infections of *P. falciparum*. Thus, signaling via erythrocyte β2-AR and Gαs activation appears to be conserved across parasite species.

Although we found some degree of precoupling of β2-AR to Gαs in erythrocytes (evidenced by efficacy of neutral antagonists in vitro), there appeared to be stimulation of receptors during infection in vivo. How erythrocyte Gαs-coupled receptors are stimulated during malarial infection in vivo remains to be understood. One possibility is that catecholamine levels are augmented during infection, resulting in stimulation of receptors such as β2-AR. An increase of catecholamines may come from an elevation of the sympathetic response or the production of catecholamine-like molecules by the malaria parasite. This may explain why antagonists like propranolol are effective at reducing parasitemias in vivo.

The use of erythrocyte G-protein raft-associated signaling mechanisms in malarial entry and/or establishment of the vacuole may provide a reason why (i) erythrocyte rafts are required and (ii) their resident proteins are internalized in infection (*8*, *9*, *20*). Raft-dependent G-protein signaling has been demonstrated in cells (*21*). Although the physiological functions of G protein receptors and their associated signaling mechanisms in erythrocytes are not well understood, an emerging idea is that they may contribute to interactions with endothelial cells (*22*). Because both Gαs and β2-AR were internalized and associated with the vacuolar parasite, their activation in malarial infection may regulate a step of vacuole formation that is conserved across parasite species. This may explain why the same antagonists inhibit infection by human malarias like *P. falciparum* and rodent malaria parasites like *P. berghei*. Signaling via G proteins rapidly reorganizes the cellular cytoskeleton in nucleated mammalian cells (*23*, *24*). In *P. falciparum*–infected erythrocytes, nearly all skeletal components and attached integral proteins associated with the host plasma membrane are excluded from the vacuole. Further parasite entry culminating in intravacuolar residence occurs within minutes. Thus, signaling via GPCR may underlie the rapid and dynamic reorganization of submembranous cytoskeleton required for infection of the nonendocytic, mature erythrocyte by this major human pathogen.

References and Notes

1. L. H. Miller, D. I. Baruch, K. Marsh, O. K. Doumbo, *Nature* **415**, 673 (2002).
2. J. A. Dvorak, L. H. Miller, W. C. Whitehouse, T. Shiroshi, *Science* **187**, 748 (1975).
3. H. E. Hamm, *J. Biol. Chem.* **273**, 669 (1998).
4. S. R. Neves, P. T. Ram, R. Iyengar, *Science* **296**, 1636 (2002).
5. K. L. Pierce, R. T. Premont, R. J. Lefkowitz, *Nature Rev. Mol. Cell Biol.* **3**, 639 (2002).
6. S. L. Schrier, *Clin. Haematol.* **14**, 1 (1985).
7. J. A. Chasis, M. Prenant, A. Leung, N. Mohandas, *Blood* **74**, 1112 (1989).
8. S. A. Lauer *et al.*, *EMBO J.* **19**, 3556 (2000).
9. B. U. Samuel *et al.*, *J. Biol. Chem.* **276**, 29319 (2001).
10. A. Gilchrist *et al.*, *J. Biol. Chem.* **273**, 14912 (1998).
11. Single-letter abbreviations for the amino acid residues are as follows: E, Glu; H, His; L, Leu; M, Met; Q, Gln; R, Arg; and Y, Tyr.
12. A. Gilchrist, M. Bunemann, A. Li, M. M. Hosey, H. E. Hamm, *J. Biol. Chem.* **274**, 6610 (1999).
13. M. J. Gardner *et al.*, *Nature* **419**, 498 (2002).
14. M. Dyer, K. Day, *Mol. Biochem. Parasitol.* **110**, 437 (2000).
15. S. A. Desai, R. L. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2045 (1997).
16. T. Kenakin, *Annu. Rev. Pharmacol. Toxicol.* **42**, 349 (2002).
17. R. A. Bond *et al.*, *Nature* **374**, 272 (1995).
18. C. Maack *et al.*, *Br. J. Pharmacol.* **132**, 1817 (2001).
19. W. Peters, B. L. Robinson, *Ann. Trop. Med. Parasitol.* **78**, 561 (1984).
20. E. Nagao, K. B. Seydel, J. A. Dvorak, *Exp. Parasitol.* **102**, 57 (2002).
21. J. M. Green *et al.*, *J. Cell. Biol.* **146**, 673 (1999).
22. J. E. Brittain, K. J. Mlinar, C. S. Anderson, E. P. Orringer, L. V. Parise, *J. Clin. Investig.* **107**, 1555 (2001).
23. S. Etienne-Manneville, A. Hall, *Nature* **240**, 629 (2002).
24. J. F. Vanhauwe *et al.*, *J. Biol. Chem.* **277**, 34143 (2002).
25. Materials and methods are available as supporting material on *Science* Online.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5640/1734/DC1
Materials and Methods
Figs. S1 to S4
Table S1

