likelihood function:

$$\left(\frac{r^2}{r^2+4r+1}\right)^a\left(\frac{4r}{r^2+4r+1}\right)^b\left(\frac{1}{r^2+4r+1}\right)^c\left(\frac{r}{r+1}\right)^k\left(\frac{1}{r+1}\right)^{m-k}$$

where r represents the ratio of Htg^+ probability given YJM145 genotype over that given S288c; other variables as above. For Htg competitions, t-tests were performed to determine the significance of differences in means. Percentage growth deficiency was calculated as $100(\log C-\log B)/(\log C-\log A)$, where A corresponds to c.f.u. ml^{-1} of the control, B to that of the hemizygote, and C to that of the hybrid.

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Inhibition of climbing fibres is a signal for the extinction of conditioned eyelid responses

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A fundamental tenet of cerebellar learning theories asserts that climbing fibre afferents from the inferior olive provide a teaching signal that promotes the gradual adaptation of movements[1–3]. Data from several forms of motor learning provide support for this tenet[4–8]. In pavlovian eyelid conditioning, for example, where a tone is repeatedly paired with a reinforcing unconditioned stimulus like periorbital stimulation, the unconditioned stimulus promotes acquisition of conditioned eyelid responses by activating climbing fibres[9–12]. Climbing fibre activity elicited by an unconditioned stimulus is inhibited during the expression of conditioned responses[9–11]—consistent with the inhibitory projection from the cerebellum to inferior olive[6,13]. Here, we show that inhibition of climbing fibres serves as a teaching signal for extinction, where learning not to respond is signalled by presenting a tone without the unconditioned stimulus. We used reversible infusion of synaptic receptor antagonists to show that blocking inhibitory input to the climbing fibres prevents extinction of the conditioned response, whereas blocking excitatory input induces extinction. These results, combined with analysis of climbing fibre activity in a computer simulation of the cerebellar–olivary system[14–16], suggest that transient inhibition of climbing fibres below their background level is the signal that drives extinction.

To examine how inhibitory inputs to the climbing fibres contribute to extinction of the conditioned response, we infused the GABA (γ-aminobutyric acid) antagonist picrotoxin into the contralateral inferior olive of well trained rabbits undergoing extinction. Twenty rabbits were initially included in the study, of which four were found through histological methods to have cannula placements in the correct region of the inferior olive (two of these four placements are shown in the left column of Fig. 1d). Thus all of our figures and findings report data from these four animals. Rabbits were trained initially for five daily sessions (108 tone plus unconditioned stimulus trials per session) until they had acquired robust eyelid responses. Subsequently, each rabbit received a total of three daily extinction sessions (108 tone alone trials per session) with a different treatment each day (no infusion, continuous artificial cerebrospinal fluid (ACSF) infusion, or continuous infusion with picrotoxin (150 μM, 0.1 μl min^{-1})). To maintain high response levels before infusion, each extinction session was preceded by two daily training sessions of the tone plus unconditioned stimulus. With no infusion or infusion with ACSF, the four rabbits showed normal extinction of responses within the 108-trial session (Fig. 1a). In contrast, during intra-olivary infusion of picrotoxin, the same four rabbits maintained robust response levels throughout the entire extinction session (Fig. 1b). A within subjects analysis of variance and subsequent F-test for simple effects revealed that the rates of extinction for no infusion and ACSF infusions were indistinguishable ($F_{11,99} = 0.97$, not significant (NS)), and that both were significantly different from the response rate during picrotoxin infusion ($F_{11,99} = 17.22$ (no infusion) and 16.90 (ACSF), $P < 0.001$) (Fig. 1c).

