A cerebelloloivary signal for negative prediction error is sufficient to cause extinction of associative motor learning

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The brain generates negative prediction error (NPE) signals to trigger extinction, a type of inhibitory learning that is responsible for suppressing learned behaviors when they are no longer useful. Neurons encoding NPE have been reported in multiple brain regions. Here, we use an optogenetic approach to demonstrate that GABAergic cerebelloloivary neurons can generate a powerful NPE signal, capable of causing extinction of conditioned motor responses on its own.

When an expected event does not happen, the brain generates an NPE signal that is used to optimize our actions by triggering neural mechanisms of extinction, an adaptive form of inhibitory learning responsible for the gradual suppression and eventual elimination of maladaptive behaviors. Previous work has suggested that neurons in the inferior olive (IO) encode an NPE signal during cerebellar-dependent learning tasks. Support for this hypothesis comes from eyeblink conditioning studies showing that IO neurons are briefly inhibited when an aversive eye puff is unexpectedly omitted. However, we do not know if this reported brief inhibition of the IO is sufficient for extinction of the eyelink response (conditioned response, CR), partly because previous attempts to examine this question have relied on electrical stimulation, pharmacological inactivation or lesions to suppress IO activity. Because these manipulations are not cell-type-specific and in some cases lack temporal precision, they can result in abnormal activation of mossy fiber inputs to the cerebellum, or cause widespread destabilization of olivocerebellar circuits which leads to general deficits in motor performance and has made previous experimental results difficult to interpret.

We used an optogenetic approach to inhibit eye-puff-driven responses in the IO of mice (Fig. 1). Because direct inhibition of IO neurons with hyperpolarizing opsins was impractical in our experiments (Extended Data Fig. 1), we opted for an approach that exploits the natural circuitry of the brain. A virus (AAV5-HSyn-hChR2(E123A)-EYFP) was injected into the right cerebellar nuclei, resulting in expression of ChR2 in somata of the cerebellar nucleus neurons (Fig. 1a–d), and in the GABAergic axon terminals of projections from the cerebellar nuclei to the contralateral IO (cerebellar nucleo-olivary pathway, CNIO; Fig. 1e–h). To validate the approach, we delivered airpuffs to the right eye while simultaneously photostimulating the inhibitory CNIO pathway via an optical fiber implanted above the left IO at the level of the dorsal accessory olive (Fig. 1c; see Extended Data Fig. 2 for a diagram of the anatomy and Extended Data Fig. 3 for histological analysis of optical fiber placement). This approach likely confines the effects of photostimulation to the IO because non-CNIO neurons expressing ChR2 around the AAV injection site are too far to be directly activated by the laser light (>2.5 mm), and because the axons of CNIO neurons have no known collaterals and are thought to project only to the IO.

The responses of individual IO neurons to the airpuff, which were measured by recording climbing fiber-driven complex spikes in Purkinje cells (‘Record’, Fig. 1a), were strongly suppressed by CNIO terminal photostimulation (Fig. 1i–j), although the IO was not always completely silenced and a small response remained in some cases (Fig. 1l). When the suppression of eye-puff-driven responses resulted in a pause of IO firing, it was often followed by a rebound in the poststimulation period (Fig. 1k,l), reminiscent of the pattern of IO activity previously observed when an expected eye puff was omitted during normal extinction trials (Extended Data Fig. 4). We will refer to photostimulation of GABAergic CNIO terminals in the IO as ‘CNIO stimulation’ for the remainder of this paper.

To test whether CNIO stimulation can serve as an NPE and effectively drive extinction, mice expressing ChR2-EYFP in CNIO axon terminals were trained in an eyelink conditioning task (‘ChR2’ mice; see Methods for task details). After the mice learned to make eyelink CRs and performance stabilized (Fig. 2a,b, ‘Training’), we continued to deliver normal conditioning trials while adding a brief CNIO stimulation pulse during the eye puff (200-ms pulse starting 20 ms before airpuff trigger; Fig. 2a,b, ‘ChR2’). Over multiple sessions of CNIO stimulation, ChR2 mice showed a gradual decay in CR probability (Fig. 2b) and CR amplitude (Fig. 2c,d). This decline in CR performance was also observed in wild-type (WT) mice undergoing extinction training with unpaired stimulus presentations (‘WT’ mice; Fig. 2e,f), and had a similar time course (Fig. 2b). Further analysis of CR performance in the ChR2 mice revealed three additional behavioral hallmarks of normal extinction. First, ChR2 mice made more CRs at the beginning of a CNIO stimulation session than at the end of the preceding session (Extended Data Fig. 5a,b), a clear sign of spontaneous recovery behavior commonly observed during normal extinction (Extended Data Fig. 5c,d). Second, when normal conditioning sessions resumed after 10 d of CNIO stimulation, ChR2 mice showed retention of the extinction memory on the first day of reacquisition, making very few CRs initially (Fig. 2b), and performing at a lower CR amplitude than in the last training session (Fig. 2b). Third, with further conditioning, ChR2 mice then displayed ‘savings’ (Extended Data Fig. 5e,f): CRs were relearned faster than during initial acquisition (see Extended Data Fig. 5g,h for a comparison with savings after normal extinction with unpaired stimulus presentations in WT mice).

