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Climbing fibers encode a temporal-difference prediction error during cerebellar learning in mice

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Climbing fiber inputs to Purkinje cells are thought to be involved in generating the instructive signals that drive cerebellar learning. To investigate how these instructive signals are encoded, we recorded the activity of individual climbing fibers during cerebellum-dependent eyeblink conditioning in mice. We found that climbing fibers signaled both the unexpected delivery and the unexpected omission of the periocular airpuff that served as the instructive signal for eyeblink conditioning. In addition, we observed that climbing fibers activated by periocular airpuffs also responded to stimuli from other sensory modalities if those stimuli were novel or if they predicted that the periocular airpuff was about to be presented. This pattern of climbing fiber activity is markedly similar to the responses of dopamine neurons during reinforcement learning, which have been shown to encode a particular type of instructive signal known as a temporal difference prediction error.

Climbing fibers originating in the inferior olive project to the cerebellar cortex[1], where they are thought to provide the instructive signals necessary for cerebellar learning[2–4]. Some of the strongest support for this hypothesis comes from studies of Pavlovian eyeblink conditioning[5–8], a cerebellar task in which animals learn to close the eyelid in response to a conditioned stimulus (CS) such as an LED light if it is repeatedly paired with a blink-eliciting unconditioned stimulus (US) such as a periocular airpuff. Consistent with their presumed role as 'teachers', climbing fibers carry signals about the instructive US in this associative learning task[9–11]. Furthermore, direct electrical stimulation of climbing fibers can serve as the US during conditioning, providing a teaching signal that is as effective as periocular stimulation[12].

It has been suggested that the teaching signals transmitted by climbing fibers are encoded as prediction errors in cerebellar learning tasks[4,13–15]. During eyeblink conditioning, for example, climbing fibers fire if the US is presented unexpectedly[9–11] (positive prediction error) and they are inhibited if an expected US is omitted[11] (negative prediction error). This type of error coding can be used to generate an effective teaching signal[16,17] by alerting the brain that current expectations about the likelihood of the instructive US are incorrect and need to be updated. Indeed, climbing fiber signals about positive and negative US prediction errors feature prominently in many computational models of cerebellar-dependent conditioning[15,18,19].

Prediction error signals about the US are well suited for driving simple forms of associative learning, such as first-order acquisition and extinction of the conditioned eyelid response[15–19]. However, for higher order learning in which animals must learn from non-primary reinforcers such as the CS, teaching signals related to the US are not enough[20]. Theories based on the influential temporal difference (TD) model[20,21] have proposed that higher order instructive signals must also alert the brain about the CS events that reliably predict the occurrence of the US. Such CS-triggered signals have been found in midbrain dopamine neurons during reinforcement learning tasks[14,22,23]. We sought to examine whether climbing fibers may encode the same type of predictive TD signals during cerebellar-dependent associative learning.

Taking advantage of a new system for eyeblink conditioning in head-fixed mice[24], we examined the neural coding of prediction errors in climbing fibers. On the basis of the predictions of the TD model, we hypothesized that, in addition to their well-known activation by an unexpected US, climbing fibers should also fire in response to presentations of the CS at the end of conditioning, after the primary association between the CS and the US has been established.

RESULTS

Monitoring climbing fibers during eyeblink conditioning

We sought to examine the signals that climbing fibers send to Purkinje cells during cerebellar learning and to evaluate whether these signals conform to the predictions of the TD model[20,21]. We used a head-fixed apparatus to train mice in a simple delay eyeblink conditioning task that is critically dependent on the cerebellum (**Fig. 1a**,**b**)[24–26]. Daily conditioning sessions comprised 100–200 trials in which a CS such as a tone or an LED light was followed after 220 ms by an aversive airpuff directed at the eye, which served as the instructive blink-eliciting US. All mice ($n = 7$) learned to make well-timed conditioned responses (CR) over the course of 10–15 conditioning sessions; that is, they learned to blink in response to the CS, closing their eyelids in anticipation of the aversive periocular airpuff.

To measure climbing fiber signals on any given conditioning session, we lowered an electrode into an identified eyeblink region of cerebellar cortex[27,28] and recorded the extracellular activity of individual Purkinje cells (**Fig. 1b**). Each activation of the powerful climbing fiber input resulted in a massive depolarization of the postsynaptic Purkinje cell[2,4], which could be detected in the raw extracellular record as a characteristic complex spike[29] (Cspk; **Fig. 1a**). The waveform