Unlike experimental manipulations that abolish responses, block learning, or cause extinction, this impairment in extinction of the

conditioned response observed with reversible infusion of picrotoxin provides many internal controls, because response is maintained throughout the infusion. For example, the effects cannot be due to blockade of tone or unconditioned stimulus pathways by picrotoxin. The former would have abolished responses, and the latter is irrelevant as the unconditioned stimulus was omitted during the extinction session. Furthermore, infusion of picrotoxin did not block pathways necessary for response expression or cause a nonspecific performance deficit, as both of these would have abolished responses. Although picrotoxin might produce cerebellar malfunction as a consequence of a tonic increase in climbing fibre activity, infusion of picrotoxin into the inferior olive at the same concentration used here has been shown to increase climbing fibre activity only slightly, to approximately 2 Hz (ref. 17), which is less than the 5–10 Hz stimulation required to turn off Purkinje cells[18]. Finally, the possibility that the responses seen after infusion of picrotoxin are not conditioned responses can be excluded, because they retained their adaptive (that is, learned) timing throughout the extinction session. These controls, together with the observation that effective cannula placements were restricted to the rostromedial portions of the dorsal accessory olive (the region that is activated by the unconditioned stimulus during eyelid conditioning[10,12]; Fig. 1d, two out of the four effective placements are shown in the left column), suggest that picrotoxin prevented the extinction of conditioned responses by blocking inhibitory transmission in the inferior olive.

Next, we examined how excitatory input to the climbing fibres contributes to the maintenance and extinction of conditioned eyelid responses. The same four rabbits with cannula placements in the dorsal accessory olive were re-trained after the final extinction session, and then were given intra-olivary infusions of either ACSF or the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-receptor antagonist NBQX during two separate tone plus unconditioned stimulus test sessions. ACSF infusion did not affect the high level of response during the first tone plus unconditioned stimulus test session (Fig. 2a). In contrast, continuous infusion of NBQX (150 μM, 0.1 μl min^{-1}) during a subsequent tone plus unconditioned stimulus session resulted in the gradual disappearance of conditioned eyelid responses, until only the reflex response to the reinforcing unconditioned stimulus remained (Fig. 2b). The decline in conditioned response during infusion of NBQX was statistically indistinguishable from that observed during normal tone-alone extinction training in the same four animals ($F_{11,99}$ = 0.50, NS) (Fig. 2c, black line).

These data alone, however, cannot distinguish between an extinction-like decline in response that depends on the presentation of tone-alone trials, and a delayed abolition of conditioned responses due to slow diffusion of NBQX to a site necessary for response expression. To distinguish between these possibilities, the same four rabbits were re-trained and re-tested with an additional tone plus unconditional stimulus session that started 40 min after the continuous infusion of NBQX had begun. During the first test session (in which infusion of NBQX was not delayed), eyelid responses had disappeared completely 40 min after the beginning of the infusion (approximately 60 trials, Fig. 2b). Thus, if NBQX were causing a time-dependent effect, no response would be expected at the time when the second NBQX session began. However, all four rabbits

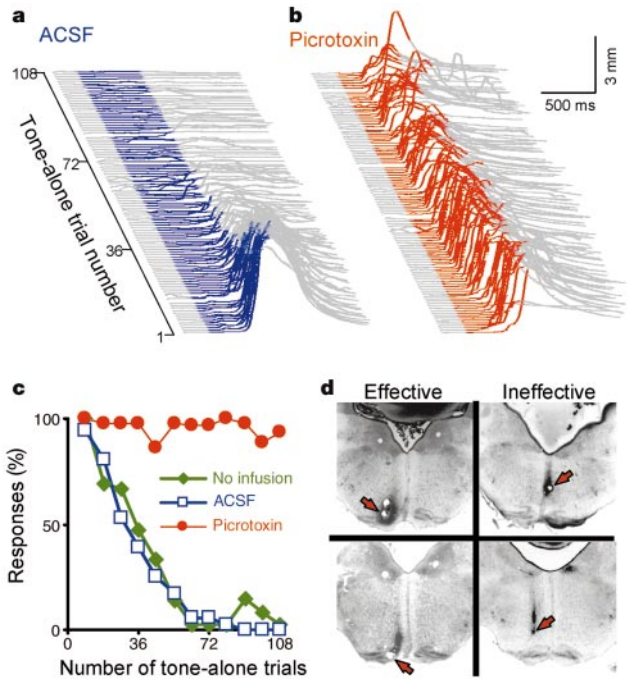

Figure 1 Infusion of picrotoxin into the inferior olive prevented extinction of conditioned responses. **a**, During tone-alone training with infusion of ACSF, conditioned responses gradually extinguished. Each trace represents the average response of the four rabbits for that particular trial. The blue section indicates tone presentation. **b**, With picrotoxin infusion the same four rabbits maintained high response levels throughout the session. The red section indicates tone presentation. **c**, Summary of response percentage for ACSF, picrotoxin, and no infusion sessions for the four rabbits with effective cannula placements. **d**, Four representative cannula placements (arrows) for two of the four animals whose data are shown in **a**–**c** (left column) and for two of the 16 animals with ineffective cannula placements (right column).

