11. J. T. Gray, D. W. Celander, C. M. Price, T. R. Cech, *Cell* **67**, 807 (1991).
12. The *O. nova* α subunit by itself preferentially binds the 3′ terminus of a telomeric oligonucleotide, but also binds single-stranded telomeric DNA internally (*11*).
13. G. Fang, T. R. Cech, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6056 (1993).
14. M. P. Horvath, V. L. Schweiker, J. M. Bevilacqua, J. A. Ruggles, S. C. Schultz, *Cell* **95**, 963 (1998).
15. W. Wang, R. Skopp, M. Scofield, C. Price, *Nucleic Acids Res.* **20**, 6621 (1992).
16. J. D. Griffith *et al.*, *Cell* **97**, 503 (1999).
17. Heterozygous diploids (h^+/h^- *leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3-D1 ade6-M210/ade6-M216 pot1*$^+$*/pot1::kan*^{r}) were constructed by replacing the entire ORF of *pot1*$^+$ with the kanamycin resistance gene as described (*34*).
18. T. M. Nakamura, J. P. Cooper, T. R. Cech, *Science* **282**, 493 (1998).
19. P. Baumann, T. R. Cech, data not shown.
20. T. Naito, A. Matsuura, F. Ishikawa, *Nature Genet.* **20**, 203 (1998).
21. The *S. pombe pot1*$^+$ ORF plus an NH_2-teminal His_6 tag and COOH-terminal V5/His_6 tag was expressed in *E. coli* strain M15 (pRep4) using tryptone phosphate media. After induction (0.8 mM isopropyl-β-D-thiogalactopyranoside) for 6 hours at 24°C, cells were harvested and resuspended in 50 mM NaH_2PO_4 (pH 8.0), 0.1 M NaCl, 2 mM imidazole, 10% glycerol, 0.2% Tween-20, 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, to which lysozyme (0.5 mg/ml) was then added. After 30 min, the concentration of NaCl was increased to 0.6 M, genomic DNA was sheared by sonication, and cell debris was removed by centrifugation. The supernatant was incubated at 4°C for 90 min with Ni-NTA resin (Qiagen), which was then loaded onto a column and washed sequentially with P buffer [50 mM NaH_2PO_4 (pH 8.0), 0.6 M NaCl, 10% glycerol, 0.2% Tween-20, and 5 mM β-mercaptoethanol] containing increasing concentrations of imidazole. Pot1p eluted around 90 mM imidazole. Pot1p-containing fractions were dialyzed against T buffer [50 mM tris-HCl (pH 8.0), 10% glycerol, 0.5 mM EDTA, and 0.5 mM dithiothreitol] containing 0.2 M KCl, and Pot1p was further purified on a Q-sepharose column (Pharmacia) using a linear gradient of KCl (0.2 to 1 M). The 22-kD Pot1p fragment was found in the flowthrough, whereas Pot1p eluted around 0.5 M KCl. The protein was dialyzed against T buffer plus 0.2 M KCl and stored at −80°C.
22. Identical results were obtained when hemagglutinin-tagged Pot1p was expressed in *S. pombe* and cell-free extracts were used in bandshift assays (*19*).
23. G. Fang, J. T. Gray, T. R. Cech, *Genes Dev.* **7**, 870 (1993).
24. J. M. Petersen *et al.*, *Science* **269**, 1866 (1995).
25. See *Science* Online (www.sciencemag.org/cgi/content/full/292/5519/1171/DC1).
26. T. M. Nakamura *et al.*, *Science* **277**, 955 (1997).
27. M. Meyerson *et al.*, *Cell* **90**, 785 (1997).
28. A. Kilian *et al.*, *Hum. Mol. Genet.* **6**, 2011 (1997).
29. Human *POT1* was amplified by polymerase chain reaction from ovary cDNA and cloned into the pQE30 expression vector. The protein was purified over Ni-NTA resin under the same conditions as SpPot1p. The human protein eluted around 135 mM imidazole.
30. S. J. Froelich-Ammon, B. A. Dickinson, J. M. Bevilacqua, S. C. Schultz, T. R. Cech, *Genes Dev.* **12**, 1504 (1998).
31. A. G. Murzin, *EMBO J.* **12**, 861 (1993).
32. Yet another state may involve a complex with heterogeneous nuclear ribonucleoproteins, several of which have properties consistent with a role in telomere maintenance (*35–38*).
33. Pot1p might also bind the displaced single strand of a D-loop within a t-loop, although the protein does have a preference for binding terminal rather than internal repeats.
34. P. Baumann, T. R. Cech, *Mol. Biol. Cell* **11**, 3265 (2000).
35. F. Ishikawa, M. J. Matunis, G. Dreyfuss, T. R. Cech, *Mol. Cell. Biol.* **13**, 4301 (1993).
36. S. J. McKay, H. Cooke, *Nucleic Acids Res.* **20**, 6461 (1992).
37. H. LaBranche *et al.*, *Nature Genet.* **19**, 199 (1998).
38. A. Eversole, N. Maizels, *Mol. Cell. Biol.* **20**, 5425 (2000).
39. G. W. Fang, T. R. Cech, *Nucleic Acids Res.* **19**, 5515 (1991).
40. J. D. Prescott, M. L. DuBois, D. M. Prescott, *Chromosoma* **107**, 293 (1998).
41. J. B. Fan *et al.*, *Nucleic Acids Res.* **17**, 2801 (1989).