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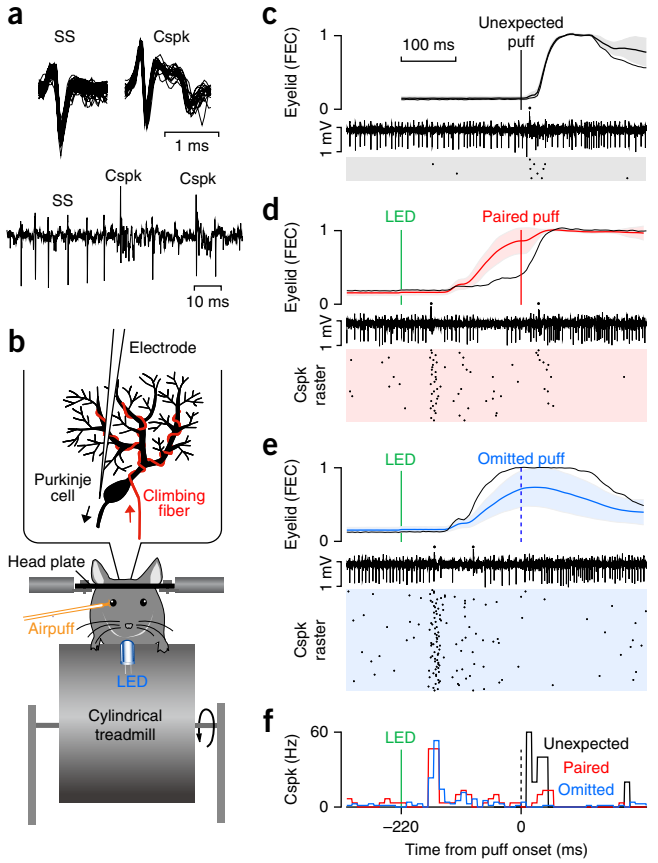


Figure 1 Experimental design and approach. (**a**,**b**) Examples of waveforms (top, **a**) and raw extracellular signal (bottom, **a**) for SSs and Cspks fired by a representative Purkinje cell during eyeblink conditioning in a treadmill apparatus for head-fixed mice (**b**). (**c**–**e**) Eyelid movements (mean (line) ± s.d. (shaded region)) and simultaneously recorded Cspks of the Purkinje cell in **a** in trials with unexpected periocular airpuff (**c**), paired LED and periocular airpuff (**d**), and LED without periocular airpuff (**e**). FEC, fraction eyelid closure. Raw extracellular traces in **c**–**e** show Cspks (dots) in an example trial (thin black eyelid trace). (**f**) Peristimulus time histograms (bin size = 10 ms) for the Cspks fired in the trials corresponding to the three raster plots of **c**–**e**.

of the climbing fiber–driven Cspk could be distinguished from normal Purkinje cell action potentials known as simple spikes[29] (SS; **Fig. 1a**). Because each Purkinje cell is known to receive input from a single climbing fiber[1,4] (**Fig. 1b**), the Cspks fired by a well-isolated Purkinje cell provide a straightforward way to measure the activity of an individual climbing fiber.

To evaluate the coding of prediction error signals, we first trained the mice and then examined the climbing fiber–driven Cspks of individual Purkinje cells in daily sessions with three types of trials: unexpected presentations of the periocular airpuff US (unexpected; **Fig. 1c**,**f**), paired trials in which the CS (tone or LED) was presented 220 ms before the US, as was the case for normal conditioning trials during training (paired; **Fig. 1d**,**f**), and trials in which the CS was presented by itself, without the US (omitted; **Fig. 1e**,**f**). Note that, even in well-trained mice, there was considerable trial-by-trial variation in performance (**Fig. 1d**,**e**) and that the size of the eyeblink CR before the US was presented could range from very small in some individual trials (**Fig. 1d**) to very large in others (**Fig. 1e**).

Climbing fibers encode US-related prediction errors

Our first analysis was designed to examine climbing fiber–driven Cspks in the 120-ms time window after the periocular airpuff (US period; **Fig. 2a**). We will refer to Cspks in this US period as $Cspk_{US}$. Because climbing fibers do not modulate their firing rate much and typically fire just once in response to each stimulus presentation[2], we computed the activity of each climbing fiber by constructing a peristimulus time histogram that averages the number of Cspks fired by the same Purkinje cell across multiple trials (**Fig. 1f** and Online Methods).

Consistent with previous reports[10,11,28], we found that the climbing fiber input of many Purkinje cells ($n = 34$) in the eyeblink region provides a bidirectional signal that encodes both positive and negative prediction errors about the US[14]: the probability of a $Cspk_{US}$ was higher than baseline when the periocular airpuff was presented unexpectedly (unexpected, Wilcoxon signed rank test, $P < 0.001$ with Bonferroni correction for five comparisons; **Figs. 1c**,**f** and **2a**,**b**,**d**) and in paired conditioning trials in which the mouse failed to make a CR (paired no CR, Wilcoxon signed rank test, $P < 0.001$ with Bonferroni correction for five comparisons; **Figs. 1d** and **2d**). In contrast, the probability of a $Cspk_{US}$ was lower than baseline in trials in which the mouse made a CR and the periocular airpuff was omitted (omitted; **Figs. 1e**,**f** and **2a**,**c**). Note that the reduction of climbing fiber activity in the omitted trials was very reliable (omitted big CR, Wilcoxon signed rank test, $P < 0.001$ with Bonferroni correction for five comparisons; **Fig. 2d**), but the size of the modulation was small because climbing fibers fired at very low rates around 1 Hz during baseline[2].

