conditioning, in this instance for the animals that were trained for five sessions using

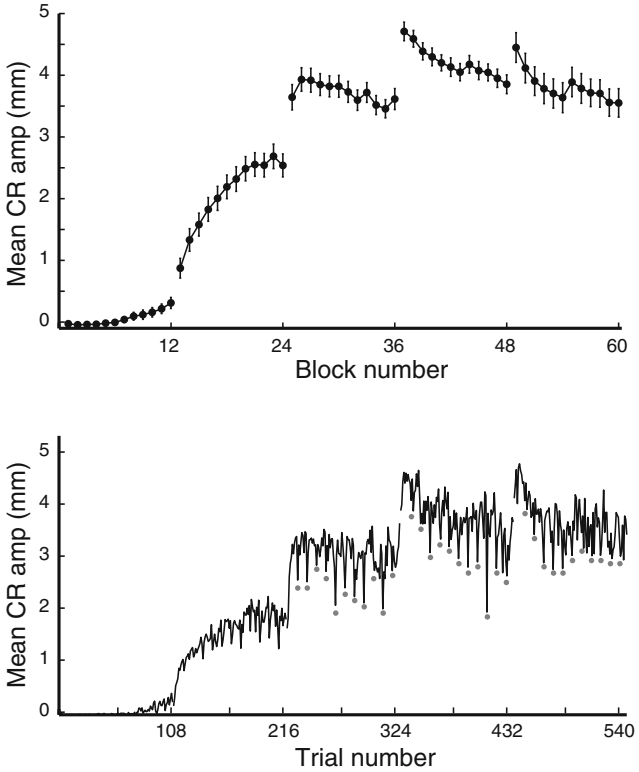

Fig. 1 **a** The acquisition of conditioned eyelid responses using a 500-ms ISI is reflected in a systematic increase in mean response amplitude over 5 days of training. Each training session was comprised of 12 blocks of nine trials, with each block comprised of eight CS+US paired trials and one CS-alone test trial. Each data point is the mean of the block average across 99 subjects trained in this way. There are characteristic features of this behavior reported in previous papers such as the decline in amplitude within each session and recovery seen as an increase in amplitude at the beginning of each session. **b** The same data plotted as the average of the individual trials across 39 subjects. This reveals that there is a noticeable decrease in amplitude in the trials following the CS-alone trials (trials after CS-alone trials indicated by *gray circles* for the last three sessions). These animals were trained with the CS-alone trials at the beginning of each block, and the remaining subjects, whose data were omitted in panel (**b**), were trained with CS-alone trials at the end of each block. These animals showed the same trend: a decrease in amplitude in the trials following a CS-alone test trial (not shown)

a tone CS and an ISI of 500 ms. As is not uncommon for eyelid conditioning experiments, these data are expressed as mean amplitude for each block of nine trials, in this instance 12 blocks per training session with the five training sessions indicated by each separation in the graphs. The mean CR amplitude grows over the 5 days of training with characteristics that have been observed and analyzed previously. For example, after initial training, there is a clear decrease in CR amplitude within sessions (sessions 3–5) that recovers between sessions, consistent with the short-term plasticity processes that have been shown to operate to decrease CR amplitude [15, 16]. Figure 1b shows the same data from approximately half of these animals where the data are expressed as averages of single trials across the five sessions. These data are from tone CS and ISI 500 animals that received CS-alone trials at the beginning of each block; similar results were observed from the remaining animals where the CS-alone trial was at the end of each block (not shown). Expressed as averages of single trials, we can now see that there are regular decreases in response amplitude during sessions with robust responding (the last three sessions in this case). These smaller amplitude responses are from the trials that immediately followed the CS-alone trials and are indicated with gray circles below the average curves in the last three sessions. In the remainder of this manuscript, we analyze this effect in eight different groups of animals: (1) four trained with a tone CS and one of four different ISIs (200, 250, 500, and 1000 ms), and (2) four groups trained with mossy fiber stimulation as the CS, and using one of four different ISIs (250, 500, 750, and 1500 ms).