17 July 2003; accepted 11 August 2003

Cerebellar LTD and Learning-Dependent Timing of Conditioned Eyelid Responses

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Mammals can be trained to make a conditioned movement at a precise time, which is correlated to the interval between the conditioned stimulus and unconditioned stimulus during the learning. This learning-dependent timing has been shown to depend on an intact cerebellar cortex, but which cellular process is responsible for this form of learning remains to be demonstrated. Here, we show that protein kinase C–dependent long-term depression in Purkinje cells is necessary for learning-dependent timing of Pavlovian-conditioned eyeblink responses.

Precise timing of movements is crucial for survival, and the central nervous system continuously tries to optimize this timing. Timing of movements can be learned, for example, by conditioning an eyelid response to a conditioned stimulus (CS), such as a tone, which continues until an unconditioned stimulus (US), such as an electrical shock or a corneal air puff, ceases. In this paradigm, the timing of the eyelid response is ultimately determined by the interstimulus interval (ISI) between the onset of the CS and the onset of the US. The exact timing of conditioned responses depends on plasticity in the cerebellar cortex (*1*–*3*). Yet, it is not clear which cellular processes are responsible for the timing properties of conditioned responses. Several mutant mice have been bred in which induction of long-term depression (LTD) (*4*) at the parallel fiber–Purkinje cell synapse is impaired, but so far it has not been possible to investigate whether this form of LTD contributes to learning-dependent timing (*5*–*7*). The deficit in these mutants either was not cell-specific or was contaminated by aberrations in motor performance, and/or the temporospatial resolution of the eyelid recording method was insufficient to detect timing differences in mice. Here, we used transgenic mice in which parallel fiber LTD is impaired in Purkinje cells in vitro and in vivo by the inhibition of protein kinase C (PKC) and in which no motor performance or excitability deficits have been detected (L7-PKCi mutants) (*8*–*10*). We subjected wild-type mice and the transgenic mice to a novel method of eyelid recording, the magnetic distance measurement technique (*11*). This method allows us to determine accurately the position of the eyelid of a mouse over time by generating a local magnetic field that moves with the eyelid and that is picked up by an aligned field–sensitive chip while the animal is freely moving.

Adult L7-PKCi mutants (C57/Bl6 background; $n = 24$) and wild-type littermates ($n = 24$) were anesthetized with an oxygenated mixture of nitrous oxide and halothane, and a premade connector (SamTec;

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www.samtec.com) was placed with a pedestal of dental cement on the skull. A magnet embedded in silicon was implanted in a pocket dissected in the eyelid and a giant magnetoresistive sensor chip (NVE; www.nve.com) was placed over the upper eyelid such that the distance between the magnet and sensor was 2 mm when the eyelid was closed and the axis of sensitivity was aligned with the north-south axis of the magnet when the eyelid was halfway closed. Two insulated copper wires with a diameter of 30 μm were placed underneath the skin that was close to the lateral corner of the eyelids to provide the US. The US electrodes were connected to computer-controlled stimulus-isolation units (Dagan S910; www.dagan.com), which created biphasic constant-current electrical shocks (30 ms, 166-Hz pulses). The strength of the shocks was controlled by an unconditioned response–based feedback mechanism (with a maximum shock strength of 1 mA) to prevent fear-conditioned responses (*11*).