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Relapse to Cocaine-Seeking After Hippocampal Theta Burst Stimulation

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Treatment efforts for cocaine addiction are hampered by high relapse rates. To map brain areas underlying relapse, we used electrical brain stimulation and intracranial injection of pharmacological compounds after extinction of cocaine self-administration behavior in rats. Electrical stimulation of the hippocampus containing glutamatergic fibers, but not the medial forebrain bundle containing dopaminergic fibers, elicited cocaine-seeking behavior dependent on glutamate in the ventral tegmental area. This suggests a role for glutamatergic neurotransmission in relapse to cocaine abuse. The medial forebrain bundle electrodes supported intense electrical self-stimulation. These findings suggest a dissociation of neural systems subserving positive reinforcement (self-stimulation) and incentive motivation (relapse).

Cocaine addiction is a chronic brain disorder with psychosocial and neurobiological determinants (*1*). Treatment efforts are hampered by relapse (*2*). Imaging techniques have been applied to study the neural substrates of cocaine craving (*3–6*). These studies, although informative, address subjective craving, not actual relapse. They are correlational, not causal, and they take place in laboratory settings, not the actual context of the cocaine experience. Complementary approaches to mapping brain areas underlying relapse are therefore desirable.

Reinstatement of cocaine-seeking behav-

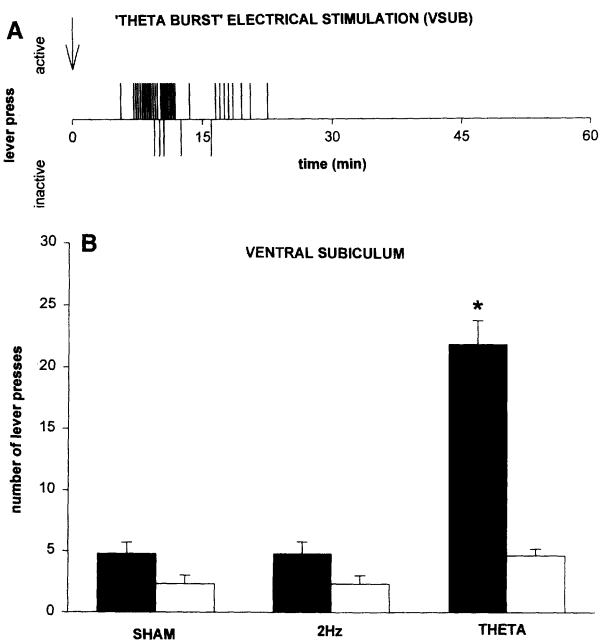

Fig. 1. (**A**) Effect of VSUB theta burst stimulation (arrow) on reinstatement in an individual rat. Upward bars: active lever presses; downward bars: inactive lever presses. For clarity, only the first hour of the 3-hour session is shown. (**B**) Effect of different patterns of VSUB electrical stimulation in a group of rats ($n = 9$). The black bars show "active" lever presses (mean ± SEM), the white bars "inactive" lever presses (mean ± SEM). During "sham" stimulation, no actual stimulation was delivered. 2 Hz: 2-Hz repetitive stimulation; THETA: stimulation in "theta burst" rhythm. Asterisk indicates significant difference compared with sham and 2-Hz groups ($^*P < 0.00001$). There were no significant differences in inactive lever presses among sham, 2-Hz, and theta burst treatment groups.

ior in the rat has high validity for relapse in the human addict (*7–9*). Reinstatement and relapse are operant responses. The rat and human share common triggers of relapse: cocaine (*10*, *11*), stress (*12*, *13*), and stimuli conditioned to cocaine (*6*, *14*, *15*). Thus, reinstatement in the rat is an objective measure of relapse with predictive value for the human.