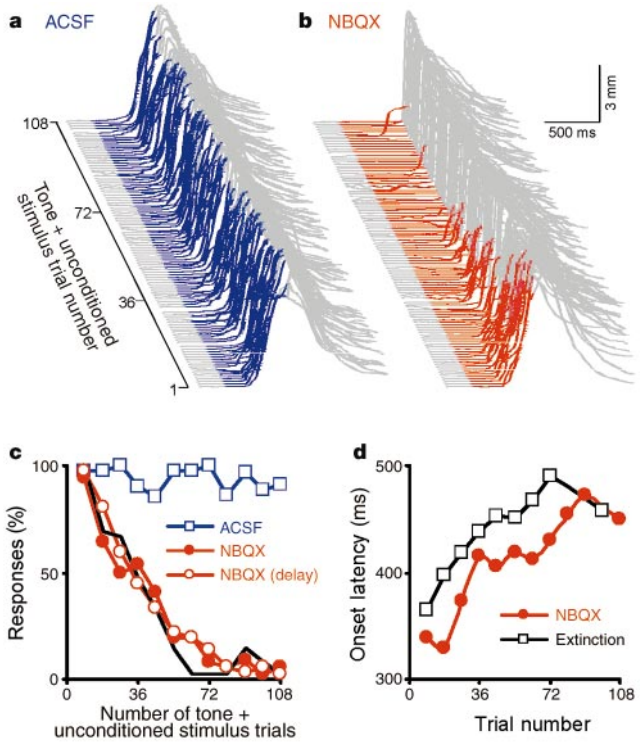

Figure 2 Infusion of NBQX into the inferior olive caused extinction of conditioned responses during tone plus unconditioned stimulus trials. **a**, Normal levels of conditioned response during tone plus unconditioned stimulus sessions were not affected by continuous ACSF infusion into the inferior olive. As in Fig. 1, traces are the average of the four rabbits with successful cannula placement. The blue section indicates tone presentation. **b**, With NBQX infusion, conditioned responses gradually decline until only reflex responses remain. The red section indicates tone presentation. **c**, The rate of response decrement was similar during normal extinction (black line) and during tone plus unconditioned stimulus trials given immediately after or 40 min after infusion of NBQX (see Methods). **d**, Concomitant with the decline in response, NBQX infusion produced a gradual increase in response latency similar to normal extinction.

responded during the initial trials of the second NBQX session, and the subsequent decline of eyelid responses was indistinguishable from the first NBQX test session ($F_{11,99} = 0.34$, NS) (Fig. 2c). NBQX infusion also produced changes in response timing that were indistinguishable from those that occur in extinction. During normal extinction, the latency to onset of the conditioned responses increases progressively as responses disappear gradually (Fig. 2d, black squares). We observed similar changes in onset latency during infusion of NBQX (Fig. 2d, red circles). Together, these findings suggest that intra-olivary infusion of NBQX produced trial-dependent extinction of conditioned responses and not time-dependent abolition.

A previously described computer simulation of the cerebellar-olivary system provides a working hypothesis to interpret the present infusion data[14–16]. Figure 3a summarizes the connectivity of the simulation that is relevant. Empirical evidence indicates that inhibitory and excitatory inputs to the inferior olive (Fig. 3a) combine with intrinsic cellular properties to regulate climbing fibre activity to approximately 1 Hz (refs 6, 19). We have shown previously that in the simulation, background climbing fibre activity is naturally self-regulated to approximately 1.2 Hz (Fig. 3b, red line), an equilibrium level at which no learning occurs[14–16]. As such, both acquisition and extinction of the conditioned response require that climbing fibre activity deviate from the equilibrium level during the simulated tone plus unconditioned stimulus or tone-alone trials. Whereas the increases in climbing fibre activity that promote acquisition of the conditioned stimulus