The eyelid responses of the wild-type mice and L7-PKCi mutants were conditioned to a tone as the CS (10 kHz, gradually increased over 40 ms to 78 dB) during daily training sessions of 10 blocks of 10 trials. The blocks consisted of 1 US-alone trial, 8 paired trials, and 1 CS-alone trial, and the trials were separated by a random intertrial interval in the range of 20 to 40 s (*11*). In the first group of experiments, the onsets of the CS and US were separated by an ISI of 350 ms. After four paired training sessions (T-1 to T-4) the percentage of conditioned responses, their average peak amplitude, and their average peak velocity in wild types reached levels of 80%, 0.68 mm, and 32.6 mm/s, respectively, whereas those in LTD-deficient mice had values of only 30%, 0.44 mm, and 19.6 mm/s, respectively (Fig. 1). All of these values were significantly different [$P < 0.01$ for all three values; multivariate analysis of variance (MANOVA) for repeated measures; adjusted for multiple comparisons by Bonferroni correction]. The percentage of conditioned responses and the peak amplitude and peak velocity in the mutants were not significantly different from those of wild types during the first two training sessions. When the CS and US were randomly paired instead of paired with a fixed ISI, neither the wild types nor the mutants showed a significant increase of conditioned responses during the training. After three subsequent daily extinction sessions, all conditioned responses disappeared in the wild-type and LTD-deficient mice.

The timing properties of the conditioned responses showed the same pattern as the rate of conditioning. Although the latency to the peak of the amplitude of the conditioned eyelid response in wild types was still indistinguishable from that of the mutant after the second training session T-2, it was significantly longer than that of the mutant at T-4 ($P < 0.05$; MANOVA) (Fig. 2A). The same relation held true for the latency to the peak of the velocity of the conditioned eyelid response ($P < 0.01$; MANOVA). When the training sessions were continued for another 2 days, the timing of the conditioned responses in the mutant did not improve (the intervals between the peak amplitude and the US on day 5 and day 6 remained as high as 243 and 259 ms, respectively). Moreover, the timing of the remaining conditioned responses in wild types during extinction sessions gradually returned to baseline levels, whereas those in the mutants remained fixed. Thus the timing of the eyelid response of wild types can be optimized effectively by training such that the peak is at the moment of the US onset, whereas that of LTD-deficient mutants is fixed during both conditioning and extinction. The latencies to the onset of the eyelid responses were not influenced by the training.

If the timing is LTD-dependent, then the latencies to peak amplitude and peak velocity in the L7-PKCi mutant should not be influenced by the length of the ISI. Indeed, increasing the ISI from 350 to 500 ms did not significantly elevate the mutants' latency to peak amplitude or to peak velocity ($P > 0.93$ for both values; MANOVA) at T-4 (Fig. 2B). In contrast, the same increase of the ISI in wild types robustly increased their average latency to peak amplitude by 167 ms and increased their latency to peak velocity by