We examined the SS activity of the same group of Purkinje cells (**Fig. 2a**) to gain some insight about the mechanisms underlying the coding of prediction errors in climbing fibers. Purkinje cells reduced their SS firing rate prior to the US period, but only in trials with a CR (big CR; **Fig. 2a**), and not when the mouse failed to make a

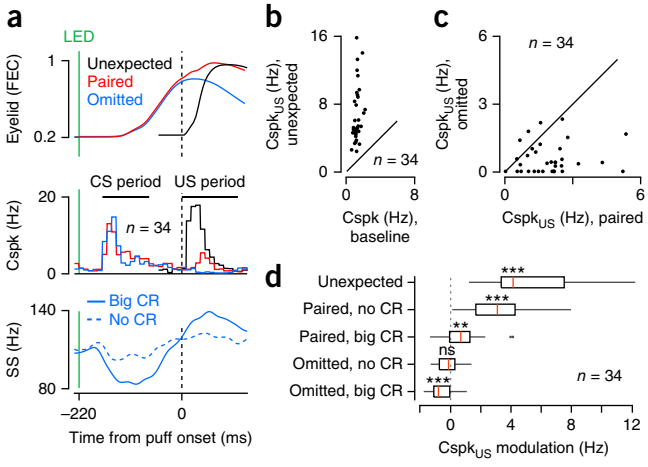


Figure 2 Climbing fiber responses in the US period. (**a**) Population averages of eyelid movements and simultaneously recorded Cspks and SSs in trials with unexpected periocular airpuff (black), paired LED and periocular airpuff (red), and LED without periocular airpuff (blue). For SS, data in LED trials without airpuff are plotted separately for trials with a conditioned eyelid response (big CR) and trials without (no CR). Time windows for CS and US analysis are indicated. (**b**–**d**) The probability of a Cspk during baseline and in the US period of different types of trials is plotted for all individual Purkinje cells (**b**,**c**) and summarized for the population (**d**). In **d**, modulation of Cspk activity in the US period is relative to baseline. Median (red line), interquartile range (box), data range (whisker), and outliers (dots). An outlier was omitted from the unexpected puff condition (first row). $^{**}P < 0.01$, $^{***}P < 0.001$, after using Bonferroni test to correct for five comparisons; ns indicates not significant.

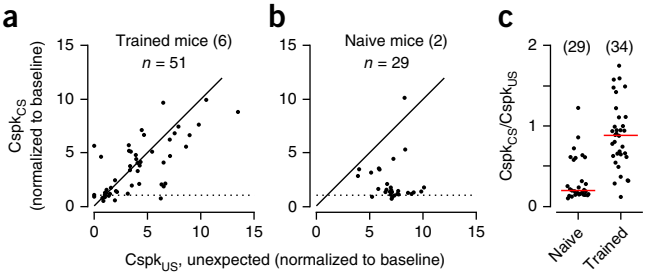
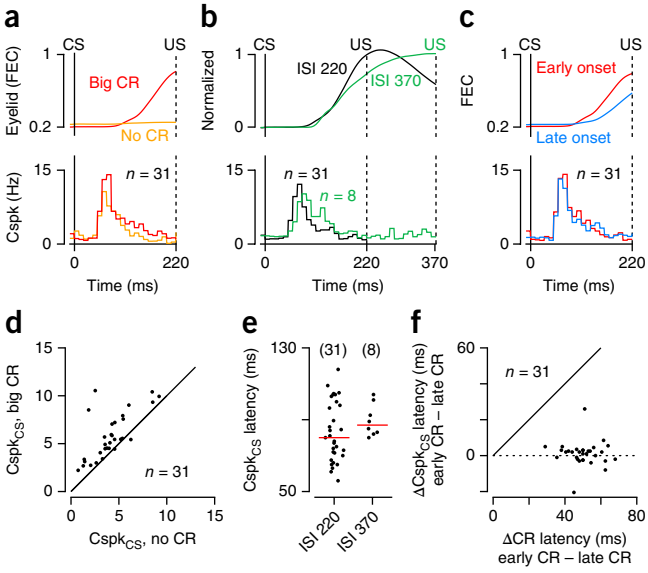


Figure 3 Climbing fiber responses in the CS period. (**a**,**b**) Probability of Cspk activity in the CS period relative to the Cspk probability after unexpected delivery of periocular airpuff, plotted for all individual Purkinje cells in well-trained mice (**a**), and in naive mice (**b**). All of the Cspk responses have been normalized to the Cspk activity of each Purkinje cell during baseline. (**c**) The responsiveness of climbing fibers to the CS in well-trained and naive mice is plotted as a ratio of Cspk response probabilities to the CS and to the unexpected presentation of the periocular airpuff stimulus. Data for individual Purkinje cells (dots) and median (red line) are shown. Numbers in parentheses indicate the number of mice used (**a**,**b**) and the number of cells recorded (**c**).

CR (no CR; **Fig. 2a**). It has been suggested that such a reduction in SS firing rate could be used to inhibit climbing fiber activity at the time of the US[18,30] via a double inhibitory pathway from Purkinje cells to neurons in the deep cerebellar nuclei and then to the inferior olive where climbing fibers originate[31]. In this model, climbing fiber activity during the US period is modulated by two inputs converging in the inferior olive: excitation from US-driven trigeminal neurons[32,33] and inhibition from CR-related neurons in the deep cerebellar nucleus[10,30,34]. This anatomical organization could help to explain why climbing fiber activity was highest in positive prediction error trials when there was no CR-related inhibition of the inferior olive at the time of the US (unexpected and paired no CR; **Fig. 2d**), reduced when US-related excitation was counterbalanced by CR-related inhibition (paired big CR; **Fig. 2d**), near baseline in the absence of US-related and CR-related inputs (omitted no CR; **Fig. 2d**), and suppressed below baseline in negative prediction trials in which CR-related inhibition of the inferior olive occurred without the US (omitted big CR; **Fig. 2d**).