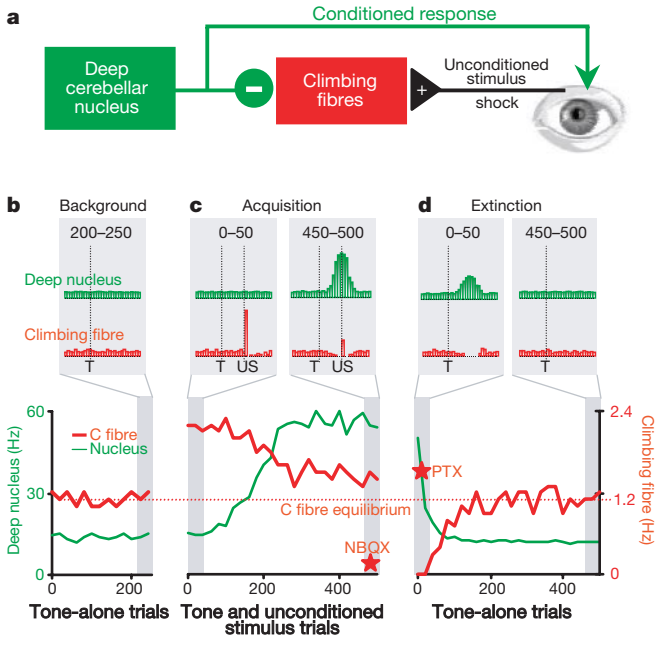

Figure 3 Activity of simulated climbing fibres and cerebellar nucleus cells during acquisition and extinction training. **a**, Schematic representation of climbing fibre afferents. **b**–**d**, Climbing fibre (red) and nucleus cell (green) activity (charts along the bottom), and activity from the training trials (histograms along the top). T, presentation of the tone; US, presentation of the unconditioned stimulus. **b**, Before training, climbing fibre and nucleus activity are relatively stable. **c**, During acquisition training, climbing fibre activity during the tone plus unconditioned stimulus trials is initially higher owing to the unconditioned stimulus. As the simulation learns, increased nucleus cell activity during the tone inhibits climbing fibre activity to near normal levels. **d**, At the outset of extinction training, climbing fibre activity during presentation of the tone is below normal owing to strong inhibition from the nucleus cells in the absence of excitation from the unconditioned stimulus. NBQX causes extinction by bringing climbing fibre activity below the normal level (star labelled NBQX), whereas picrotoxin blocks extinction by preventing climbing fibre activity from decreasing (star labelled PTX).

follow fairly intuitively from the activation of excitatory inputs by the unconditioned stimulus (Fig. 3a, unconditioned stimulus input), the simulation suggests factors that may decrease climbing fibre activity below equilibrium to promote extinction of the conditioned response.

The changes in climbing fibre activity over simulated acquisition and extinction training demonstrate an interpretation of the infusion results (red line in Fig. 3c, d). We began by training the simulation with 500 tone plus unconditioned stimulus trials (Fig. 3c). Initially, the unconditioned stimulus engaged the excitatory input to the inferior olive and was able to activate climbing fibres reliably (Fig. 3c, red histogram for trials 0–50) because cerebellar output was low (Fig. 3c, green histogram for trials 0–50) and thus the inhibitory input to the climbing fibres was weak. This reliable activation of climbing fibres by the unconditioned stimulus promoted the acquisition of conditioned responses (indicated by a gradual increase in cerebellar output; Fig. 3c, green line). However, the ability of the unconditioned stimulus to activate climbing fibres decreased as conditioned responses were gradually acquired (Fig. 3c, red line; compare red histograms in the left and right panels). The reason for this decline is that climbing fibres are increasingly inhibited by the increased cerebellar output that generates the conditioned response. This yielded climbing fibre activity near the equilibrium level in well trained simulations, despite the excitatory drive from the unconditioned stimulus (Fig. 3c, red histogram for trials 450–500), consistent with the behaviour of climbing fibres seen in several recording studies[9–11].