This effect following CS-alone trials is observed more clearly when data are collapsed across all blocks for all training sessions that satisfied the criterion of greater than 70 % CRs over the session, and then collapsed across all animals in the same CS/ISI group (Fig. 2). All data points in the upper panels of Fig. 2 are normalized to the mean CR amplitude of the CS-alone response and the responses from the two preceding trials. Each graph shows these three trials and the eight paired CS + US trials that followed the CS-alone trial (indicated by the red arrow in each panel). The data for the four ISI groups trained using a tone CS are shown in Fig. 2a, with the ISI groups color-coded as indicated. The upper panel shows that there was a decrease in mean CR amplitude in the trial following a CS-alone trial and that this effect is clearly larger for the longer two ISIs (ISI 500 and 1000) that were tested. This same panel also shows that over the subsequent seven CS + US trials, the CR amplitude gradually increases. The horizontal black bar indicates the trials for which there was a significant overall decrease in CR amplitude as compared to the CS-alone trial. The asterisk indicates the trials for which the decrease for the different ISIs were significantly different. These changes in the mean CR amplitude could potentially be accounted for entirely by a decrease in CR likelihood. In order to examine this possibility, we performed the same analyses for the CR likelihood measure (Fig. 2a, middle panel). Here the change following the CS-alone trial is clearly smaller than the changes in amplitude, suggesting that the effect on amplitude is at least not entirely explained by an increase in CR failure following a CS-alone trial. The histogram at the bottom of Fig. 2a shows for each of the four ISIs the mean change in response amplitude (in mm) between trials just preceding and immediately following the CS-alone trial; a negative value indicates the response amplitude decreased after the CS-alone trial. The decrease in amplitude is significant for the majority of ISIs (two-tailed t test, $p<0.05$ for ISI 250 and $p<0.01$ for ISIs 500 and 1000), and differences between ISI groups are indicated by horizontal bars (one-way ANOVA,