Climbing fibers responses to the CS in well-trained mice

Previous electrophysiology studies[9–11,30], including the analysis presented above (**Fig. 2b–d**), have focused exclusively on climbing fiber signals around the time of the instructive US. However, to comply with the requirements of the TD model[20,21], climbing fibers must fire in response to stimuli such as the CS that reliably predict the occurrence of the US. To assess whether climbing fibers meet this criterion, we examined Cspks in a 100-ms time window starting 0–50 ms after the LED or tone stimulus used as the CS (CS period; **Fig. 2a**). We will refer to Cspks in this CS period as $Cspk_{CS}$.



Presentation of the CS reliably elicited Cspks in well-trained mice (**Fig. 2a**). The probability of a $Cspk_{CS}$ was higher in Purkinje cells that also had a reliable Cspk response to unexpected periocular airpuffs ($R = 0.72$; **Fig. 3a**). In contrast, the probability of a $Cspk_{CS}$ was near baseline in the majority of Purkinje cells recorded in naive mice, even those that fired Cspks reliably in response to unexpected presentation of the periocular airpuff (**Fig. 3b**). Thus, Purkinje cells in this region of cerebellar cortex fired Cspks much more robustly to the unexpected US than to the CS in naive mice (naive; **Fig. 3c**), and equally well to both stimuli after training (trained; **Fig. 3c**). These results are consistent with a hypothesis in which the majority of climbing fibers are initially unresponsive to the CS, and gradually acquire a response during conditioning, as the CS becomes predictive of the instructive US. However, it is clear that the CS can activate some climbing fibers even in naive mice (**Fig. 3b**,**c**). We will return to this observation below.

In addition to providing the teaching signals necessary for cerebellar learning, it has been suggested that climbing fibers also contribute to the ongoing control of movement timing[35]. We performed three analyses to assess whether there is a relationship between $Cspk_{CS}$ activity and the CR-related movement of the eyelid (**Fig. 4**). First, we found that, in most Purkinje cells, the probability of a $Cspk_{CS}$ was only marginally higher in trials with a big CR than in trials without a CR (Wilcoxon signed rank test, $P < 0.001$; **Fig. 4a**,**d**). Second, we recorded an additional eight Purkinje cells in a mouse trained with a 370-ms CS-US interstimulus interval (ISI; **Fig. 4b**,**e**) and found that the latency of the $Cspk_{CS}$ fell in the range observed for Purkinje cells in mice trained with the 220-ms interval (Wilcoxon rank sum test, $P = 0.22$; **Fig. 4e**), even though the temporal profile of the CR was different[24,36] (**Fig. 4c**). Third, we confirmed that the latency of the $Cspk_{CS}$ did not depend on the latency of the CR and was essentially the same for trials in which the eyelid started closing early or late (**Fig. 4c**); that is, the difference in $Cspk_{CS}$ latency between trials with early CRs and late CRs was near zero for most of the Purkinje cells (Wilcoxon signed rank test, $P = 0.14$; **Fig. 4f**). Thus, as expected for a TD prediction error signal[20,21], climbing fibers fired at a fixed latency after the CS in well-trained mice regardless of the expected time of

Figure 4 Complex spikes in CS period are not driven by eyelid movement. (**a**,**d**) Comparison of population-averaged Cspk activity in trials with (big CR) and without (no CR) a conditioned eyelid response (**a**), and the corresponding Cspk response in the CS period for all individual Purkinje cells (**d**). (**b**,**e**) Comparison of population-averaged Cspk activity in mice trained with a 220-ms ISI (ISI 220) and a mouse trained with a 370-ms ISI (ISI 370) (**b**), and the corresponding median Cspk latency in the CS period for all individual Purkinje cells (dots) and population median (red line) (**e**). Eyelid traces in **b** have been normalized to provide direct comparison of movement time course. (**c**) Comparison of population-averaged Cspk activity in trials with early-onset and late-onset CR movements. Numbers in parentheses indicate the number of cells recorded. (**f**) The difference between the latency of the Cspk in trials with early-onset and late-onset CRs is plotted for all individual Purkinje cells.

