1:10,000 dilution of peroxidase-conjugated mouse antibody to rabbit IgG (Jackson ImmunoResearch), washed, and developed with enhanced chemiluminescence (ECL) (Amersham). The exposure times needed for ECL detection ranged from 10 s to 5 min, a period of time too brief to detect signals from bound ^{125}I.
26. CD19-containing immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose filters. We used lysates of *Escherichia coli* containing recombinant p85 fused to the epitope tag KT3 at a protein concentration of 1 μg/ml to probe blocked nitrocellulose filters [A. Klippel, J. Escobedo, W. Fantl, L. Williams, *Mol. Cell. Biol.* **12**, 1451 (1992)]. The filters were sequentially incubated with a 1:1250 dilution of mouse antibodies to KT3 and peroxidase-conjugated rabbit antibodies to mouse IgG (Jackson ImmunoResearch) and then developed with the ECL reagent.
27. We thank B. Drucker for the 4G10 antibody, A. Klippel and L. Williams for the p85-KT3 recombinant protein and the antibody to KT3, A. Choi for assistance with the Betascope, and L. Cantley for the use of his high-performance liquid chromatography (HPLC) facility. Supported by grants AI22833 and AI28191 from the National Institute of Allergy and Infectious Diseases of NIH (to D.T.F.), by the Medical Scientist Training Program (number 5T32GM07309 to D.A.T.), and by the Arthritis Foundation (to R.H.C.).

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Localization of a Memory Trace in the Mammalian Brain

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The localization of sites of memory formation within the brain has proven to be a formidable task even for simple forms of learning and memory. In order to localize a particular site of memory formation within the brain, the rabbit eyeblink response was classically conditioned while regions of the cerebellum or red nucleus were temporarily inactivated by microinfusions of the γ-aminobutyric acid agonist muscimol. Cerebellar inactivation completely blocked learning but had no effect on subsequent learning after inactivation, whereas red nucleus inactivation did not prevent learning but did block the expression of conditioned responses. The site of memory formation for this learned response thus appears to be localized within the cerebellum.

A primary goal of neuroscience and psychology is to acquire an understanding of the mechanisms that underlie long-term memory formation, storage, and retrieval in the mammalian, particularly the human, brain (*1*). An essential prerequisite is identification of the sites within the brain where particular memories, either distributed or localized, are created and stored (the memory trace). To date, precise identification of loci for memory storage has remained elusive (*2*, *3*). We report here evidence for localization of a long-term memory trace in the mammalian brain, specifically within the cerebellum.

We used local microinfusions of the γ-aminobutyric acid (GABA) agonist muscimol to reversibly inactivate select regions of the brain during the training of rabbits in order to localize the memory trace. Muscimol is known to temporarily inhibit activity of neurons that express $GABA_A$ receptors by hyperpolarizing somata and dendrites by increasing a Cl^- conductance (*4*). Muscimol has been used for reversible inactivation of brain regions, including the cerebellum (*5*) and the red nucleus (*6*), but generally has not been used to localize memory traces.

The logic underlying the use of reversible lesions to localize sites of memory formation is as follows. Naïve animals are trained while a region of the brain is inactivated. If this inactivated region is part of the circuitry essential for a given form of learning and memory, then expression of any learned response will be prevented during the inactivation training. If learning occurs during inactivation, as evidenced by the immediate expression of learned responses in training after inactivation, then the site or sites of memory trace formation must be afferent (upstream) to the region of inactivation in the essential circuitry. If no learning occurs during inactivation training, then the region of inactivation must be the site of memory formation or a mandatory afferent projecting ultimately to the site of memory formation.