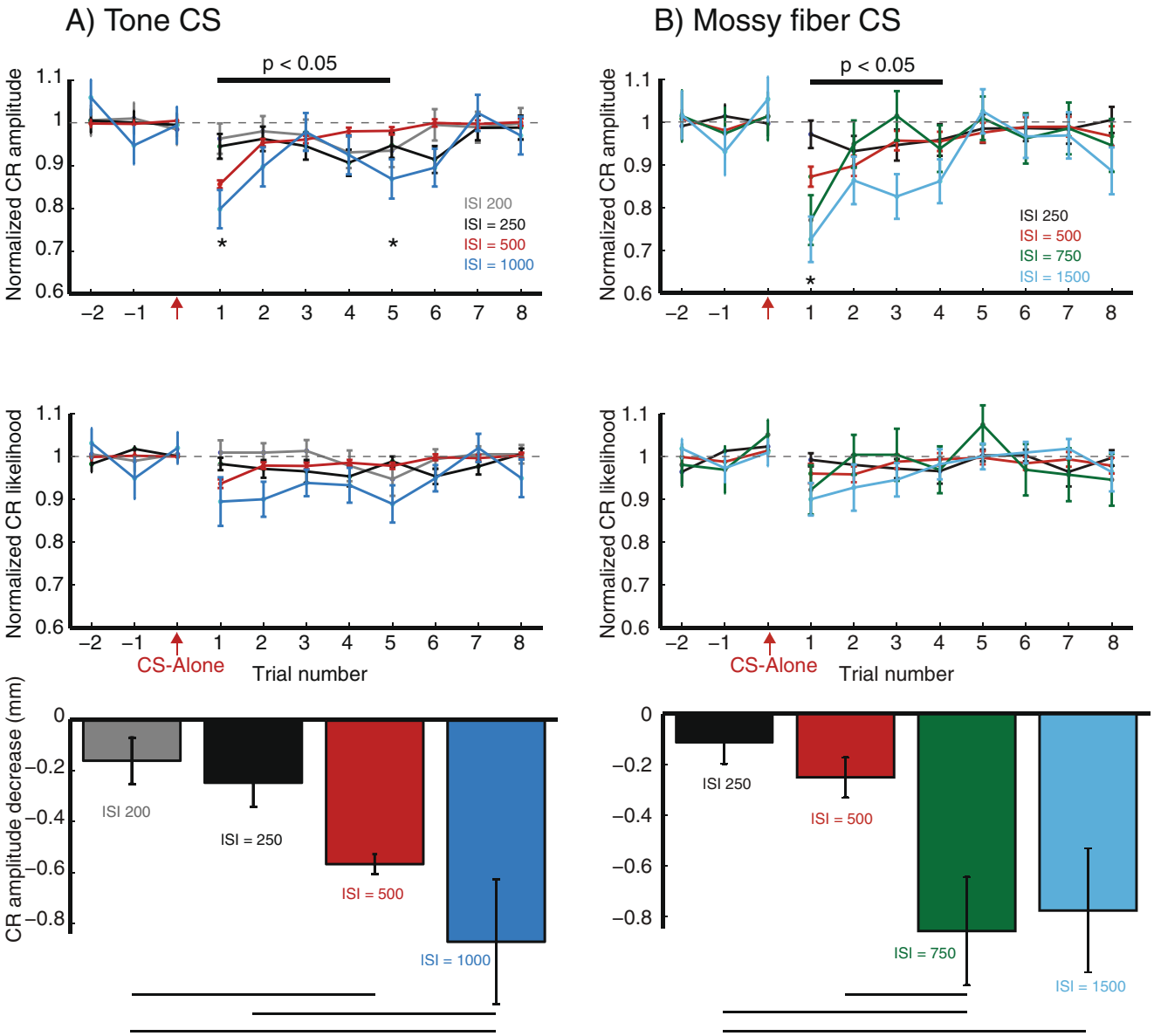

Fig. 2 The decrease in response amplitude following CS-alone trials is seen in animals trained using a tone CS (**a**) and with animals trained using mossy fiber stimulation as the CS (**b**). The *upper panels* show the effects of CS-alone trials on CR amplitude by averaging across all CS-alone trials for all animals. Each data point is normalized to the average amplitude (for that block, see the "Methods" section) of the CS-alone trial and the two trials that preceded it. The *red arrow* indicates the CS-alone trials. Each graph therefore shows the amplitude of the CS-alone trial, the two trials that preceded it, and the eight trials that followed it. There are small differences in trials -2 and -1 versus 7 and 8 because, for each session, either the first CS-alone trial was omitted if it was the very first of the session or the last CS-alone trial was omitted if it was the very last trial of the session. The *middle panels* show CR likelihood data normalized in the same fashion. The *bottom graphs* show for the different ISI groups the mean change of CR amplitude (in mm) in the trial that followed a CS-alone trial compared to the trial preceding it. **a** Using a tone CS, there was a significant decrease in CR amplitude after a CS-alone trial (*upper panel*) that (1) recovered over the next eight paired CS+US trials and (2) generally increased in amplitude as the ISI increased. Four ISIs were used with a tone CS, 200 ms (*gray*), 250 ms (*black*), 500 ms (*red*), and 1000 ms (*blue*). The *middle panel* shows that effects are smaller with the CR likelihood measure, indicating that the decrease in CR amplitude following CS-alone trials is not entirely explained by an increase in CR failures. The *bottom panel* shows that there were significant differences for each ISI and between the ISIs as indicated by the *horizontal bars* and *asterisks*. **b** The same general results were seen in animals trained with mossy fiber stimulation as the CS rather than a tone CS. The *upper panel* shows the normalized amplitude data, with clear decreases in the amplitude of the trials following the CS-alone trial. These decreases were larger for longer ISIs and recovered over the following eight CS+US paired trials. The *middle panel* shows, as with the tone CS, that the effects were much smaller when expressed as normalized CR likelihood. The bar graph at the *bottom* shows again that the mean decrease in amplitude is significant for each ISI and increases across the ISIs. Significant differences between ISI groups are shown with *horizontal bars* and *asterisks*. As with the tone CS data, *black* indicates ISI 250 ms and *red* indicates ISI=500 ms. With mossy fiber stimulation animals, there were two new ISIs, *green* indicates ISI=750 ms, and *cyan* indicates ISI=1500 ms (Color figure online)

$F_{(3,369)}=5.19$, $p=0.002$). The four groups trained using mossy fiber stimulation as the CS showed essentially the same pattern of results (Fig. 2b). The top panel of Fig. 2b shows a similar decrease in CR amplitude following CS-alone trials as was seen with the tone CS. These results are also similar in that the largest effects on amplitude were seen with the longer ISIs. Also similar to the tone CS data, there was a much smaller effect observed in the CR likelihood measure (middle panel, Fig. 2b). The histogram at the bottom of Fig. 2b compares the decrease in CR amplitude across the four ISIs tested. ISIs are color-coded, and ISIs tested for both tone and mossy fiber CSs maintain color coding (black = ISI 250, red = ISI 500 ms). As with tone, CR amplitude decreases were significant for the majority of ISIs (two-tailed *t* test, $p<0.01$ for ISIs 500, 750, and 1500), and the larger decreases in CR amplitude were observed with the longer ISIs (one-way ANOVA, $F_{(3,57)}=4.11$, $p=0.011$)

Together, these data show statistically reliable decreases in CR amplitude following CS-alone trials. We observed the same pattern independent of whether the CS was a tone or mossy fiber stimulation, eliminating the possibility that the cause of the single-trial effect can be upstream of the cerebellum. This effect increases with the ISI, consistent with observations that the rate of extinction in delay eyelid conditioning is quite slow for shorter ISIs and is much faster for longer ISIs (Ohyama and Mauk, unpublished). The incremental nature of cerebellar learning is illustrated in Fig. 2, which shows how the mean CR amplitude or likelihood returns to the baseline level with additional CS + US trials. While these increases are generally consistent with the "climbing fiber is present" portion of the Medina and Lisberger study, we do not pursue it further here because we have no way at the level of behavior to parse trials where there was a complex spike present from those where it was absent. Overall, this pattern of results is consistent with the "climbing fiber absent" portion of the Medina and Lisberger observation of single-trial changes in behavior, with the addition that, in this case, these changes correlate with the variations in the rate of learning (extinction) over different experimental conditions.