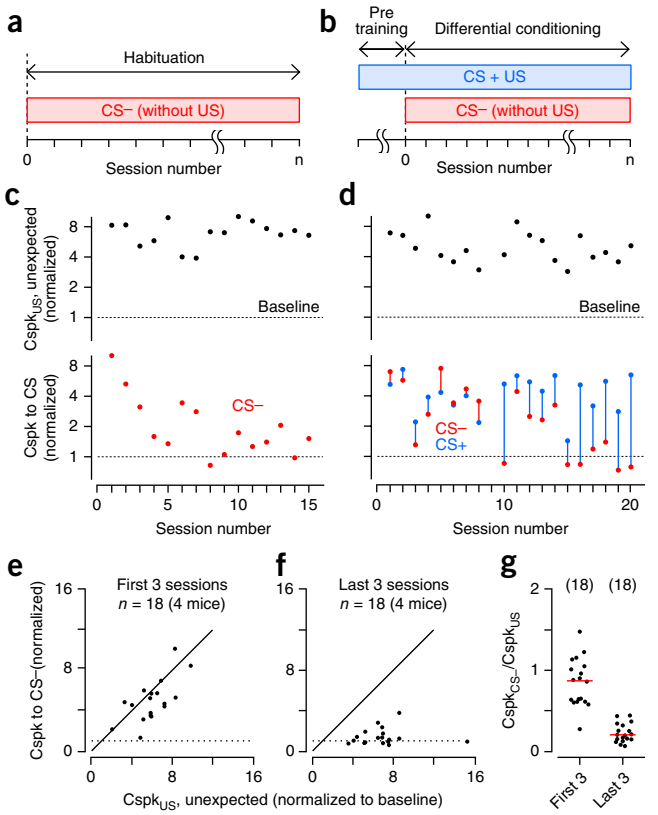


Figure 5 Climbing fiber responses to novel stimuli. (**a**,**b**) Experimental design for mice in habituation (**a**) and differential conditioning (**b**) groups. (**c**,**d**) Probability of Cspk response to the unexpected airpuff (black) and to the CS+ (blue) and CS– (red) over the course of multiple sessions of habituation (**c**) or differential conditioning (**d**). Each session shows data for a different Purkinje cell. All Cspk responses have been normalized to the Cspk activity of each Purkinje cell during baseline. (**e**,**f**) Probability of Cspk response to the CS– in the first three (**e**) and last three (**f**) recording sessions. The data for tone and LED stimuli and for habituation and differential conditioning sessions were combined. (**g**) The responsiveness of climbing fibers to the CS– in the first three and the last three sessions is plotted as a ratio of the Cspk response probabilities to the CS– and to the unexpected presentation of the periocular airpuff stimulus. Data for individual Purkinje cells (dots) and median (red line) are shown. Numbers in parentheses indicate the number of cells recorded.

the US or the timing of the CR. Furthermore, we can rule out the possibility that Cspk$_{CS}$s are driven by CR-related movement of the eyelid, as they were clearly present both in trials with and without a CR (**Fig. 4a**,**d**).

Climbing fiber responses to novel stimuli

In recent computational studies based on the TD model, instructive signals incorporate a component related to the novelty of the CS[37,38]. We performed two additional experiments to assess whether there is a relationship between climbing fiber activity and stimulus novelty ($n = 4$ mice): habituation training, in which naive mice received daily sessions comprising 20–50 repeated presentations of a tone or LED stimulus (CS–) without the periocular puff (**Fig. 5a**), and differential conditioning, in which mice were first trained by pairing one stimulus (CS+) with the periocular puff and, after learning, received daily sessions in which the same CS+ continued to be paired with the airpuff in 75% of all trials, and a different CS– was presented without the airpuff in 25% of all trials (**Fig. 5b**). None of the mice made eyelid movements in response to the CS– in any of our experiments (**Supplementary Fig. 1**).

Figure 5c,**d** summarizes the Cspk responses of Purkinje cells recorded in two representative mice during habituation and differential conditioning (one Purkinje cell per session). Purkinje cells recorded in the first few sessions of habituation (**Fig. 5c**) and differential conditioning (**Fig. 5d**) fired Cspks reliably in response to the CS–. In contrast, Purkinje cells recorded in subsequent sessions were much less likely to fire Cspks to the CS– than to the CS+ or to unexpected presentations of the periocular airpuff (**Fig. 5c**,**d** and **Supplementary Fig. 1c**,**f**,**i**). Pooling the data for all the mice revealed a relationship between climbing fiber activity and the novelty of the CS– (**Fig. 5e**–**g**): the CS– triggered a Cspk with high probability when it was relatively novel, that is, in the first three sessions of habituation or differential conditioning (Wilcoxon signed rank test, $P < 0.001$; **Fig. 5e**,**g**), but not in the last three sessions when it had been presented many times and was more familiar (Wilcoxon signed rank test, $P = 0.09$; **Fig. 5f**,**g**). The same result was obtained by comparing the Cspk responses to the CS– in the first two and the last two sessions, or in the first five and the last five sessions (**Supplementary Fig. 2**). This finding explains our previous observation that the CS can trigger climbing fiber–driven Cspks in some Purkinje cells even in naive mice (**Fig. 3b**,**c**). Indeed, the eight responsive Purkinje cells shown in **Figure 3b**,**c** were all recorded in the first two days of experiments, during initial exposure to the CS.

DISCUSSSION

We examined the activity of climbing fibers during eyeblink conditioning in mice by recording the Cspks of Purkinje cells in an eyelid region of cerebellar cortex. Our results confirm those of previous reports[9–11], suggesting that many climbing fibers in this region signal both the unexpected delivery and the unexpected omission of the periocular airpuff that serves as the instructive US. In addition, we found that, under certain conditions, the same climbing fibers can also fire in response to a CS from a different sensory modality, such as a tone or an LED light. Here we discuss the implications of these results for understanding the neural representation of instructive signals during cerebellar learning.