To utilize reversible inactivation, one must first identify the brain circuitry essential (necessary and sufficient) for a given form of learning and memory. This has largely been achieved for one form of basic associative memory in animal studies, aversive classical conditioning of discrete behavioral responses—for example, eyeblink and limb flection (*2*, *7*). In brief, the results of lesion, recording, and stimulation studies indicate that the conditioned stimulus (CS) pathway includes sensory relay nuclei, the pontine nuclei, and mossy fiber projections to the cerebellum; the unconditioned stimulus (US) pathway includes somatosensory relay nuclei, the inferior olive, and its climbing fiber projections to the cerebellum; and the conditioned response (CR) pathway includes the cerebellum, its projection from the interpositus nucleus to the red nucleus, and red nucleus projections to premotor and motor nuclei (*8*, *9*). Unilateral cerebellar lesions in humans completely prevent learning of the eyeblink CR ipsilateral (but not contralateral) to the lesion (*10*).

We used microinfusions of muscimol into the ipsilateral lateral cerebellum or the contralateral red nucleus to temporarily inactivate these structures during acquisition of the classically conditioned eyeblink response of the rabbit. Behavioral training comprised 11 daily sessions in which a tone CS (350 ms, 1 KHz, 85 dB) was paired with a coterminating corneal airpuff US (100 ms, 2.1 N/cm^2) (*11*). One hour before each of the first six sessions, animals received an infusion of (i) muscimol into the ipsilateral lateral cerebellum ($n = 6$), (ii) saline (vehicle) into the ipsilateral lateral cerebellum ($n = 6$), or (iii) muscimol into the contralateral red nucleus ($n = 6$) (*12*). No infusions were administered on days 7 to 10 of training. All animals received 3 days of rest between sessions 6 and 7 to ensure no lingering effects of infusion. On day 11, all animals were infused with muscimol to test retention.

Control animals that were treated with saline had fully learned the CR by the third day of infusion training. Neither the animals infused with muscimol into the cerebellum nor those infused in the red nucleus showed any appreciable number of CRs during the 6 days of infusion training (Fig. 1A). At the beginning of training after the infusion sessions (day 7), the animals infused with muscimol in the cerebellum showed no signs of having learned and subsequently learned at exactly the same rate as the saline-treated control animals had learned on the first 4 days of training (Fig. 1B). In marked contrast, the animals that had had muscimol infused into the red nucleus showed asymptotic learning from the beginning of training after the infusion (Fig. 1B). Infusions of muscimol on day 11 into the cerebellum (including the control animals that had previously been infused with saline) or the red nucleus reversibly abolished the CRs of all animals. In all cases, infusions of muscimol into the cerebellum or the red nucleus during acquisition had no effect on UR amplitudes relative to controls (*13*) (Fig. 1C).

Locations of the cannula tips for all animals are shown in Fig. 2A. All cerebellar cannulae were aimed at the anterior interpositus nucleus (AIN). After all training, four animals (three infused in the cerebellum, one in the red nucleus) received infusions of [^{3}H]muscimol equiva-

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lent to the doses infused during training. An autoradiograph of the largest distribution of [^{3}H]muscimol diffusion in the cerebellum is shown in Fig. 2B. This pattern of diffusion, extending throughout the AIN and upwards into the cerebellar cortex, was typical in all cases. These infusions inactivated both the AIN as well as much of hemispheric lobule VI and neighboring regions of the cortex. In no instance did the cerebellar infusions result in labeling outside the cerebellum. Red nucleus infusion resulted in labeling throughout the magnocellular division with minimal labeling outside the nucleus.

In summary, animals trained with muscimol infused into the cerebellum did not express CRs, did not learn at all, and subsequently learned as though naïve to the training situation. Further, they showed no learning impairment during training after the infusions, which indicates that repeated muscimol infusions did not cause significant damage to the tissue. On the other hand, the animals trained with muscimol infused into the red nucleus did not express CRs at all during infusion training but in fact fully learned the CR during this training. This result demonstrates that normal learning can occur with the complete absence of performance of the learned response during training. In no instance did the effective cerebellar or red nucleus infusion have any effect on reflex responses to the US, either on paired trials or on trials with the US alone. After the training that followed infusion, all animals, including all saline-treated controls, were given retention tests with muscimol infusions (day 11). In all cases, the drug produced complete and reversible abolition of the CR, which demonstrates that the cerebellum is necessary for both acquisition as well as long-term retention of the learned response. These results support conclusions from reversible inactivation cooling studies of the cerebellum and red nucleus (*14*) and the results of lidocaine studies (*15*) and contradict results reported by Welsh and Harvey, who used lidocaine for reversible inactivation of the cerebellum during transfer training from a light to a tone CS (*16*).