To characterize the nature of the changes in CRs produced by single CS-alone trials, we calculated frequency histograms of the differences between CR amplitudes in the trials just preceding and immediately following CS-alone trials (Fig. 3a, b). Here a negative value indicates a decrease in amplitude compared to the preceding CS-alone trial. The histograms are arranged with ISI increasing from left to right and are staggered so that the two ISIs used for both mossy fiber stimulation (Fig. 3a) and tone CS (Fig. 3b) animals are aligned vertically (ISI=250 ms shown in the gray box and ISI=500 in the lighter gray box). For each of the eight panels in Fig. 3a, b, zero change is denoted with a vertical dotted line and also in each panel the mean (black arrow) and the median (gray arrow) are less than zero, indicating a decrease in response amplitude. Distributions for each ISI and CS are skewed in the negative direction reflecting that the decrease in CR amplitude after CS-alone trials is a general property of cerebellar learning.

Because the trial-to-trial variability of CR amplitude increases with the ISI, we compared the changes in CR amplitude following a CS-alone trial with the population of trial-to-trial changes in CR amplitude for each ISI. Figure 3c shows frequency histogram of the population trial-to-trial change in CR amplitude. These were calculated by randomly sampling pairs of trials that a trial in between them from the same sessions used to calculate the effects of CS-alone trials. Figure 3d shows the differences between the population distributions and the CS-alone distributions for five groups. Each ISI involved in this population analysis is shown with a unique color other than black (cyan = ISI 200, green = ISI 250, blue = ISI 500, gray = ISI 1000, and red = ISI 500 with mossy fiber stimulation as the CS). These difference distributions (CS distribution minus population distribution) show that for all groups, except ISI 200, trials following CS-alone trials demonstrate more frequent decreases in CR amplitude and fewer increases in CR amplitude than would be expected from the population changes. Moreover, the panels in Fig. 3d show that the likelihood of larger decreases in CR amplitude increase with the ISI.

Next, we asked how CS-alone trials affect the time course or temporal profile of the CRs at each ISI. Figure 4 shows average response profiles averaged across the four ISIs used in tone training (Fig. 4a) and the four ISIs used in training where the CS was stimulation of mossy fibers (Fig. 4b). The top panel in Fig. 4a shows average response profiles for the CS-alone trials (black traces) and for the paired CS + US trials that preceded them (green traces). Four pairs of traces are shown, one each for the four ISIs as indicated. These traces show that the CS-alone trials and those that precede them are almost indistinguishable through the whole eyelid trajectory prior to the US, having the same CR onset and amplitude. The shaded region of each trace indicates 95 % confidence intervals. The only notable difference in the four pairs of traces is the reflex response to the US that occurs on the CS + US paired trials. The reflex response for ISI 200 has been omitted to avoid overlap with the ISI 250 traces. In contrast, the lower panel of Fig. 4a shows the small but significant differences between the CS-alone trials (black traces) and the trials that immediately followed (red traces). At the longer two ISIs, there are clear differences in the time profiles and subsequent amplitude of the CRs, while the onset of the responses remain consistent. The same data, with essentially the same pattern of results, from animals trained with mossy fiber stimulation as the CS are shown in Fig. 4b.

The mean amplitude of CRs on the trial after the CS-alone trial shows a consistent decrease that then recovers to normal levels during the subsequent eight paired CS + US trials. Still,