Our analyses suggest that the gradual decay in CR performance of ChR2 mice in Fig. 2 is the result of an extinction process. We performed five control experiments to rule out alternative explanations: (1) Mice expressing EYFP or no fluorescent protein in CNIO terminals were trained in five control experiments to rule out alternative explanations.
axon terminals (‘Control’ mice; see Extended Data Fig. 3 for histological analysis) showed no effects of photostimulation on CR probability (Fig. 2b). Thus, the light or the heat generated by the photostimulation did not cause ChR2 mice to stop making CRs. (2) Following the optogenetic experiments, all ChR2 mice reacquired the CR and attained prestimulation performance levels (Fig. 2b,g,h; ‘Training’ versus ‘Retraining’), indicating that CNIO stimulation did not cause permanent damage to the olivo-cerebellar circuits that are critical for eyeblink conditioning. (3) Impairments in processing the eye puff stimulus could also be ruled out because CNIO stimulation had no effect on the amplitude of reflexive blinks in ChR2 mice (Extended Data Fig. 6a,b), and although its duration was shortened (Extended Data Fig. 6c), similar changes in reflex duration did not affect CR probability during normal training (Extended Data Fig. 6d,e). (4) CNIO stimulation did not weaken the ongoing motor drive for the CR directly because ChR2 mice that received CNIO stimulation throughout the entire CR window (200 ms before eye puff) made more frequent and bigger CRs (Extended Data Fig. 7a–f), and produced reflexive eyelid movements after the tone that were more vigorous than normal, including bigger β-startles (Extended Data Fig. 7g–i) with shortened latency (Extended Data Fig. 7j). (5) Finally, the two experiments shown in Fig. 3 demonstrate that the repeated delivery of brief but frequent CNIO stimulation pulses did not cause dysregulation of activity in downstream Purkinje cells, which is known to produce impairments in CRs that can be mistaken for extinction learning. First, the cerebellum remained

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**Fig. 1 | Inhibiting the IO in vivo.** a–h, Analysis of opsins expression in the sites of AAV-hSyn-Chr2-eYFP injection in the cerebellar nuclei (a–d), and optical fiber implant in the IO (e–h). Schematic drawings show the cerebellar sites of virus injection and single-unit recordings (a) and the fiber implant site in the brainstem (e). Confocal images from one example mouse (from n = 11 total mice), showing Chr2-EYFP expression (green) in somata of cerebellar nucleus neurons (b, arrowhead) and CNIO terminal axons in the IO (f). Fluorescent Nissl stain (magenta) indicates location of cell bodies in the cerebellar nucleus (c) and IO (g). Colocalization of Chr2-EYFP and Nissl is shown in cells of the cerebellar nucleus (d, white), but not in the IO (h). i, Top: raw extracellularly recorded signals showing simple spikes and complex spikes (CSpks, ^) from an example trial. Bottom: CSpk raster plots for an example Purkinje cell for control condition (i) and during photostimulation of CNIO terminals in the IO (j, blue shading; 11.63 mW mm⁻², 200-ms laser pulse starting 20 ms before the airpuff trigger). j, Peristimulus time histograms (bin size = 10 ms) for the CSpks in the two raster plots of i and j. k, Suppression of CSpk responses to airpuff by CNIO stimulation (blue) (n = 11 neurons from 4 mice; two-tailed paired t-test, t = 6.54, d.f. = 10, P = 6.56 × 10⁻⁵, Cohen's d = 2.58). CSpk rates were normalized to pretrial baseline. ***P < 0.001. br, bregma.
**Fig. 2** | CN$_{ac}$ stimulation during eye puff stimulus. **a**, Schematic showing the relative timing of stimulus presentations and laser pulses in the different phases of the experiments. **b**, CR probability (median ± MAD) before (left, 'Training'), during (gray shaded area) and after (right, 'Retraining') the sessions with optogenetic stimulation in ChR2 (blue circles, $n = 6$ mice, reduction in CR probability was significant: repeated measures ANOVA, Friedman's statistic $F_r = 32.17$, d.f. $= 6$, $P = 1.51 \times 10^{-5}$, Kendall's $W = 0.81$) and control (red squares, $n = 5$ mice, no change in CR probability: Friedman's ANOVA, $F_r = 5.23$, d.f. $= 6$, $P = 0.51$) groups, or sessions with extinction in the WT group ($n = 5$ mice, black circles). An nparLD analysis showed that the ChR2 and WT mice exhibited no differences in their learning rates ($n = 10$ mice, nparLD, Friedman's ANOVA, $F_{group \times session} = 1.56$, d.f. $= 2.40$, $P = 0.20$). After the laser manipulation, mice in the ChR2 group produced very few CRs ('retraining', mean ± s.e.m., $1.7 \pm 1.1$ CRs in the first 10 trials).