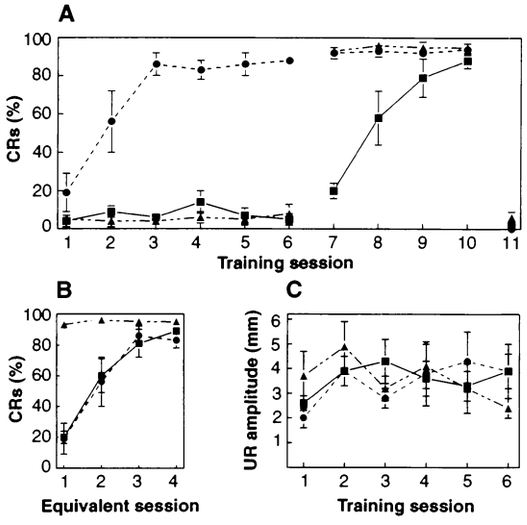

Fig. 1. Effect of muscimol infusion on CRs and URs. (**A**) All animals received an infusion before training on sessions 1 to 6. The cerebellar group (■) (n = 6) received muscimol infusions into the ipsilateral lateral cerebellum, the red nucleus group (▲) (n = 6) received muscimol in the contralateral red nucleus, and the saline group (●) (n = 6) received 1 μl of saline vehicle into the ipsilateral lateral cerebellum. No infusions were administered on days 7 to 10. All animals received muscimol infusions before session 11. Data are expressed as percent CRs averaged over all animals in each group for each training session. (**B**) Percent CRs for sessions 1 to 4 of the saline group and sessions 7 to 10 of the cerebellar and red nucleus groups. (**C**) UR amplitudes on airpuff-only test trials during the six sessions in which infusions were administered. There were no significant differences between groups on these days. All data points are means ± SEM. Symbols are the same for all charts.