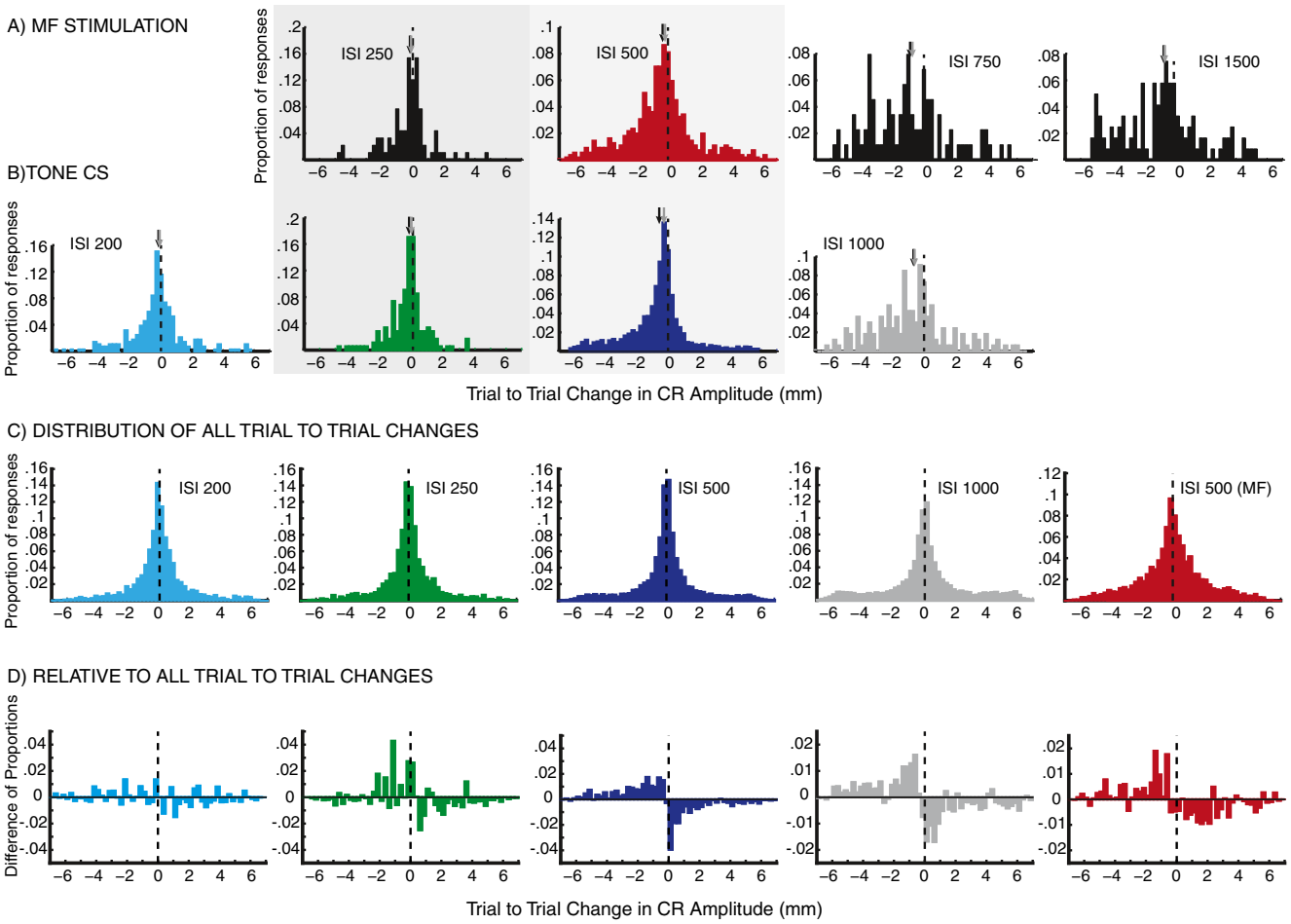

Fig. 3 Changes in CR amplitude following CS-alone trials are expressed as frequency histograms of the difference between the amplitude of CRs just preceding and immediately following CS-alone trials—negative values indicate smaller responses on the trial following the CS-alone trial. Separate histograms are shown for each ISI with data from animals using mossy fiber stimulation as the CS along the *top row* (**a**) and data from animals trained with the tone CS along the *second row* **b**. In panels (**a**) and (**b**), the histograms are arranged with ISI increasing from left to right and are staggered so that the two ISIs used for both tone and mossy fiber stimulation animals are aligned vertically (ISI=250 ms shown in the *gray box* and ISI=500 in the *lighter gray box*). For each of the eight panels, zero change is denoted with a *vertical dotted line* and also in each panel the mean (*black arrow*) and the median (*gray arrow*) are less than zero, indicating a decrease in response amplitude. **c**, **d** The changes in CR amplitude following CS-alone trials can be better understood when compared to the typical change in CR amplitude for trial pairs of randomly selected trials that have a trial in between them (to match the comparison, a trial following CS-alone versus the trial prior to the CS-alone trial). **c** These population frequency histograms were constructed for five groups, four tone-CS groups (ISI 200=*cyan*, ISI 250=*green*, ISI 500=*blue*, and ISI 1000=*gray*), and the one mossy fiber-CS group for which there was enough data to perform this analysis (ISI 500=*red*). These five histograms were constructed by randomly selecting with replacement a total of 10,000 pairs of trials from the same sessions used for panels (**a**) and (**b**). These figures reveal the unsurprising result that there is more variability in CR amplitude for longer ISIs than for shorter ISIs. **d** To compare the changes in CR amplitude after CS-alone trials with the typical (population) variability, the population frequency histograms in (**c**) were subtracted from the relevant (color-coded) frequency histograms in (**a**) and (**b**). For example, the *red histogram* in panel (**c**) was subtracted from the *red histogram* in panel (**a**) to produce the *red difference histogram* in panel (**d**), which is for the mossy fiber stimulation CS and ISI=500 the change in CR amplitude produced by CS-alone trials normalized to the typical changes between trials that is observed in the same sessions. In general, these difference histograms show that CR amplitude decreases more often than expected after CS-alone trials and increases less often than expected. The results for ISI 200 are the exception, where it is difficult to see a significant trend. Comparing across ISIs, these difference histograms show that there tends to be small decreases in amplitude at shorter ISIs and larger decreases in amplitude for longer ISIs (Color figure online)

looking only at the mean values of CR amplitude conceals the question of how the entire distribution of CRs is influenced by the CS-alone trial. To address this question, we used heat maps to illustrate how the distribution of CR amplitudes changes after the CS-alone trial (Fig. 5). For all ISIs and both tone and mossy fiber stimulation CSs, we calculated the mean distribution of CR amplitudes on CS-alone trials and the two trials preceding it and plotted changes from that baseline distribution on trials following the CS-alone trial. The single-trial effect on the distribution of CR amplitudes is not clear at short ISIs, but starting from ISI 500, a common trend emerges. For both types of CSs, following the CS-alone trial, there is an