As recording data have shown that the ability of the unconditioned stimulus to activate climbing fibres is inhibited during a conditioned response[9–11], the simulations suggest that a simple extension of this hypothesis may explain the present infusion results: that is, strong inhibition during a conditioned response at a time when the unconditioned stimulus is omitted (as with extinction training) drives climbing fibre activity below its equilibrium level, thereby signalling the induction of plasticity responsible for extinction. Omitting the unconditioned stimulus after acquisition, or blocking the excitatory input to the climbing fibre as with NBQX infusion, tipped the balance in favour of inhibition, and thus promoted extinction by causing climbing fibre activity during the trial to fall well below equilibrium (Fig. 3c, red star labelled NBQX for NBQX infusion; Fig. 3d, red histogram for trials 0–50 for normal extinction). Conversely, although omitting the unconditioned stimulus during extinction training removed the strong excitatory input from the unconditioned stimulus, simulated infusion of picrotoxin also blocked the strong inhibitory input associated with making a conditioned response. Thus, climbing fibre activity did not decrease below the spontaneous level during the trial (Fig. 3d, red star labelled PTX), and extinction was prevented. This interpretation of the effects of picrotoxin may also explain why manipulations that block cerebellar nucleus output directly also prevent extinction[20]. Thus, our infusion data support the hypothesis that the induction of extinction is signalled by a decrease in climbing fibre activity during the tone, and that this decrease is produced by strong response-associated inhibition occurring in the absence of the excitatory drive from the omitted unconditioned stimulus.

The simulation incorporated a site of plasticity in the cerebellar cortex on the basis of observations that co-activation of granule cells and climbing fibres induces long-term depression (LTD) of granule to Purkinje synapses[21,22], whereas granule activity alone induces long-term potentiation (LTP)[22]. Thus, the bi-directional modulation of climbing fibre activity shown in Fig. 3 was responsible for acquisition and extinction of eyelid responses through the induction of LTD and LTP, respectively. This hypothesis seemingly contradicts evidence supporting the widely held view that extinction does not simply reverse changes that mediate acquisition. The evidence most frequently cited against 'unlearning' mechanisms of extinction is the phenomenon of savings, where relearning after

extinction is faster than original learning. We have shown recently, however, that a site of plasticity outside of the cerebellar cortex (possibly the cerebellar nuclei) is resistant to extinction and contributes to savings[16]. At least for cerebellar learning, therefore, phenomena such as savings are not inconsistent with the idea that acquisition and extinction can involve LTP and LTD at the same set of synapses given that there are at least two sites of plasticity.

The behaviour of climbing fibres during simulated acquisition and extinction is similar to the bi-directional modulation of activity observed in the dopamine neurons of the basal ganglia in response to unexpected reward or to the omission of expected reward[23,24]. In addition, the predicted contribution of response-associated inhibition in signalling extinction shares important features with the proposed role of feedback inhibition in the regulation of fear conditioning[25]. Thus, spontaneous activity tightly regulated by feedback inhibition, and the integration of excitatory inputs with feedback inhibition to modulate this activity in both directions, may represent general mechanisms for bi-directional learning. □

Methods

Subjects

We obtained data from 4 male New Zealand albino rabbits (*Oryctolagus cuniculus*), each weighing 2.5–3.0 kg (in addition, 16 rabbits were not included in the study because histological examination revealed that the cannulas were misplaced). The animals were individually housed and given food and water *ad libitum*.

Surgery

All animals were prepared with a cannula implanted in the inferior olive and with a head bolt cemented to the skull. Animals were pre-anaesthetized with 5 mg kg^{-1} acepromazine, and their skulls were immobilized in a stereotaxic restrainer. Aanesthesia was maintained with isoflurane (2–3% mixed in oxygen), and sterile procedures were used during the placement of the cannulas. The head was positioned with lambda 1.5 mm ventral to bregma. A cannula (Plastics One) consisting of a 26-gauge stainless steel guide sheath was placed at stereotaxic coordinates corresponding to the dorsal accessory olive (from the lambda landmark on the skull: 1 mm anterior, 0.7 mm lateral, 21.9–23.2 mm ventral). After placement, the cannula and head bolt were secured to the skull with dental acrylic. In addition, two stainless steel stimulating electrodes were chronically implanted in the periorbital musles rostral and caudal to the eye. Treatment of the animals and surgical procedures were in accordance with the National Institutes of Health Guidelines, and were approved by the University of Texas, Houston, animal welfare committee.