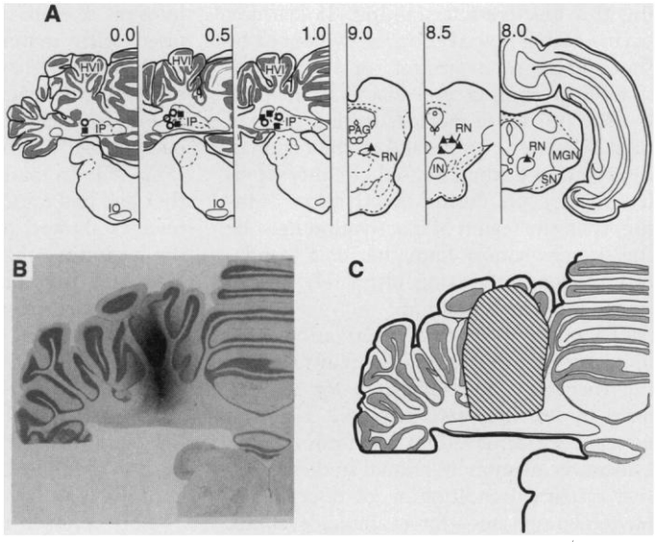

Fig. 2. Localization of inactivated regions. (**A**) Locations of all cannulae tips. Filled squares, cerebellar muscimol cannulae; open circles, saline controls; filled triangles, red nucleus cannulae. The numbers above the first three sections are the distances (in millimeters) rostral to lambda; above the last three are distances caudal from the bregma skull sutures. HVI, hemispheric lobule VI; IN, interpeduncular nucleus; IP, interpositus nucleus; IO, inferior olive; MGN, medial geniculate nucleus; PAG, periaqueductal grey; RN, red nucleus; SN, substantia nigra. (**B**) Digitized image of an autoradiograph showing the greatest extent of [^{3}H]muscimol diffusion in the lateral cerebellum. Labeling encompasses dorsal aspects of the anterior interpositus and the overlying cortex, including lobule HVI. In no instance was any labeling found outside the cerebellum. The autoradiograph is shown superimposed upon the Nissl-stained section from which it was exposed. (**C**) Outline drawing of micrograph in (B). Hatched area delineates maximal extent of [^{3}H]muscimol diffusion.

These results permit the following conclusions. (i) The memory trace cannot be formed and stored in the red nucleus; inactivation of the red nucleus during training, which completely prevented expression of the CR, did not prevent or interfere with learning at all. (ii) The memory trace cannot be formed solely before the cerebellar area of inactivation in the essential circuit or in the reflex pathways; otherwise, learning would have occurred during cerebellar infusion training and no learning at all occurred.

The mandatory CR pathway has been identified: interpositus nucleus via superior cerebellar peduncle to magnocellular red nucleus to motor nuclei. In brief, neurons in the critical region of the interpositus, where unilateral lesions completely and permanently abolish acquisition, retention, and relearning of the ipsilateral CR with no effect on the UR, develop a learning-induced increase in discharge frequency that forms an amplitude–time course model of the behavioral CR that precedes and predicts the occurrence and form of the CR both within trials and over the trials of training (*8*, *17*). Microstimulation of the critical interpositus region elicits eyeblinks in untrained animals and lesion of the superior cerebellar peduncle (projection from the interpositus to the red nucleus) abolishes this stimulation-elicited response; the CR circuit is hard-wired from interpositus to behavior (*17*, *18*). Such lesions also abolish the CR learned to peripheral CSs (*19*). Unit recordings from the critical region of the red nucleus (in the magnocellular division) develop the same learning-induced model of the CR as do neurons in the

interpositus, and very small lesions in this region of the red nucleus also abolish the CR with no effect on the UR (*9, 18*). In trained animals, lidocaine or cold-probe inactivation of the interpositus abolishes both the behavioral CR and the learning-induced neuronal model in the red nucleus; inactivation of the red nucleus abolishes the behavioral CR but has no effect on the learning-induced neuronal model in the interpositus (*14, 20*). Our results demonstrate that the memory trace must be formed at or beyond the cerebellar site of inactivation but before the red nucleus. On the basis of these results and data cited (*15*), we conclude that the memory trace for eyeblink conditioning must be localized to the ipsilateral lateral cerebellum. Our findings strongly support the hypothesis that the memory traces for learned movements are formed and stored in the cerebellum (*21*).