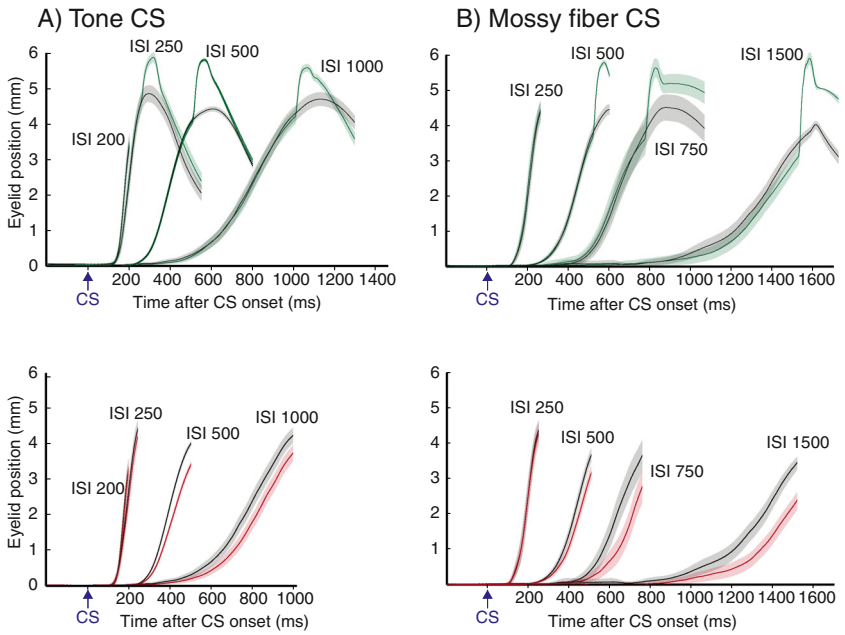

Fig. 4 The decrease in CR amplitude seen in the trials following CS-alone trials is also seen in averaged response profiles. For each panel, the abscissa shows time in milliseconds following CS onset (indicated by *arrow*) and the ordinate is mean CR amplitude in millimeters. Each sweep in these four panels is the average response profile over all responses across all animals for the ISI indicated. In all cases, the shaded portion of the sweep depicts the 95 % confidence interval for the data. The *top panels* compare for each ISI the average response profile of the CS-alone trials (*black traces*) and the trials preceding each CS-alone trial (*green traces*). The *bottom two panels* compare for each ISI the average response profile for the CS-alone trials (again in *black*) compared to the trials following each CS-alone trial (*red traces*). **a** The *upper panel* shows that CS-alone trials were almost indistinguishable from the responses in the paired trials that preceded them. The only regions of non-overlap in the responses are the reflex responses to the US that occur on the paired trials (the reflex response for ISI 200 was omitted to avoid overlap with the responses from ISI 250). These data also clearly show the robust timing differences between the responses at different ISIs. Only the mean responses for ISIs 200 and 250 ms are not obviously different. The *bottom panel* in contrast shows small but significant decreases in amplitude of CRs that follow CS-alone trials, especially at the longer two ISIs. **b** The same general pattern of results was seen from animals where the CS was stimulation of mossy fibers. The *upper panel* shows the similarity between CS-alone trials and the trials that precede them whereas the *lower panel* shows clear decreases in the amplitude of trials that follow CS-alone trials. As with the tone CS data, the decrease in response amplitude was larger for the longer ISIs (Color figure online)

increase in the frequency of smaller amplitude responses and a decrease in the frequency of large amplitude responses. As the length of the ISI increases, this effect becomes especially strong and persists over several trials following the CS-alone trial. These plots demonstrate how specifically CS-alone trials decrease the mean amplitude of CRs in trials immediately after them and how the distribution of CR amplitudes recovers to the baseline level as paired CS + US training within the block of nine trials accumulates.