We found that climbing fibers encode a prediction error signal that satisfies three basic principles of TD models[20,21]: climbing fibers fire in response to unexpected presentation of the US (positive prediction error), climbing fibers are inhibited if an expected US is omitted (negative prediction error), and climbing fibers fire in response to the predictive CS in well-trained mice, after the relationship between the CS and the US has been learned. According to the TD model[20,21], the response to the CS should develop gradually during conditioning, progressively increasing in strength as learning proceeds and the association between the CS and US is established. We were unable to test this hypothesis directly because cerebellar-dependent eyeblink conditioning in mice is a slow learning process[24], and it is not currently feasible to maintain good isolation and track the activity of the same climbing fiber over long periods of time. Until the right tools become available, we can at least note that, in our experiments, climbing fibers with similar responses to the unexpected presentation of the US fired much more reliably to the CS in well-trained mice than in naive mice. This finding is consistent with the hypothesis that climbing fiber responses to the CS start out relatively weak and get progressively stronger during learning, as predicted by TD models[20,21].

Our experiments also revealed that climbing fibers often fired in response to the initial presentations of the CS in naive mice. Because

this response habituates if the CS continues to be repeatedly presented without the US, we interpret it as a signal related to stimulus novelty. Novelty signals were not part of the original TD model[20,21], but they have been recently incorporated into the general TD framework, where they are important for triggering exploratory and orienting actions that help to determine the meaning of stimuli with high potential importance[37,38]. Thus, in our working hypothesis, climbing fibers have the capacity to multiplex: they generate novelty signals about stimuli whose meaning is yet to be determined, as well as prediction error signals about stimuli whose meaning is known or has been learned. An alternative hypothesis that can account for many of our observations is that climbing fibers encode a signal about stimulus salience, which is thought to be important for associative learning[39]. However, our results indicate that climbing fibers do more than provide saliency information. For example, we have shown that climbing fiber activity is suppressed below baseline when an expected US is omitted, which is a hallmark of a negative prediction error signal, but it's difficult to explain with a pure saliency code.

Climbing fiber responses to visual and auditory CSs were obvious and highly prevalent in our experiments, but they are conspicuously absent in previous studies of eyeblink conditioning[9–11]. In fact, we could only find one preliminary report (Edgley, S.A., Mostofi, A. & Holtzman, T. *Soc. Neurosci. Abstr.* 786.4, 2010) and three publications that briefly mention in passing what may have been occasional CS-related responses[10,40,41]. There are two important features of our experimental approach that could help to explain this apparent discrepancy. First, we only examined the activity of climbing fibers that project to an identified eyeblink region of cerebellar cortex[27,28] and that responded reliably to unexpected presentations of the periocular airpuff stimulus. This selection is likely to be important because the response properties of climbing fibers can vary widely depending on their exact location in the anatomical microzonal organization of the cerebellar cortex[28]. Second, we performed all of our experiments in head-fixed mice that were free to locomote on top of a treadmill[24], whereas others before us have often immobilized their subjects[9–11] or used a decerebrate preparation to increase recording stability[11]. Whether any of these methodological considerations can account for the lack of CS-related climbing fiber responses in previous studies remains an open question. However, we note that climbing fiber responses to peripheral stimulation are strongly modulated by behavioral state[42] and differ greatly between resting and walking conditions[43,44].

Our results provide some clues as to the origin of the multimodal responses of climbing fibers to somatosensory, visual and auditory stimuli. All our recordings targeted Purkinje cells in a small eyeblink region of cerebellar cortex that receives climbing fiber inputs from neurons located in three subdivisions of the IO[28]: the dorsal accessory olive, the medial accessory olive and the medial dorsal olive. Neurons in these three subdivisions of the IO appear to be well positioned for multisensory processing, as they receive converging inputs from proprioceptive and cutaneous receptors via ascending spino-olivary pathways and from multiple sensorimotor areas of the cortex via descending cerebro-olivary pathways[3,45,46]. In addition, some cells in the medial accessory olive respond to flashes of light and tapping sounds[47], possibly via activation of inputs from the superior colliculus[48]. Notably, we found that climbing fibers of well-trained mice fired in response to auditory or visual CSs even in trials in which the mouse failed to make a conditioned eyelid movement. This observation rules out the possibility that what may have looked like a sensory response to the CS was really a proprioceptive response or a reafference response driven by the neurons or muscles that control the eyelid. However, a non-eyelid motor source related to orienting or startle movements to the CS is still possible[41].

Our results are disruptive and call for a major revision of existing theories about the function of climbing fibers during cerebellar learning tasks such as eyeblink conditioning. In the prevailing view, climbing fibers carry a prediction error signal about the instructive US[5–8,15,18,19]. The anatomy supports such a view because it is well-known that the neurons of the inferior olive (IO) that send their climbing fiber axons to eyeblink regions of the cerebellar cortex receive a hardwired excitatory input from US-responsive areas in the trigeminal nucleus[32,33]. In addition, the same IO neurons also receive an inhibitory input from cells of the deep cerebellar nucleus that fire during the CR[10,30,34], thereby establishing an anatomical basis for computing positive- and negative-prediction error signals about the US; that is, IO cells will be excited by the US when it is not predicted, and they will be inhibited by CR-related activity if a predicted US is omitted. This anatomical arrangement of excitatory and inhibitory synaptic inputs in the IO is the foundation for current models of cerebellar learning based on the 'comparator' hypothesis[15,18,19]. However, none of the existing theories take into account our discovery that the same climbing fibers that carry error-related information about the instructive US also respond to visual and auditory CSs if these stimuli are novel or if they have been previously conditioned.