REFERENCES AND NOTES

1. J. H. Byrne, *Physiol. Rev.* **67**, 329 (1987); D. G. Lavond, J. J. Kim, R. F. Thompson, *Annu. Rev. Psychol.*, in press.
2. R. F. Thompson, *Science* **233**, 941 (1986).
3. R. D. Hawkins, G. A. Clark, E. R. Kandel, in *Handbook of Physiology*, F. Plum, Ed. (American Physiological Society, Bethesda, MD, 1987), vol. 5, pp. 25–83; L. R. Squire, *Memory and Brain* (Oxford Univ. Press, New York, 1987).
4. R. R. Matsumoto, *Brain Res. Rev.* **14**, 203 (1989).
5. J. Van-Neerven, O. Pompeiano, H. Collewijn, *Arch. Ital. Biol.* **127**, 243 (1989); J. G. Keating and W. T. Thach, *Soc. Neurosci. Abstr.* **17**, 1381 (1991); J. W. Mink and W. T. Thach, *J. Neurophysiol.* **65**, 330 (1991).
6. J. H. Martin and C. Ghez, *Behav. Brain Res.* **28**, 217 (1988).
7. N. H. Donegan, R. W. Lowry, R. F. Thompson, *Soc. Neurosci. Abstr.* **9**, 331 (1983); T. J. Voneida, *ibid.* **16**, 270 (1990).
8. J. E. Steinmetz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3531 (1987); J. L. Lewis, J. J. LoTurco, P. R. Solomon, *Behav. Neurosci.* **101**, 151 (1987); D. A. McCormick, J. E. Steinmetz, R. F. Thompson, *Brain Res.* **359**, 120 (1985); M. D. Mauk, J. E. Steinmetz, R. F. Thompson, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5349 (1986); G. A. Clark, D. A. McCormick, D. G. Lavond, R. F. Thompson, *Brain Res.* **291**, 125 (1984); C. H. Yeo, M. J. Hardiman, M. Glickstein, *Exp. Brain Res.* **60**, 87 (1985); J. E. Steinmetz, D. G. Lavond, D. Ivkovich, C. G. Logan, R. F. Thompson, *J. Neurosci.* **12**, 4403 (1992); J. F. Disterhoft, K. J. Quinn, C. Weiss, M. T. Shipley, *ibid.* **5**, 941 (1985).
9. M. E. Rosenfield, A. Dovydaitis, J. W. Moore, *Physiol. Behav.* **34**, 751 (1983); M. E. Rosenfield and J. W. Moore, *Behav. Brain Res.* **10**, 393 (1983); *ibid.* **17**, 77 (1985).
10. R. H. Lye, D. J. O'Boyle, R. T. Ramsden, W. Schady, *J. Physiol. (London)* **403**, 58P (1988).
11. Details of the surgical cannula implant procedures and behavioral recording apparatus have been published (*20*). Surgical anesthesia was ketamine (60 mg per kilogram of body weight), xylazine (8 mg/kg), and halothane (1 to 3% in oxygen). Each training session consisted of 100 trials: 80 paired trials with 10 tone-alone and 10 airpuff-alone test trials evenly distributed throughout the session. Intertrial intervals ranged between 20 to 40 s (mean, 30 s). Behavioral responses were recorded with a minitorque potentiometer attached to the animal's nictitating membrane by means of surgical suture. A CR was defined as a response greater than 0.5 mm after the CS onset but preceding the US onset.
12. Infusion doses were (i) cerebellar muscimol, 14 nmol in 1 μl of saline vehicle (pH adjusted to 7.4); (ii) saline controls, 1 μl of saline vehicle; or (iii) red nucleus muscimol, 1.3 nmol in 0.75 μl of saline vehicle. All infusion rates were 0.3 μl per minute. The cerebellar muscimol dose was chosen on the basis of previous work to reliably label both the interpositus-dentate nucleus and overlying cerebellar cortex; this dose had no observable behavioral effect on any animal. Likewise, the red nucleus dose was chosen to reliably label the nucleus with minimal spread to other regions. With this dose, some of the animals displayed a slight head tilt to the side contralateral to the infusion and a tendency to walk in a circular direction contralateral to infusion. This effect disappeared within 3 to 4 hours of infusion. All other observable behaviors (for example, grooming, feeding, exploration) were unaffected. Although the cerebellar dose used in this study was greater than the red nucleus dose, we have also trained five rabbits with much lower doses (0.7 nmol) in the cerebellum with identical results.
13. Analysis of variance of UR amplitudes revealed no significant differences between muscimol and saline infusion groups across the 6 days of training during infusion on either paired [$F(2,15) = 0.13$; not significant] or airpuff-alone trials [$F(2,15) = 0.01$; not significant].
14. R. E. Clark and D. G. Lavond, *Behav. Neurosci.* **107**, 264 (1993); R. E. Clark, A. A. Zhang, D. G. Lavond, *ibid.* **106**, 879 (1992).
15. A. F. Nordholm *et al.*, *ibid.*, in press. Continuous lidocaine infusion into the same cerebellar locus as muscimol completely prevented learning in naïve animals. Infusion into white matter ventral to the interpositus (output) prevented expression of CRs, but the naïve animals fully learned the CR, as evidenced in postinfusion training, which suggests that learning occurs when the output of the interpositus to its target sites is blocked.
16. J. P. Welsh and J. A. Harvey, *J. Physiol. (London)* **444**, 459 (1991). Their animals were already well trained to a light CS. They reported subsequent transfer of training to a tone CS during cerebellar lidocaine infusions; their saline controls also showed substantial transfer. In addition, cannulae placements may be critically important (*15*).
17. D. A. McCormick and R. F. Thompson, *J. Neurosci.* **4**, 2811 (1984).
18. P. F. Chapman, J. E. Steinmetz, R. F. Thompson, *Brain Res.* **442**, 97 (1988); D. A. McCormick and R. F. Thompson, *Science* **223**, 296 (1984).
19. D. A. McCormick, P. E. Guyer, R. F. Thompson, *Brain Res.* **245**, 347 (1982).
20. P. F. Chapman, J. E. Steinmetz, L. L. Sears, R. F. Thompson, *ibid.* **537**, 149 (1990).
21. J. S. Albus, *Math. Biosci.* **10**, 25 (1971); J. C. Eccles, *Brain Res.* **127**, 327 (1977); M. Ito, *The Cerebellum and Neural Control* (Raven, New York, 1984); D. Marr, *J. Physiol. (London)* **202**, 437 (1969); W. T. Thach, H. G. Goodkin, J. G. Keating, *Annu. Rev. Neurosci.* **15**, 403 (1992).