**c–h**, Averaged eyelid movement traces (**c**, **e**, **g**) and CR amplitude (**d**, **f**, **h**) during the 'laser' phase for ChR2 mice (**c**, **d**, $n = 6$ mice; Friedman's ANOVA, $F_r = 26.14$, d.f. $= 6$, $P = 2.09 \times 10^{-4}$, $W = 0.73$), during the extinction phase for WT mice (**e**, **f**, $n = 5$ mice; Friedman's ANOVA, $F_r = 19.54$, d.f. $= 6$, $P = 3.34 \times 10^{-4}$, $W = 0.65$) and during the 'retraining' phase for ChR2 mice (**g**, **h**). In **h**, two-tailed paired $t$-tests showed a significant difference between CR amplitudes on the last day of training and the first day of retraining ($n = 6$ mice, $t = 8.05$, d.f. $= 5$, $P = 4.79 \times 10^{-4}$, $d = 2.60$) but no difference between the last day of training and last day of retraining ($n = 6$ mice, $t = −0.35$, d.f. $= 5$, $P = 0.74$). The time window used to calculate CR amplitude is indicated (**c**, **e**, **g**; yellow shaded area). In all panels, boxplot center, mean; box bounds, ±s.e.m.; whiskers, distribution minimum and maximum. Family-wise alpha values were Bonferroni–Holm corrected for multiple comparisons. *$P < 0.05$, ***$P < 0.001$. MAD, median absolute deviation; NS, not significant; R, retraining session; RLast, last retraining session; T, training session; TLast, last training session; TLast-1; penultimate training session.
functional in ChR2 mice that received CNIO stimulation 350 ms after the eye puff stimulus in every conditioning trial (Fig. 3a), as evidenced by stable CR probability (Friedman’s ANOVA, Fr = 1.40, d.f. = 7, P = 0.97), averaged eyelid movement traces (F) and CR amplitude (d) were unchanged during sessions with CNIO stimulation delivered after the airpuff in ChR2 mice (n = 7 mice; Friedman ANOVA, Fr = 1.41, d.f. = 7, P = 0.96; boxplot center, mean; box bounds, ±s.e.m.; whiskers, distribution minimum and maximum). e. Comparison between the averaged trial-by-trial changes in CR amplitude (normalized to mean CR amplitude on ‘TLast’) in the ‘Laser after airpuff’ experiment (red) and the ‘Laser during airpuff’ experiment shown in Fig. 2 (blue, n = 6 mice). Individual sessions are separated from each other by vertical dashed lines. The time window used to calculate CR amplitude is indicated (c; yellow shaded area). f. Raw extracellular signal (red boxes; CSpk) and simple spike firing rate during repeated trials with CNIO stimulation for ten Purkinje cells (same cells as Fig. 1; firing rate normalized to the mean in the first five trials, filtered using a moving average with window size of one trial). f. Inset: simple spike firing rate during the first 10% (‘First’) and last 10% (‘Last’) of trials in the recording session for each cell (n = 10 neurons; two-tailed paired t-test, t = −0.14, d.f. = 9, P = 0.89). Family-wise alpha values were Bonferroni–Holm corrected for multiple comparisons.

Fig. 3 | CNIO stimulation after eye puff stimulus. a. Schematic showing the relative timing of stimulus presentations and laser pulses in the different phases of the experiment. b–d. CR probability (b) (median ± MAD, n = 7 mice, no change in CR probability; Friedman’s ANOVA, Fr = 1.40, d.f. = 7, P = 0.97), averaged eyelid movement traces (c) and CR amplitude (d) were unchanged during sessions with CNIO stimulation delivered after the airpuff in ChR2 mice (n = 7 mice; Friedman ANOVA, Fr = 1.41, d.f. = 7, P = 0.96; boxplot center, mean; box bounds, ±s.e.m.; whiskers, distribution minimum and maximum). e. Comparison between the averaged trial-by-trial changes in CR amplitude (normalized to mean CR amplitude on ‘TLast’) in the ‘Laser after airpuff’ experiment (red) and the ‘Laser during airpuff’ experiment shown in Fig. 2 (blue, n = 6 mice). Individual sessions are separated from each other by vertical dashed lines. The time window used to calculate CR amplitude is indicated (c; yellow shaded area). f. Raw extracellular signal (red boxes; CSpk) and simple spike firing rate during repeated trials with CNIO stimulation for ten Purkinje cells (same cells as Fig. 1; firing rate normalized to the mean in the first five trials, filtered using a moving average with window size of one trial). f. Inset: simple spike firing rate during the first 10% (‘First’) and last 10% (‘Last’) of trials in the recording session for each cell (n = 10 neurons; two-tailed paired t-test, t = −0.14, d.f. = 9, P = 0.89). Family-wise alpha values were Bonferroni–Holm corrected for multiple comparisons.
via inhibition of dopaminergic cells\textsuperscript{13–15}, which also have a low spontaneous firing rate that severely limits the range of NPE values that can be veridically represented\textsuperscript{16}. Further experiments will be needed to assess whether the dynamic range of NPE signals originating in the IO can be enhanced downstream in the cerebellar cortex, possibly by modulation of complex spike responses in Purkinje cell populations\textsuperscript{12}.