The pattern of climbing fiber responses that we recorded during eyeblink conditioning bears a marked resemblance to the responses of many dopamine neurons during reinforcement learning tasks[22,49,50]. We already knew that, in both cases, these responses encode positive and negative prediction error signals about the instructive US, that is, about the periocular airpuff used for eyeblink conditioning in the case of climbing fibers, and about the reward used for reinforcement learning in the case of dopamine neurons. Here we found that the similarities go beyond coding of US-related instructive signals. As with the climbing fibers recorded in our experiments, dopamine neurons in reinforcement learning tasks fire in response to auditory or visual stimuli if these stimuli are novel[22,49] or if they predict that the US is about to be presented[22,49,50]. In theories of reinforcement learning based on the TD model, these dopamine responses are important for driving higher order acquisition of approach behavior to potential reward[23,37,38,49,50]. Future experiments will help to determine whether the novelty and CS-related signals of climbing fibers may have a similar teaching role during cerebellar learning.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.O. and J.F.M. designed the research plan. S.O. performed all of the experiments and analyzed data. J.F.M. and S.O. prepared the figures and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal preparation. All procedures were performed in C57BL/6 male mice approximately 11–14 weeks of age (Jackson Laboratories, $n = 10$), following the guidelines of the US National Institutes of Health and a protocol approved by the University of Pennsylvania Animal Care and Use Committee. Before starting our experiments, we implanted a head plate and a recording chamber above the right cerebellar cortex following surgical procedures described previously[24].

Stimulus control and behavioral procedures. In all experiments, mice were head-fixed on top of a cylindrical treadmill and allowed to walk freely[24]. Facial and eyelid movements ipsilateral to the recording site were monitored with a high-speed monochrome camera (GE680, Allied Vision) under infrared illumination. Video frames (200 fps) were triggered by a Sys3 processor (RZ5, TDT) and stored with the MATLAB Video Acquisition Toolbox. A Sys3 processor (RZ5, TDT) was used to control the timing of stimuli during conditioning. The US was an airpuff of nitrogen (40 psi, 10–20 ms) delivered via a 23-gauge flat-ended needle placed ~4 mm in front of the right cornea of the mouse. The CS were either a 500-ms blue light LED positioned 8 cm in front of the mouse's face, or a 500-ms tone of white noise delivered via a speaker (4-Ω magnetic speaker, FF1, TDT) positioned 20 cm in front of the mouse. The volume of the white noise was set before the first conditioning session to just below the threshold for eliciting short-latency startle movement of the eyelid.

After 3 d of acclimatization to the cylindrical treadmill, mice were submitted to one of three behavioral procedures: (i) habituation: daily sessions comprising 20–50 unpaired presentations of the CS– (LED or tone without the US), (ii) eyeblink conditioning: daily sessions comprising 21-179 paired trials in which the CS+ (LED or tone) was presented for 500 ms and the US was delivered 220 ms after the CS+ onset (220-ms ISI). For one mouse, the ISI was 370 ms, In ~25% of the trials the US was omitted, but this fraction was increased in a few recording sessions to collect enough data for analysis, (iii) differential conditioning: mice first received 15–30 sessions of eyeblink conditioning comprising paired presentations of the CS+ (LED or tone) and the US (220-ms ISI; see above). After the mice learned to make conditioned eyelid responses to the CS+, they began receiving daily sessions comprising a mixture of trials in which the same CS+ continued to be paired with the US (approximately 75% of all trials), and CS– trials in which a different stimulus (for example, when the CS+ was a tone, the CS– was an LED, and vice versa) was presented without the US (approximately 25% of all trials). In all recording sessions, the US was delivered unexpectedly to the ipsi- and contralateral corneas in occasional trials randomly interleaved during each session. The minimum interval between trials (intertrial interval; ITI) was 7–16 s, but trials could only start if the eyelid position was stable for at least 0.6 s.

Behavioral analysis. Movement of the eyelid was calculated frame by frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, according to a procedure described previously[24]. Eyelid closure was measured in units of fraction eyelid closure (FEC), ranging from 0 (fully open) to 1 (fully closed). Trials in which FEC did not reach at least 0.1 in the ISI period were defined as no-CR trials (**Figs. 2d** and **4a**,**d**). For **Figure 4b**, the eyelid traces were normalized for each session so that the average eyelid position was 0 at the onset of the CS and 1 at the onset of the US. For **Figure 4c**, the trials of each recording session were divided into three equal-sized groups according to the onset latency of the CR: early-onset, middle-onset and late-onset CRs. CR onset was defined for each trial in the session as the time when the low-pass filtered eyelid velocity trace reached a threshold of 0.002 FEC ms^{-1}. No-CR trials were excluded from this analysis. For **Figure 4f**, the median CR latencies for the early-onset and late-onset groups were subtracted from each other for each recording session and plotted on the x axis.