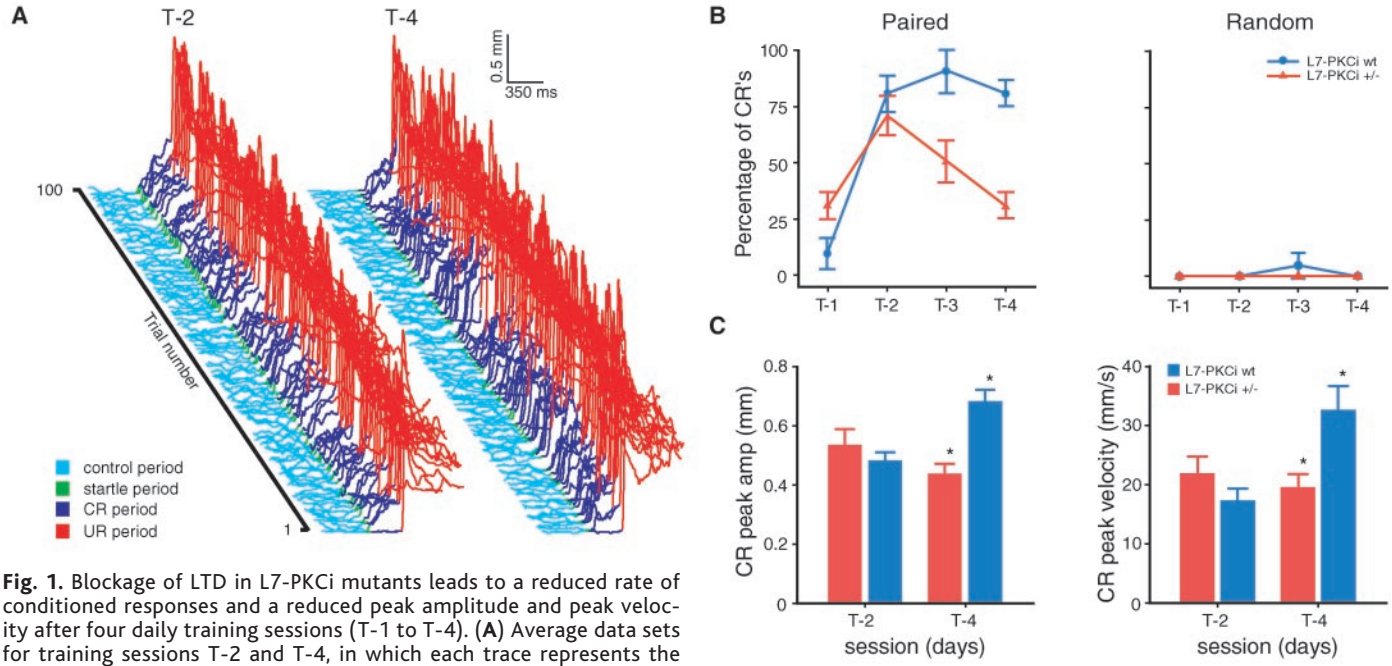

Fig. 1. Blockage of LTD in L7-PKCi mutants leads to a reduced rate of conditioned responses and a reduced peak amplitude and peak velocity after four daily training sessions (T-1 to T-4). (**A**) Average data sets for training sessions T-2 and T-4, in which each trace represents the response of wild-type (wt) animals for a particular trial. The conditioned response (CR) periods of T-2 and T-4 differ, whereas the control period, startle period, and unconditioned response (UR) period do not. (**B**) The mean percentages (±SEM) of significant CRs over four days of either paired (left) or unpaired (right) training for L7-PKCi transgenic mice and their wild-type littermates ($n = 10$ and $n = 5$ in both groups for paired and unpaired training, respectively). (**C**) As compared with L7-PKCi mutants, wild-type animals showed a significantly increased peak amplitude ($P < 0.01$; * in left panel) and a significantly increased peak velocity ($P < 0.01$; * in right panel) at T-4 but not at T-2.

141 ms ($P < 0.02$ and $P < 0.05$, respectively; MANOVA).

These data show that well-timed eyelid movements can be learned in wild-type mice but not in mice in which cerebellar LTD is blocked by the inhibition of PKC. Yet, to draw the conclusion that cerebellar LTD contributes to learning-dependent timing, it has to be shown that part of the learning that occurs in the L7-PKCi mutant does indeed have a cerebellar origin and that the well-timed components of the eyelid movements in wild types cannot occur without an intact cerebellum. We therefore investigated the conditioned responses in mutants and wild types in which the anterior interposed cerebellar nuclei were lesioned bilaterally with the use of pressure injections of 200 mmol of *N*-methyl-D-aspartate after they were trained. Although the number of responses was substantially reduced, both the L7-PKCi mutants and the wild types displayed remnant conditioned responses after the lesions (Fig. 3). However, the peak amplitudes were significantly reduced in both groups ($P < 0.01$ and $P < 0.05$ for wild types and mutants, respectively). Moreover, the mean latencies to peak amplitude and peak velocity were significantly reduced in the wild types ($P < 0.005$).

Our study provides evidence for a solution to a long-standing issue in the field of classical eyelid conditioning, i.e., whether LTD contributes to the temporal shaping of conditioned responses (*12*–*14*). Although not all components of eyelid conditioning in mice are under cerebellar control, the data demonstrate that mice without parallel fiber LTD show cerebellum-dependent conditioned responses that lack any form of temporal adaptive capacity. One cannot exclude the possibility that climbing fiber LTD, which also depends on PKC activity in vitro (*15*), is also affected in the L7-PKCi mutants. However, parallel fiber LTD but not climbing fiber LTD can be visualized with optical imaging in vivo, and the L7-PKCi mutant shows no induction of parallel fiber LTD in vivo (*10*). This evidence indicates that learning-dependent timing is probably mediated by PKC-dependent parallel fiber LTD.

During the learning of well-timed movements, the efficacy of a specific set of parallel fiber–Purkinje cell synapses may be diminished by LTD induction so that, ultimately, different ISIs trigger different time rings of different sets of granule cells and Purkinje cells (*14*, *16*), which in turn may lead to the induction of long-term potentiation or to an increase in the intrinsic excitability of the cerebellar nuclei target neurons (*17*).