It is remarkable that mice learned to stop making CRs during CN\textsubscript{IO} stimulation, even though this is a maladaptive change in behavior that leaves the cornea exposed at the time of the eye puff, and many neurons outside the IO must have been broadcasting error signals for promoting protective CRs. Indeed, error signals have been observed throughout the brain\textsuperscript{1}, including in many neurons of the cerebellum\textsuperscript{17–20}, which is the brain area responsible for making eyeblink CRs\textsuperscript{11}. We do not know how all of these signals may interact to drive adaptive changes in behavior and optimize future performance. However, our results indicate that despite the multiplicity of error signals likely to be present in the brain during our experiments, NPE signals generated in the IO take precedence. Because CN\textsubscript{IO} neurons are capable of profoundly reducing sensory-evoked responses in the IO, they appear to be ideally positioned to dictate which behaviors will ultimately be acquired or extinguished during cerebellar learning.

**Online content**

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**Methods**

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**Animals.** All procedures were approved by the College of Medicine Institutional Animal Care and Use Committee based on the guidelines of the US National Institutes of Health. Experiments were performed on male C57BL/6 mice at least 10 weeks of age (n = 23 mice) housed on a reverse light/dark cycle (8:00 lights-off to 20:00 lights-on); mice were housed in cages of up to four before surgery and were singly housed after surgery. Ambient temperature was maintained at 20–22 °C, and ambient humidity was maintained at 30–70% relative humidity. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications. Mice were randomly allocated into different groups. Two mice were removed from the experiment because task performance failed to reach criterion during the training phase.

**Surgery.** Procedures have been described previously. In brief, mice were anesthetized with isoflurane (5% by volume in O2, for induction, 1–2% by volume for maintenance; SurgiVet) and kept on a heating pad to maintain body temperature. Surgeries were carried out under sterile conditions, and mice received postoperative analgesia (0.02 ml of 0.5% bupivacaine and 2% lidocaine, subcutaneously at incision site; 5 mg/kg−1 meloxicam, subcutaneously). Fascia and obstructing muscle tissue were cleared from the skull through a midline incision, and the skull was leveled into the stereotaxic plane. Each mouse (n = 23 mice) received a head-plate implant, secured to the skull using two jeweler’s screws (relative to bregma: anterioposterior axis (AP) −0.5 mm, mediolateral axis (ML) ±1.3 mm) and C&B Metabond. The five mice used for extinction did not receive any further implants (“WT” group). All remaining mice (n = 18 mice) were implanted with an optical fiber (400-µm diameter, constructed in-house using ThorLabs FP400UR/0.22 NA) just dorsal of the left dorsal accessory olice (DAO) (relative to bregma: AP −6.6 mm, ML ±0.2 mm; relative to skull surface at AP/ML target: −5.4 mm). Mice with fiber implants also received a virus injection (200 nl, 10 nl min−1, Hamilton, 65458-02 with 30° bevel; WPI, UMP-3 pump) in the right anterior interpositus (AIP) (relative to lambda: AP −2.0 mm, ML −1.9 mm; relative to dura mater surface at AP/ML target: dorsoventral axis (DV) −2.4 mm; 10° angle posterior to anterior). For 4 of the 18 mice (“Control” group), we injected AAV5-hSyn:EF1FP (Karl Deisseroth, UNC Vector Core). For the remaining 14 mice (“ChR2” group), we injected AAV5-hSyn:ChR2(E123A)-EF1FP (Karl Deisseroth, UNC Vector Core). In the 4 mice in the ChR2 group, we opened a craniotomy over the right cerebellar cortex to record eye-puff-driven complex spikes in Purkinje cells (2.5 × 2.5 mm2; protected by a three-dimensionally printed chamber, NeuroNexus).

**Stimulus control and behavioral monitoring.** Conditioning sessions were conducted inside of a sound-attenuating chamber. During all sessions, masking white noise was delivered in the background at 65 dB. Tone stimuli (500-ms duration, 10kHz) were delivered via a speaker (Dell, AC511) positioned behind the mouse. Airpuffs were delivered via a pressure injector (ASI, MPPI-3) connected to plastic tubing terminating in a blunt-tipped, 23 G needle positioned 5 mm in front of the mouse’s right eye. A high-speed monochrome camera (Allied Vision Technologies, GE680) recorded 200 fps videos of the right side of the face (that is, ipsilateral to the virus injection site) under infrared (Bosch, EX12LD) illumination. During sessions with photostimulation, light was delivered using analyzer-controlled, 473-nm lasers (Blue Sky Research, FT42473) and 200-µm-diameter patch cables. Blue ambient illumination (5X, UScell a14122700ux012 in series, 550-µm resistance to ground, 5 V 2 A -power supply) was present throughout all sessions to mask light escaping from the junction between the patch cable and implanted ferrule during photostimulation. Because the goal of the experiment was to examine learning processes that take place over consecutive days, the same stimulus presentations and experimental conditions were maintained constant for multiple sessions before switching to a different condition.