Discussion

The current study is in part a replication of previous findings initially reported from the smooth pursuit experiments [1, 2], which described climbing-fiber-dependent changes in Purkinje cell activity and behavior on a trial-by-trial basis. Here, our proxy for recording climbing fiber input was to look at the behavioral effects of CS-alone trials, since both theoretical models predicted [17] and recording studies [6] (unpublished observations from our lab) have shown a strong decrease in the likelihood of a climbing fiber input to Purkinje cells during early CS-alone trials in extinction training. Results of the current study extend previous findings by describing how the single-trial effect interacts with a cerebellum-dependent behavior over a range of differently timed CRs trained at various ISIs. The current experiment also isolated the decrease in CR amplitude due to the single CS-alone trial effect to mechanisms inside the cerebellum through use of mossy fiber stimulation as the CS.

The main finding of this analysis is consistent with previous studies and demonstrates a statistically reliable decrease in animal performance after a single CS-alone trial, again suggesting the incremental nature of cerebellar learning. Having the data collected from a large number of animals trained at different ISIs allowed us to examine this effect in detail. Generally, we did not see any reliable differences in the single-trial effect between groups of animals trained with either tone or electrical mossy fiber stimulation as the CS. Some differences in the mean value of CR amplitude reduction or the significance level of effects between identical ISIs (Fig. 2, ISIs 250 and 500) for the two CS types were likely due to a generally