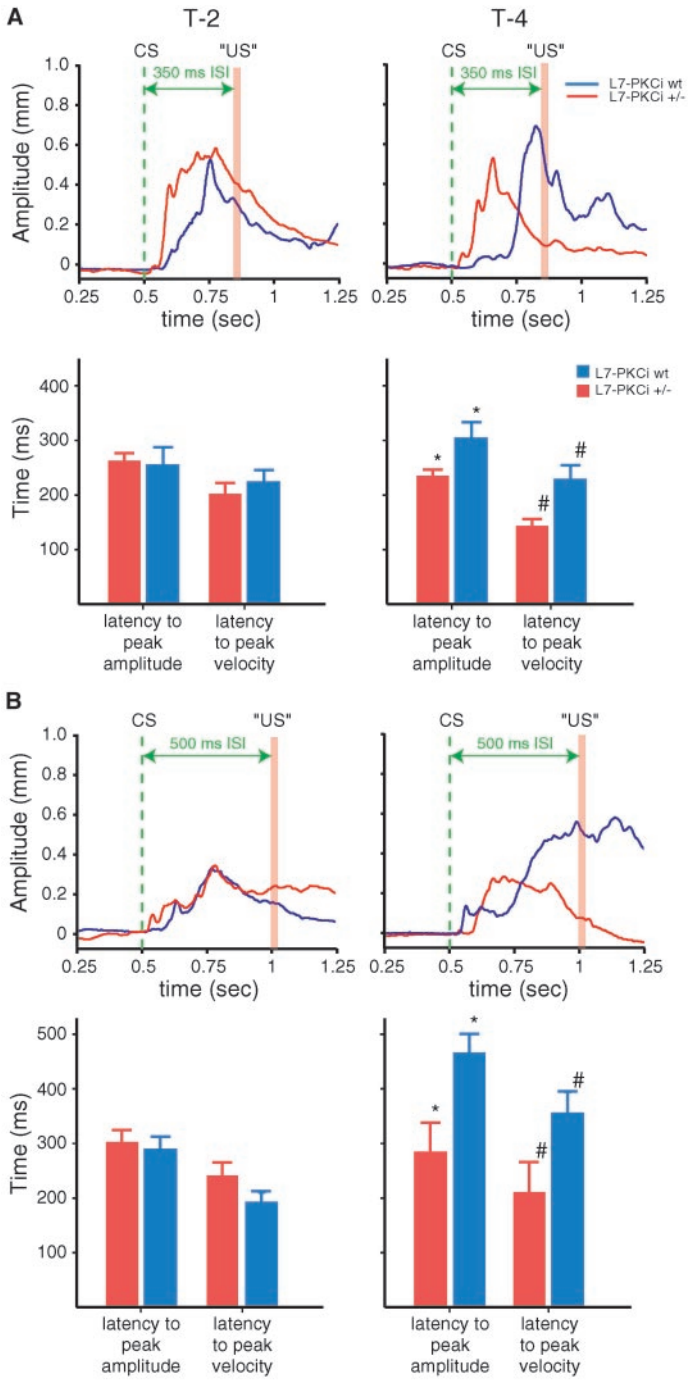

Fig. 2. Blockage of LTD in L7-PKCi mutants prevents learning-dependent timing. (**A**) Examples of average data sets for training sessions T-2 and T-4, showing the average CS-alone responses of a wild-type animal (blue) and a L7-PKCi mutant (red) (top). At T-4 but not at T-2, the wild-type animal shows well-timed responses around the moment when the US is supposed to take place. In contrast, the peak of the response of the LTD-deficient L7-PKCi mutant remains fixed in time over the training sessions. The average latencies to peak amplitude and peak velocity do not differ between wild types and mutants at T-2, but both values differ significantly at T-4 ($P < 0.05$ and 0.01, indicated by * and #, respectively; bars represent mean and SEM; $n = 10$ for both groups) (bottom). (**B**) When the CS-US interval is extended to 500 ms, the wild types (blue) but not the mutants (red) adapt the timing of their conditioned eyelid responses accordingly (top). Similar to the responses with an ISI of 350 ms, wild types and transgenics show responses with comparable timing characteristics at T-2 (left), whereas the latency to peak amplitude and latency to peak velocity of their responses are significantly different at T-4 ($P < 0.02$ and $P < 0.05$, indicated by * and #, respectively) (right). Bars represent mean and SEM ($n = 3$ for both groups).