**Acquisition of eyeblink conditioning.** Mice underwent 2d of habituation training, during which they were head-fixed on top of a cylindrical treadmill and allowed to locomote freely, as they would be during all future experiments. At the beginning of all subsequent sessions, mice received a blink-eliciting airpuff (8.5 peak PSI, 30-ms trigger duration) to calibrate eyelid position measurements. On the first day of conditioning, up to 15 presentations of 10-kHz tones were delivered (60+ s inter-trial interval (ITI)) at different volumes (65–95 dB) to find the lowest intensity that reliably evoked an eyelid startle in each mouse. This tone volume was used for the rest of the experiment. Conditioning sessions consisted of 100 tone + airpuff trials per day (200-ms ISI, 15–20-s ITI; 30- ms airpuff duration), unless mice exhibited signs of excessive stress and sessions were terminated early. After each acquisition session, performance criterion of 50% CRs (see ‘Behavioral data analysis’ below for CR criteria) on 3 consecutive days), airpuff intensity was systematically decreased in nine of the mice, to shorten the duration of the reflex blink and examine the impact on CR probability. Before initiating either photostimulation or extinction sessions, the airpuff intensity was reset in all mice to just above threshold for maintaining >50% CRs, and kept at that level for at least 2 d to verify that the duration of the reflex blink was ~200 ms and that conditioned responding was stable (~10% CRs).

**Photostimulation experiments.** During photostimulation experiments, tone + airpuff stimuli continued to be presented as above, and a brief pulse of light (200 ms, 473-nm wavelength) was delivered via the implanted optical fiber at a particular time in every trial. We performed three experiments that were identical, except for the time in the trial at which the photostimulation pulse was delivered: (1) photostimulation during puff (onset 20 ms before airpuff trigger); (2) photostimulation after puff (onset 350 ms after the airpuff trigger, after tone + airpuff offset); and (3) photostimulation before puff (onset 200 ms before the airpuff trigger, simultaneous with tone onset). The same timing for the photostimulation pulse was used for up to 10 consecutive days. Between experiments with different photostimulation times, no time was returned to normal conditioning trials to re-establish baseline performance. To estimate the level of irradiance reaching the DAO, we first calculated the light power at the base of the tether by taking into account the input laser power and discounting this value by the percentage loss due to light transmission through each optical fiber. Then, for each mouse, we measured the distance between the DAO and a lens marking the tip of the optical fiber (marking lesion deliberately made at the end of the experiment by 30–85-nm photostimulation), and used this measurement to estimate irradiance in the DAO based on the known degree of irradiance decay over distance in mammalian brain tissue. Our estimates of irradiance in the DAO ranged from 7.57 to 30.5 mW mm−2 (corresponding to 32.97–75.00 mW at the base of the tether in optical fibers, with transmission efficiency values ranging from 76% to 92%). Note that, because the optical fiber tip was implanted just dorsal to the IO, which is >2 mm ventral of and posterior to the cerebellar nuclei, light irradiance at the level of the AAV injection site would have been negligible (an estimated 0.34–0.75 mW mm−2 in the caudal pole of the posterior cerebellum). To examine reacquisition, after the last day of photostimulation all mice received 3–5 consecutive days of training with behavioral sessions consisting of 100 tone + airpuff trials.

**Extinction experiment.** We used an unpaired stimulus protocol to examine normal extinction in five WT mice that were not used for any of the photostimulation experiments. For 10 consecutive days, the mice received daily sessions with 200 trials each (7–10s ITI). On odd-numbered days, the tone was presented alone. On even-numbered trials, the airpuff was presented alone. This unpaired extinction protocol provides a more suitable comparison group than other extinction protocols based on just tone presentations because it degrades the contingency between the tether and the airpuff while maintaining the same total number of tone and airpuff presentations per session as in the experimental groups. To examine reacquisition, after the last day of extinction all mice received 3–5 consecutive days of additional training with behavioral sessions consisting of 100 tone + airpuff trials.

**Behavioral data analysis.** Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset). Single-unit recordings. Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset). Single-unit recordings. Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset). Single-unit recordings. Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset). Single-unit recordings. Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset). Single-unit recordings. Analyses were conducted using custom scripts written in MATLAB. Eyelid position was calculated for each video frame by counting the number of white pixels in a thresholded binary image of the eye and surrounding fur, as described previously. Eyelid latency was measured relative to the onset of the airpuff (onset 20 ms before airpuff trigger, after tone + airpuff offset).
isolation was established and periocular airpuffs were delivered to verify that the isolated cell responded to the stimulus with a CSpK. Then, a repeating block design consisting of one airpuff trial, one photostimulation trial (200-ms duration, 473-nm wavelength, 8.12–30.59 mW mm\(^{-2}\)) and one trial with airpuff and photostimulation (airpuff trigger 20 ms after laser onset, same photostimulation parameters as above) was carried out until isolation was lost (66–224 total trials). In addition, Extended Data Fig. 4 includes CSpK activity from a previously collected dataset, which contains recordings performed as described above (n=6 C57BL/6J mice, n=32 units), but collected during presentation of normal extinction trials in conditioned mice.

Single-unit data analysis. SSps and CSpsks were sorted manually for 200+ ms preceding and 1,000 ms following each trial. The different spike types were identified by their characteristic waveforms in Spike2, and trials with poor SSpk and CSpk isolation were excluded from further analyses. The presence of statistically significant CSpK rebounds after inhibition was assessed by generating a bootstrapped distribution from the pretrial baseline data of each individual neuron, using a case-resampling procedure with replacement and the Monte Carlo algorithm. A neuron was considered as exhibiting a CSpK rebound if firing rate in the 50 ms after a pause was greater than 99% of the bootstrapped distribution.