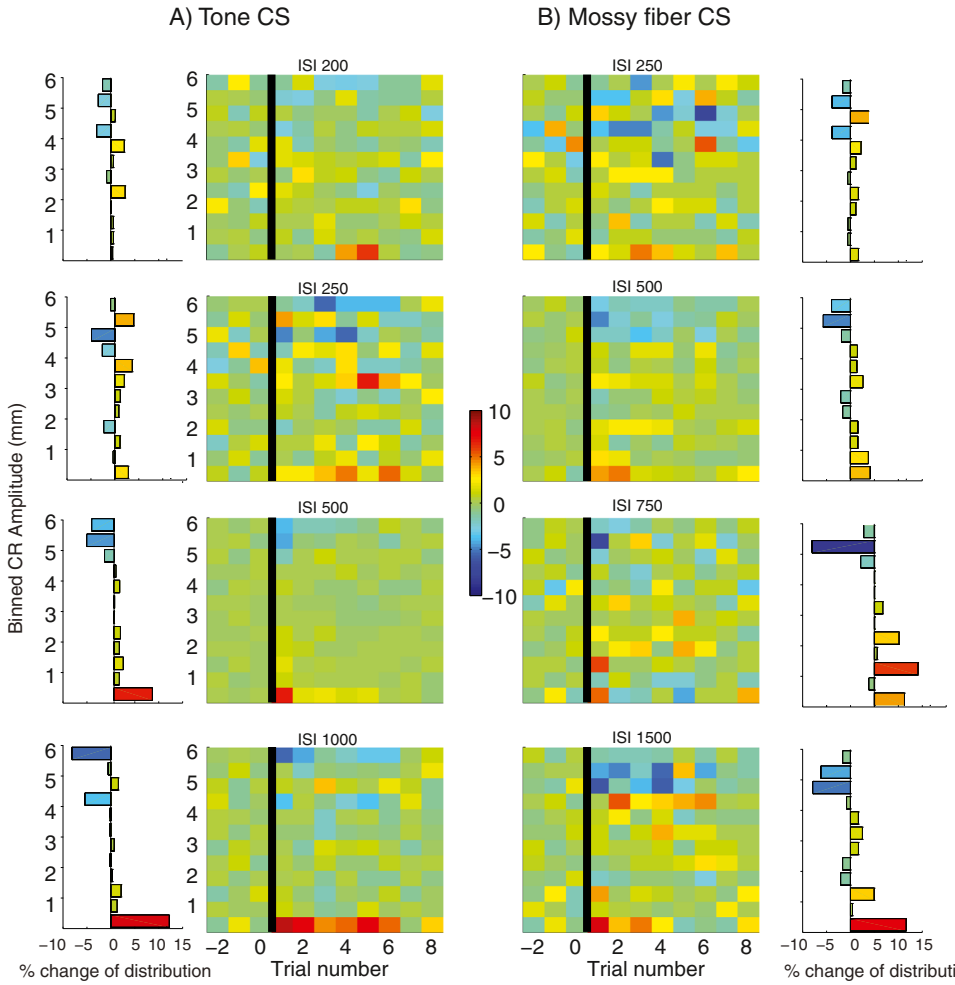

Fig. 5 Color-coded heat maps and histograms illustrate the changes in responses of various amplitudes over the eight trials following a CS-alone trial. Data for tone CS is shown at *left* (**a**) and data for mossy fiber stimulation is shown at *right* (**b**). The amplitudes of all responses were binned (0.5-mm bin width), with bins ranging from 0 to .5 mm at the bottom of each graph and 5.5–6.0 mm along the top. Each graph represents with color the percent change of each bin of the CR amplitude distribution, with negative values denoting a decrease in the number of responses at that amplitude. Each heat map shows data from a different ISI, with ISIs increasing from top to bottom. The abscissa for each heat map shows the CS-alone trials (the bin to the left of the *black vertical lines*), the two trials that precede the CS-alone trial (far left two bins), and the eight trials that follow the CS-alone trials (all bins to the right of the *black lines*). The bar graphs to the left of the tone CS heat maps and the right of the mossy fiber CS heat maps show, with the same color coding used in the heat maps, the change in the CR amplitude distributions for the trial immediately following the CS-alone trial (notice that the colors of each bar is the same as the corresponding amplitude bin just to the right of the *black line* in each heat map). The heat map colors to the left of the *vertical bars* are generally neutral colors, showing that the responses have returned to their usual amplitudes. In contrast, the bins just to the right of the vertical bar tend to show cooler colors in the larger amplitude bins and warmer colors in the smaller amplitude bins, indicating a decrease in response amplitude after the CS-alone trials. The heat maps demonstrate, for each ISI, how the CR amplitude distribution returns to the baseline level in the trials that follow the CS-alone trial. The *color calibration bar* shows the percent change from the full distribution of response amplitudes (Color figure online)

larger number of animals trained with the tone CS. Thus, we believe that at least the main source of the single-trial effect is constrained to learning mechanisms inside the cerebellum, which is fully consistent with reported changes in Purkinje cell firing determined by the presence or absence of climbing fiber inputs from Lisberger's lab.

We observed that a single CS-alone trial affects not only CR amplitude on the following trial but also its whole average time profile (Fig. 4). It also alters the distribution of CR amplitudes by decreasing the frequency of CRs with the highest amplitudes and increasing the frequency of both non-CRs and low amplitude CRs (Fig. 5). The distribution of intermediate amplitude CRs, on the other hand, did not seem to be influenced by the CS-alone trials. That suggests a specific way in which the cerebellum is implementing a decrease in average CR amplitude: It is not the case that the whole distribution becomes skewed to change the mean CR amplitude value. Rather, the majority of the distribution of CR amplitudes is preserved with only the frequencies of the lowest and the highest amplitudes changing.

These changes in amplitude distributions for each ISI could arise in two general ways: There could be a tendency for large

responses to become non-CRs, or there could be a tendency for each response to decrease by a small amount. Figure 3 suggests that for shorter ISIs, the latter is more applicable, as the modal change is a small decrease in amplitude (and the distribution is relatively narrow). For these shorter ISIs, Figs. 3 and 5 show that most CS-alone trials produce a small decrease in CR amplitude of the next trial. However, results from the same two figures suggest that for longer ISIs, the situation is slightly different: There, decreases in CR amplitude following a CS-alone trial are larger and there is a tendency for more non-CRs. In both cases, this is generally consistent with casual observation of response changes during extinction. For shorter ISIs, we generally observe a steady decline in CR amplitude over many trials, whereas for longer ISIs, we observe decreases in amplitude that often result in the abrupt disappearance of the CR. In ongoing work, we hope to understand better the mechanisms that contribute to these differences.

Since climbing fiber suppression is the signal for extinction in eyelid conditioning [13], the single-trial effects observed in the current study are likely due to a small amount of extinction during CS-alone trials. In delay eyelid conditioning, there is a strong effect of ISI on the rate of extinction (Ohyama and Mauk, unpublished) such that extinction is quite slow for shorter ISIs and much faster for longer ISIs. For short ISIs (e.g., ISI 250) animals will not extinguish for hundreds of trials while extinction at longer ISIs (e.g. ISI 750) is often very rapid, occurring in around 20 trials, for both tone and mossy fiber stimulation conditioned animals. Consistent with the relationship between extinction and ISI, all of the effects we report here also significantly scale up with the length of the ISI. If the cerebellar mechanism for extinction and the single-trial effects observed in the current analysis are the same, the implication based on the current results would be that behavioral changes produced by cerebellar learning during extinction become faster as the ISI increases.

The current results also show that not only is the magnitude of the single-trial effect larger at the longest ISIs but also that more paired CS + US trials are needed for CRs to recover to full amplitude at long ISIs as well (Fig. 2, upper panels). It is well established that the initial rate of learning decreases with longer ISIs as animals transition from the naïve state to the learned state in eyelid conditioning [10, 16]. On the other hand, rate of extinction and changes due to single CS-alone trial effects scale in the opposite way by increasing with longer ISIs. That suggests that cerebellar learning mechanisms are arranged in a way that makes it harder to learn and faster to extinguish CRs as the ISI increases. In future studies, the cerebellar mechanisms related to the scaling rate of the single CS-alone trial effect, during extinction and acquisition with different ISIs, will need to be examined with cerebellar simulations and recordings from Purkinje cells during eyelid conditioning. It will be important, for example, to identify whether the cerebellar mechanisms underlying the single-trial effects observed during eyelid conditioning are similar to results observed during smooth pursuit learning [1, 2].

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