Conditioning

Each daily training session consisted of 12 blocks of 9 trials. For paired tone plus unconditioned stimulus sessions, each block consisted of eight paired presentations of the tone and unconditioned stimulus and one presentation of the tone by itself. The tone (1 kHz, 85 dB) was presented for 550 ms during tone-alone trials, and co-terminated with a 50 ms train of constant pulses (200 Hz, 1 ms pulse width, 2–3 mA) delivered to the periorbital electrodes during paired trials. Trials were separated by a random inter-trial interval in the range of 25–35 s. We recorded movement of the unrestrained eyelid by measuring the reflectance of an infrared light-emitting diode aimed at the eyelid.

Data analysis

Digitized sweeps of eyelid movement corresponding to the 200 ms before and 2,300 ms after the tone onset were analysed using custom software. To be counted as a conditioned response, the movement amplitude had to reach 0.3 mm within 500 ms after tone onset. Onset latency was determined by calculating the point at which the response reached the 0.3 mm criterion. Trials in which there was greater than 0.3 mm of movement during the 200 ms baseline period collected before tone onset were excluded from additional analysis.

Infusions

Infusions of ACSF, picrotoxin (150 μM), or NBQX (150 μM) were accomplished with a 33-gauge internal cannula that extended 1.2 mm beyond the tip of the guide cannula. Each infusion started 10 min before the beginning of the conditioning session at a rate of 0.1 μl min^{-1}. Then conditioning began and infusion continued throughout the session at 0.1 μl min^{-1}. In one set of experiments (NBQX delay), the conditioning session started 40 min after infusion of NBQX had begun. All compounds were dissolved in artificial cerebrospinal fluid (ACSF) consisting of (in mM): 124 NaCl, 3.0 KCl, 1 NaH_2PO_4, 1.3 H_2O, 3.0 MgCl, 10 dextrose, 10.0 HEPES (pH 7.35), 3.0 $CaCl_2$). Control experiments involved infusion of ACSF alone.

Histology

The infusion site was marked by passing a direct current anodal current (200 μA for approximately 10 s) through a small wire cut to the length of the internal cannula and exposed at the tip. Animals were killed with an overdose of sodium pentobarbital and perfused intracardially with 1.0 l of 10% formalin. Brains were embedded in an albumin gelatin mixture, and the brainstem was sectioned using a freezing microtome (80 μm sections). Tissue was mounted, stained with cresyl violet, and counterstained with Prussian blue.

Computer simulations

The circuitry of the simulation was based on the known anatomy and physiology of the cerebellar-olivary system[6,13] and the way in which this circuitry is engaged during eyelid conditioning[7,8,15]. Details about the simulation and the physiology of its connections have been presented previously[14–16], and will be described only briefly. For each simulated neuron, we used a single-compartment, integrate-and-fire representation, which calculates membrane potential based on leak and synaptic conductances. Of particuluar relevance to the present results is the connectivity of the inferior olive shown in Fig. 3a. In short, climbing fibres (shown in red) receive inhibitory input from the deep cerebellar nuclei (shown in green) and excitatory input from the reinforcing unconditioned stimulus (shown in black)[6]. Thus, presentation of the unconditioned stimulus during paired trials was simulated by adding a constant depolarizing pulse to the membrane potential of the climbing fibre. In our simulations, acquisition/extinction produce increases/decreases in cerebellar nuclei activity through the induction of plasticity in both the cerebellar cortex and cerebellar nuclei[14–16]. However, the data presented here regarding the activity of simulated climbing fibres during conditioning only require that cerebellar nuclei activity increases during acquisition (and decreases during extinction), and therefore our results do not depend on the plasticity mechanisms underlying the changes in cerebellar nuclei activity that are observed during eyelid conditioning.

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