A similar mechanism, in which timing properties are related to input specificity of Purkinje cells, has been proposed for adaptation of the vestibuloocular reflex (VOR) (*8*). Indeed, blockage of LTD impairs the level of VOR adaptation after a few hours of visuovestibular training in a frequency-specific fashion, whereas the adaptation becomes non–frequency-specific when mechanisms other than LTD can come into play (*8*, *18*). Thus, it may be a general concept for cerebellar motor learning that LTD does not only control the amplitude of trained movements but also their timing by means of an input-specific mechanism.

References and Notes

1. S. P. Perrett, B. P. Ruiz, M. D. Mauk, *J. Neurosci.* **13**, 1708 (1993).
2. K. S. Garcia, M. D. Mauk, *Neuropharmacology* **37**, 471 (1998).
3. J. F. Medina, W. L. Nores, M. D. Mauk, *Nature* **416**, 330 (2002).
4. M. Ito, *Annu. Rev. Neurosci.* **12**, 85 (1989).
5. A. Aiba *et al.*, *Cell* **79**, 377 (1994).

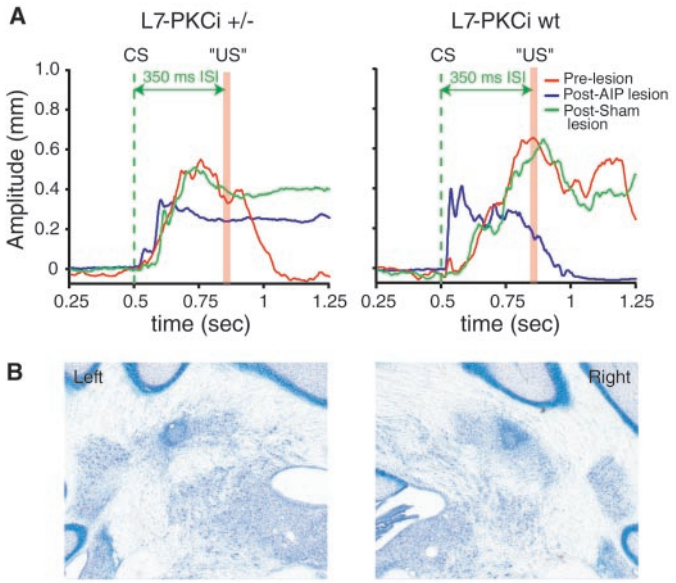

Fig. 3. Lesions of anterior interposed cerebellar nuclei (AIP) in trained animals show that conditioned responses in both L7-PKCi mutants and wild-type mice are composed of both cerebellar and extracerebellar components and that the cerebellar component is necessary for the timing of the eyelid response. (**A**) Average data sets of CS-alone responses at T-4 before the lesions (red) and after the lesions (blue) or a sham operation (green) in mutants and wild types ($n = 3$ in all cases). Conditioned responses still occur after lesions of the cerebellar nuclei in both wild types and mutants, but the amplitudes of these responses are in both cases substantially higher with intact cerebellar nuclei. (**B**) Example of lesions placed in the left and right anterior interposed cerebellar nucleus of a wild-type mouse; lesions can be recognized by the dark blue circumscribed areas in the center.

6. M. Miyata *et al.*, *Eur. J. Neurosci.* **13**, 1945 (2001).
7. K. Shibuki *et al.*, *Neuron* **16**, 587 (1996).
8. C. I. De Zeeuw *et al.*, *Neuron* **20**, 495 (1998).
9. J. Goossens *et al.*, *J. Neurosci.* **21**, 5813 (2001).
10. W. Gao *et al.*, *J. Neurosci.* **23**, 1859 (2003).
11. S. K. E. Koekkoek, W. L. Den Ouden, G. Perry, S. M. Highstein, C. I. De Zeeuw, *J. Neurophysiol.* **88**, 2124 (2002).
12. C. Yeo, G. Hesslow, *Trends Cogn. Sci.* **2**, 322 (1998).
13. K. S. Garcia, P. M. Steele, M. D. Mauk, *J. Neurosci.* **19**, 10940 (1999).
14. D. Buonomano, M. D. Mauk, *Neural Comput.* **6**, 38 (1994).
15. C. Hansel, D. Linden, *Neuron* **26**, 473 (2000).
16. W. M. Kistler, C. I. De Zeeuw, *Cerebellum* **2**, 44 (2003).
17. C. D. Aizenman, P. B. Manis, D. J. Linden, *Neuron* **21**, 827 (1998).
18. A. M. van Alphen, C. I. De Zeeuw, *Eur. J. Neurosci.* **16**, 486 (2002).

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