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Induction of $G\alpha_{i2}$-Specific Antisense RNA in Vivo Inhibits Neonatal Growth

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Guanosine triphosphate-binding regulatory proteins (G proteins) are key elements in transmembrane signaling and have been implicated as regulators of more complex biological processes such as differentiation and development. The G protein $G\alpha_{i2}$ is capable of mediating the inhibitory control of adenylylcyclase and regulates stem cell differentiation to primitive endoderm. Here an antisense RNA to $G\alpha_{i2}$ was expressed in a hybrid RNA construct whose expression was both tissue-specific and induced at birth. Transgenic mice in which the antisense construct was expressed displayed a lack of normal development in targeted organs that correlated with the absence of $G\alpha_{i2}$. The loss of $G\alpha_{i2}$ expression in adipose tissue of the transgenic mice was correlated with a rise in basal levels of adenosine 3′,5′-monophosphate (cAMP) and the loss of receptor-mediated inhibition of adenylylcyclase. These data expand our understanding of G protein function in vivo and demonstrate the necessity for $G\alpha_{i2}$ in the development of liver and fat.

G proteins propagate signals from cell surface receptors to a diverse group of effectors that includes adenylylcyclase, phospholipase C, and cation channels (*1–4*). Visual transduction, neuronal signaling, cell growth and differentiation, and metabolic pathways such as glycogenolysis and gluconeogenesis are mediated by way of G proteins. The G protein $G\alpha_{i2}$ has been implicated in the inhibition of adenylylcyclase and oncogenesis (*5–7*). In order to suppress $G\alpha_{i2}$ expression in vivo, we adopted the use of a construct to express $G\alpha_{i2}$-specific antisense RNA instead of gene disruption. To enhance the accumulation of the $G\alpha_{i2}$ antisense RNA, the target sequence was inserted in the 5′-untranslated region of the rat *phosphoenolpyruvate carboxykinase* (*PEPCK*) gene. The *PEPCK* gene was selected for three reasons. (i) The 2.8-kb hybrid *PEPCK*-$G\alpha_{i2}$ antisense RNA would be more stable than a comparatively short-lived an-

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