Histology. After mice with optical fiber implants completed the behavioral experiment, marking lesions were made via intense light stimulation through the optical fibers (Shanghai Laser & Optics Century, BL473T3-150FC, 85 mW for 30 s). After 3 d, mice were euthanized and brains were extracted. Tissue was stored at 4 °C in 4% paraformaldehyde (Affymetrix, AAJ19943K2) overnight before being transferred to a 30% sucrose solution in PBS for 3–5 d. Tissue was sectioned at 50-μm thickness on a cryostat (Leica, CM1950) and alternating stored at 4 °C in 4% paraformaldehyde (Affymetrix, AAJ19943K2) overnight. The optical fibers (Shanghai Laser & Optics Century, BL473T3-150FC, 85 mW for 30 s) and 40 ms after a pause was greater than 99% of the bootstrapped distribution.

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Author contributions

O.A.K. and J.F.M. designed the experiments. J.F.M. supervised the project. O.A.K. performed virus injection and optical fiber implant surgeries, and conducted all behavior/optogenetics experiments. S.O. performed all electrophysiology-related surgery and experiments. O.A.K. analyzed and curated all original data collected for this publication. S.O. curated data included from a previous publication. O.A.K. and J.F.M. wrote the original draft of the paper. J.F.M., O.A.K. and S.O. revised and edited the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Summary of failed experiments using inhibitory opsins in the dorsal accessory olive. a–f, Arch expression (green) in and around the IO (outlined in white) in histological sections counterstained with red fluorescent nissl (magenta). Expression of ArchT was very sparse and did not cover the region of the IO involved in eyelink conditioning (dorsal accessory olive, DAO; white arrows) in Pdx-cre mice crossed with flex-ArchT mice (a), or Pdx-cre mice that received an AAV-ArchT injection in the IO (b). (c–e) Expression of Arch3 was patchy and did not cover the DAO in CRH-cre mice crossed with either heterozygous (c) or homozygous flex-Arch3 mice (e), or in CRH-cre mice that received an AAV-Arch injection in the IO (d). (f–i) Although we obtained good expression of Arch in the DAO in wildtype mice injected with AAV1/9-αCaMKII-Arch-GFP (f), the health of 8/11 mice began to deteriorate two weeks after AAV injection, such that the mice could not tolerate eyelink conditioning sessions and had to be euthanized. Experiments were not possible in the few mice that survived (3/11 mice) because these mice were severely impaired in eyelink conditioning (g), and the performance of even the best mouse (i) was much worse than the performance of control mice (h, control data previously published in^{21}). Abbreviations: CR, conditioned response; CS, conditioned stimulus; DAO, dorsal accessory olive; DM, dorsomedial cell column; FEC, fraction eyelid closure; MAO, medial accessory olive; PO, principal olive; RF, reticular formation.
Extended Data Fig. 2 | Olivo-cerebellar circuits relevant to eyeblink conditioning. Somatosensory information about the eye puff stimulus crosses the midline and is sent to the contralateral inferior olive via the trigeminal nucleus. The inferior olive sends a predominantly contralateral projection to eyeblink-generating Purkinje cells in the cerebellar cortex via the climbing fiber pathway. Purkinje cells and cerebellar nucleus projection (CNrn) neurons control CR generation for the ipsilateral eye. Cerebellar nucleo-olivary neurons (CNio) send a GABAergic projection to the inferior olive. During the experiments described in this paper, we induced broad ChR2 expression in the cerebellar nuclei and then selectively activated the cerebello-olivary pathway by photostimulating CNio axon terminals at the level of the inferior olive. Note that CNio neurons are a distinct population, completely separate from the CNrn neurons, and for this reason, photostimulation-driven backpropagating action potentials in the CNio axons do not have direct access to the neurons that are responsible for generating the eyeblink CR.
Extended Data Fig. 3. | Opsin expression and optical fiber placement in ChR2 and control mice. a, e, Outline of nuclei around the virus injection site (a) and the fiber implant site (e) traced from a representative mouse (Chr2 1). The cerebellar and inferior olivary nuclei implicated in eyeblink conditioning are highlighted in green (anterior interpositus nucleus, AIP; dorsal accessory olive; DAO) (b–d, f–h). Coronal sections at the level of the cerebellar nuclei (b–d) and inferior olive (f–h) from mice in the Chr2 (b, d, f, h) and control (c, g) groups. A unique identifier for each mouse is shown in the bottom left corner of each photomicrograph. Cerebellar and vestibular nuclei are revealed by fluorescent nissl stain (magenta). Chr2-eYFP (green) was visible at the level of the AIP (b–d) and, with longer exposure times, the DAO (f–h). The AAV injection did not work in mouse ‘Control 5’. Lesions deliberately made at the end of the experiments to mark the location of the optical fiber tip are also visible just dorsal to the DAO (yellow dashed outline). Abbreviations: AIP, anterior interposed nucleus; cctx, cerebellar cortex; DAO, dorsal accessory olive; DM, dorsomedial cell column of the inferior olive; DC, dorsal cochlear nucleus; DN, dentate nucleus; F, fastigial nucleus; icp, inferior cerebellar peduncle; LVe, lateral vestibular nucleus; MAO, medial accessory olive; MVe, medial vestibular nucleus; P, paracochlear glial substance; PO, principal olive; py, pyramids; RF, reticular formation; VCB, vestibulocerebellar nucleus.
Extended Data Fig. 4 | CSpk activity is similar during CNIO stimulation and normal extinction. a, CSpk firing rate (normalized to pre-trial baseline) for individual neurons (heat plots) and for groups of neurons (mean, histograms) in 50-ms bins, aligned to the end of the pause during CNIO stimulation trials in Chr2 mice (a, n = 11 neurons, same as in Fig. 1) or extinction trials in wildtype mice (b, n = 32 neurons, recorded during a previous experiment\(^5\)). c, Quantification of rebound size in the 50 ms after the pauses shown in (a, b). Each dot shows the mean, normalized firing rate response in the 50 ms after a pause (rebound window) for a single neuron. Dots are filled if neurons exhibited significant rebound firing (see Online Methods). Of the neurons recorded during CNIO stimulation trials in Chr2 mice, 6/11 (55%) exhibited a significant rebound. Of the neurons recorded during extinction trials in wildtype mice, 15/32 (47%) exhibited a significant rebound. There was no significant difference between rebound firing rates of the neurons recorded in the Chr2 and the wildtype mice (n = 43 neurons, two-sided Wilcoxon rank-sum test: W = 195, p = 0.61; boxplot center: mean, box bounds: ± SEM, whiskers: distribution minimum and maximum).
Extended Data Fig. 5 | Extinction during CN$_{iO}$ stimulation displays spontaneous recovery and savings. 