Single-unit recording. Extracellular recording of simple spikes and Cspks in Purkinje cells was performed with 1–5 MΩ tungsten microelectrodes (75 μm of shaft diameter, FHC) or glass capillary electrodes (BF150-86-10, Sutter instrument) with 2–7-μm tip and 3–6-MΩ impedance (P-1000, Sutter instrument). The electrodes were controlled with a hydraulic microdrive (MMO-220A, Narishige) mounted on a three-axis manual micromanipulator (M325, WPI). The voltage signal was acquired at a 24,414-Hz sampling rate, and band-pass filtered between 0.1–10 kHz using a digital processor (RZ5, TDT).

To target the eyeblink microzone located near the floor of the primary fissure[27,28] (2,000–2,400 μm below the surface of dura matter), electrodes were directed along a 15-deg angled axis from posterior dorsal to anterior ventral, relative to the vertical plane. The eyeblink microzone could be identified by the presence of a large negative LFP signal (400–800 μV) in the molecular layer of the cerebellar cortex in response to ipsilateral periocular airpuffs (10–20 ms), and by the corresponding Cspk recorded in individual Purkinje cells. As reported previously for the eyeblink microzone in rabbits[28], contralateral periocular airpuffs did not evoke Cspks as reliably.

Electrophysiology analysis. We recorded a total of 151 Purkinje cells (29 from mice in habituation group, 51 from mice trained with the 220-ms ISI, 8 cells from a mouse trained with the 370-ms ISI, and 63 from mice in the differential conditioning group). Cspks could be isolated in all 151 Purkinje cells, and simple spikes in 122 of them. Cspks and simple spikes were sorted off-line using the threshold-crossing and template-matching algorithm of Spike2 software (Cambridge Electronic Design). For Cspks we also examined the voltage waveform traces trial-by-trial and performed additional manual sorting, blind to task condition and behavior. Finally, to confirm that the Cspks and the simple spikes originated from the same Purkinje cell, we checked that there was a 10–40-ms pause in simple spike activity after each Cspk[4]. Although climbing fibers typically fired just once in response to the CS+ and to the unexpected US, we observed occasional doublets of Cspks in quick succession (raw record in **Fig. 1a** and raster in **Fig. 1c**). Similar doublets have been observed before[51]. Also note that after firing to the CS+, the refractory period of climbing fibers (approximately 100 ms)[4] is too brief to affect how the climbing fiber will fire in response to the US (which is delivered 220–370 ms after the CS, after the refractory period is over).

A Cspk peristimulus time histogram (PSTH) was constructed for each climbing fiber and for each trial type (see example PSTH's for unexpected, paired and omitted trials types of a representative climbing fiber in **Fig. 1f**). Climbing fiber activity in the PSTH was expressed as frequency in Hz, by adding all the Cspks fired in each time bin and dividing by the number of trials multiplied by the bin size (0.01 s). We took three measurements from each PSTH: (i) the spontaneous (baseline) frequency of the climbing fiber in the 500-ms time window before the trials started, (ii) the frequency of the climbing fiber in the 120-ms window after delivery (or omission) of the US, and (iii) the frequency of the climbing fiber in the 100-ms time window after delivery of the tone CS, or 50 ms after delivery of the LED CS to take into account longer delays related to visual processing. The Cspk response to the CS or the unexpected US was considered to be statistically significant if it was at least two s.d. higher than the baseline frequency. Of the 51 cells in mice trained with a 220-ms ISI, 34 had a statistically significant Cspk response to the unexpected presentation of the US (analyzed in **Figs. 2** and **3c**), and 31 had a statistically significant Cspk response to the CS (analyzed in **Fig. 4**). In addition, statistically significant responses to the unexpected US were found in 29 cells from mice in the naive/habituation group (analyzed in **Figs. 3b** and **5e–g**; note that, in **Fig. 5e–g**, only a fraction of the 29 cells were recorded in the first three or the last three sessions), eight cells from a mouse trained with the 370-ms ISI (analyzed in **Fig. 4b**,**e**), and 45 cells from mice in the differential conditioning group (analyzed in **Fig. 5e–g**, note that only a fraction of these 45 cells were recorded in the first three or the last three sessions). The frequency of each climbing fiber was normalized by its baseline frequency for **Figures 3a**,**b**, **4d** and **5e**,**f**.

Statistical analysis. Mean and within-session variability (s.d.) are displayed for eyelid traces in **Figure 1d**,**e**. Average of all sessions is displayed for eyelid traces and Cspk histograms in **Figures 2a** and **4a–c**. All statistical analyses were performed using the Statistics toolbox in MATLAB. We applied the Bonferroni correction for multiple comparisons In **Figure 2d**, to test the difference of Cspk modulation (firing frequency above baseline) from zero. We used nonparametric statistical tests without assuming normality, except for data in **Figure 3a**, where we used Pearson's correlation coefficient. All tests were two-sided. No randomization was used, but mice were assigned to specific experimental group without bias and no animals were excluded. The experimenter was blind to task condition and behavior during spike sorting and further analyses.

A **Supplementary Methods Checklist** is available.

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