**a**, CR probability (median±MAD) measured in blocks of 20 trials during the final training session (white background) and repeated CN$_{iO}$ stimulation sessions (gray shaded background) in Chr2 mice ($n=6$ mice). Sessions are separated from each other by vertical dotted lines, and the line of best fit to the 5 median values in each session is shown.

**b**, CR probability in the last block of 10 trials in a session was significantly lower than in the first block of 10 trials in the next one for the first 5 sessions with CN$_{iO}$ stimulation for the mice shown in panel **a** ($n=6$ mice; two-tailed paired t-test: $t=3.63$, $df=5$, $p=0.015$, $d=1.55$).

**c**, Same as (**a, b**), but for wildtype mice undergoing extinction training (gray shaded background in panel **c**; $n=5$ mice; two-tailed paired t-test: $t=5.53$, $df=4$, $p=0.005$, $d=2.12$).

**e**, CR probability (median±MAD), and **f**, number of sessions to acquire the task (mean±SEM), during initial training (filled symbols) and retraining (open symbols) of Chr2 mice before and after CN$_{iO}$ stimulation ($n=6$ mice, training: $8\pm1.06$ sessions, retraining: $5\pm0.86$ sessions, one-tailed paired t-test: $t=3.22$, $df=5$, $p=0.01$, $d=1.25$).

**g**, Same as (**e, f**), but for initial training and retraining of wildtype mice before and after extinction ($n=5$ mice, training: $6.5\pm1.6$ sessions, retraining: $3.6\pm0.68$ sessions, one-tailed paired t-test: $t=2.62$, $df=4$, $p=0.03$, $d=0.67$). Family-wise alpha values were Bonferroni-Holm corrected for multiple comparisons. In panels **b, d, f, h**, boxplot center: mean, box bounds: ±SEM, whiskers: distribution minimum and maximum. *$p<0.05$. 

- $n$: number of mice.
- $t$, $df$, $p$, and $d$: t-test statistics, degrees of freedom, and effect size, respectively.
Extended Data Fig. 6 | Extinction during CNio stimulation is not caused by impaired processing of eye puff stimulus. a, Averaged reflex eyelid responses to the airpuff (unconditioned response, Ur; mean ± SEM). b, Ur peak amplitude (n = 7 mice; two-tailed paired t-test: t = 1.41, df = 6, p = 0.21), and c, Ur duration in sessions with (blue) and without (black) CNio stimulation for Chr2 mice (n = 7 mice; two-tailed paired t-test: t = 5.51, df = 6, p = 0.001, d = 1.25). Only trials without eyelid movements preceding the airpuff are included. d, UrS (mean ± SEM) from 9 mice, during training sessions in which the intensity of the eye puff was systematically changed to generate long UrS (black) or short UrS (red). UrS during CNio stimulation sessions in (a) are duplicated for comparison (blue). e, Cr probability (open circles) and Ur duration (filled circles; mean ± SEM) for the mice in (d), shown during a training session with long UrS (black) and the following 5 consecutive training sessions, in which the eye puff intensity was set to generate shorter UrS (red). In spite of shorter UrS (filled red circles; n = 9 mice, Friedman’s ANOVA: F = 12.71, df = 4, p = 0.013, W = 0.35), Cr probability remained unchanged (open red circles; n = 9 mice, Friedman’s ANOVA: F = 5.07, df = 4, p = 0.28). Family-wise alpha values were Bonferroni-Holm corrected for multiple comparisons. *p < 0.05.
Extended Data Fig. 7 | CN_2 stimulation before the airpuff does not impair CR performance. **a**, Schematic showing relative timing of stimulus presentations and laser pulses in the different phases of the experiment. **b**, CR probability (median ± MAD) in the last training session (‘TLast’) and sessions during CN_2 stimulation (gray shaded area) in Chr2 mice (n = 6 mice; CR probability increased during CN_2 stimulation, Friedman’s ANOVA: Fr = 14.86, df = 6, p = 0.02, W = 0.36). **c**, Averaged eyelid movement traces, and **d**, CR amplitude during sessions with CN_2 stimulation increased significantly (n = 6 mice; Friedman’s ANOVA: Fr = 24.50, df = 6, p = 4.22 × 10^{-5}, W = 0.68). Repeating the analysis in (c–d) but excluding trials without CRs revealed that the increase in CR amplitude during CN_2 stimulation shown in (d) is not simply driven by a higher CR probability – CRs were significantly larger (e–f; n = 6 mice; Friedman’s ANOVA: Fr = 20.0, df = 6, p = 0.003, W = 0.56). The time window used to calculate CR amplitude is indicated (c, e; yellow shaded area). **g**, Averaged eyelid position, and **h**, eyelid velocity traces for the last training session and subsequent sessions with CN_2 stimulation. The gray shaded area highlights the β-startle window, which follows the α-startle and precedes the CR. **i**, β-startle amplitude was increased (n = 6 mice; Friedman’s ANOVA: Fr = 29.71, df = 6, p = 4.45 × 10^{-5}, W = 0.82), and **j**, latency was shortened (n = 6 mice; Friedman’s ANOVA: Fr = 25.57, df = 6, p = 2.67 × 10^{-4}, W = 0.71) excluding trials without a β-startle. Family-wise alpha was Bonferroni-Holm adjusted for multiple comparisons. In panels d, f, i, j, boxplot center: mean, box bounds: ± SEM, whiskers: distribution minimum and maximum). **p < 0.01, ***p < 0.001.
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Software and code

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Data collection  Eyelid movements were monitored and trial parameters were recorded using Neuroblinks (http://github.com/blinklab), custom MATLAB (Mathworks version 2018b) software developed in this laboratory for conducting eyeblink conditioning experiments. Single-unit recording data were collected using a multichannel acquisition system (Tucker Davis Technologies, Sys3, RPvdsEX). Photomicrographs were collected using NIS-Elements Confocal edition and Zen blue edition. When photomicrographs were exported for publication, they were opened in NIS-Elements Viewer for Windows (version 4) or Zen (version blue edition).

Data analysis  Behavior data and single-unit recordings were analyzed with custom MATLAB (version 2018b) and R (version 3.6.0) scripts. R scripts made use of the following packages:
  nparLD, coin, effsize, nlme, lmmeans, tidyverse, ggpubr, rstatix, car, multcompView, & sjstats.
  The code used to analyze the data in this study are available at www.github.com/blinklab/KimOhmaeMedina.

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**Sample size**

No statistical methods were used to predetermine sample sizes. The number of mice used is comparable to numbers used for similar studies (e.g., Ohmae & Medina, 2015 Nature Neuroscience; Chang et al., 2015 Nature Neuroscience) and is justified by the size of the effects shown in the paper (Kendall’s W=0.73 and 0.81 out of a possible 1.0 for the main effects reported in Fig. 2).

**Data exclusions**

Mice that failed to acquire the eyeblink conditioning task to criterion levels (n = 2) were removed from the study, as this learning deficit would have, by definition, precluded these animals from exhibiting extinction learning.

**Replication**

All attempts at replication were successful. Below is a list of experiments and the number of times the experiment was conducted:

- Effects of photostimulation on PC responses to airpuffs: 2 times
- Effects of photostimulation during the airpuff on ChR2 mouse conditioned responding: 2 times
- Effects of photostimulation during the airpuff on EYFP mouse conditioned responding: 1 time
- Effects of removing the US on WT mouse conditioned responding: 2 times
- Effects of photostimulation after the airpuff on ChR2 mouse conditioned responding: 2 times
- Effects of photostimulation during the conditioned response on ChR2 mouse conditioned responding: 2 times
- Histological analysis of virus expression in ChR2 mice: 3 times
- Histological analysis of virus expression in EYFP mice: 1 time

**Randomization**

Mice were randomly allocated into different groups.

**Blinding**

Data collection and analysis were not performed blind to the conditions of the experiments. Our experimental design provided a within-animal control, and because comparisons were not required between different groups of mice, it was not necessary to be blind as to the group allocation of any individual mouse. In addition, analyses were based on automated scripts applied across experimental conditions and thus were not subject to any experimenter bias.

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- n/a
- ☒ ChIP-seq
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**Laboratory animals**

All experiments were performed during the dark cycle using adult (>p60) male mice (wild-type C57b16/J, n = 23), randomly selected from breeding litters. All mice were housed in a vivarium with inverted light/dark cycles in cages of up to 4 mice before surgery, and were singly housed after surgery. Ambient temperature was maintained between 68-72 degrees F, and ambient humidity was maintained between 30-70% RH.

**Wild animals**

This study did not involve wild animals.

**Field-collected samples**

This study did not involve field-collected samples.
The Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine approved all experimental procedures, which were in accordance with National Institutes of Health guidelines.

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