The environmental context of the cocaine experience is a powerful determinant of cocaine-seeking behavior in the rat and human (*15*, *16*). The hippocampus underlies the learning of associations between the environmental context and unconditioned stimuli (*17–19*) (e.g., cocaine). Stimulation of the ventral subiculum (VSUB) of the hippocampal formation induces long-lasting dopamine (DA) release in the nucleus accumbens (NAC) (*20–23*) and enhances the firing of mesolimbic DA neurons that originate in the ventral tegmental area (VTA) (*23*, *24*). NAC DA has been implicated in reinstatement (*25–27*). Hence, VSUB stimulation may elicit reinstatement.

We catheterized the jugular vein of Long-Evans rats and implanted an electrode into the VSUB, cerebellum (CBL), or medial forebrain bundle (MFB) (*28*). The rats self-administered cocaine intravenously (i.v.) during daily 3-hour sessions in operant chambers equipped with one "active" and one "inactive" lever. Active lever presses resulted in cocaine delivery (1.0 mg/kg per infusion) and a light signal; inactive lever presses were counted but had no further consequence. After 1 week of stable cocaine self-administration, saline was substituted for cocaine. The lever pressing progressively diminished, a behavioral phenomenon called "extinction." The extinction period varied between 7 and 20 days. When the extinction criterion was met (three consecutive sessions with fewer than 10 lever presses), priming electrical stimulations were administered (*28*).

Neither sham nor 2-Hz VSUB stimulation elicited reinstatement (Fig. 1B). However, brief (8 s) theta burst stimulation mimicking rhythms recorded electroencephalographically in the hippocampus (*29*) elicited reinstatement (Fig. 1, A and B). All reinstated lever presses occurred after the end of electrical stimulation; no lever presses occurred during the stimulation (Fig. 1A).

VSUB stimulation enhances VTA DA neuron firing (*23*, *24*). NAC DA increase after VSUB stimulation depends on VTA glutamate (GLU), because the NAC DA increase is blocked by the nonselective ionotropic GLU receptor antagonist kynurenic acid (KYN) applied into the VTA (*23*). Therefore, reinstatement after VSUB stimulation may depend on VTA GLU neurotransmission. Microinjection of KYN (50 nmol/0.5 μl), but not 0.5 μl vehicle (VEH), into the VTA blocked reinstatement after VSUB burst stimulation (Fig. 2) (*30*). This suggests the involvement of the AMPA and/or *N*-methyl-D-aspartate (NMDA) ionotropic GLU receptor in reinstatement. The ionotropic GLU receptor agonist NMDA in the VTA enhances NAC DA (*31*, *32*). Microinjection of NMDA (83 pmol/0.5 μl), but not 0.5 μl VEH, into the VTA of the same subjects elicited reinstatement (Fig. 2).

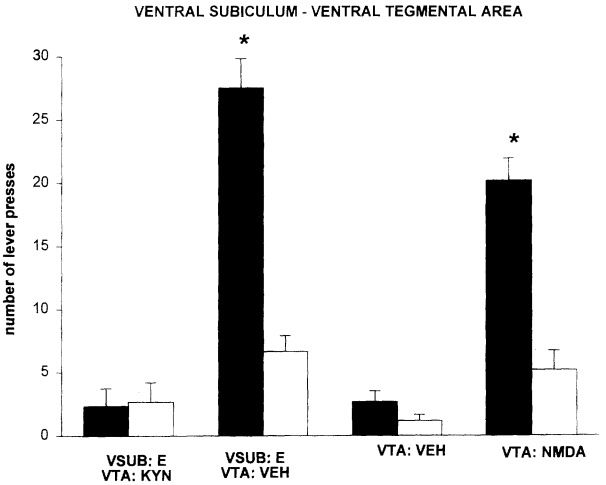

Fig. 2. Effect of microinjection of VEH (0.5 μl) or KYN (50 nmol/0.5 μl) into the VTA on reinstatement elicited by VSUB theta burst stimulation (n = 6); effect of microinjection of VEH (0.5 μl) or NMDA (83 pmol/0.5 μl) into the VTA on reinstatement (n = 6). VEH: vehicle; KYN: kynurenic acid; E: theta burst stimulation. Legend as in Fig. 1B. Asterisks indicate significant difference compared with the VEH only group (*P < 0.00001) (active lever presses).

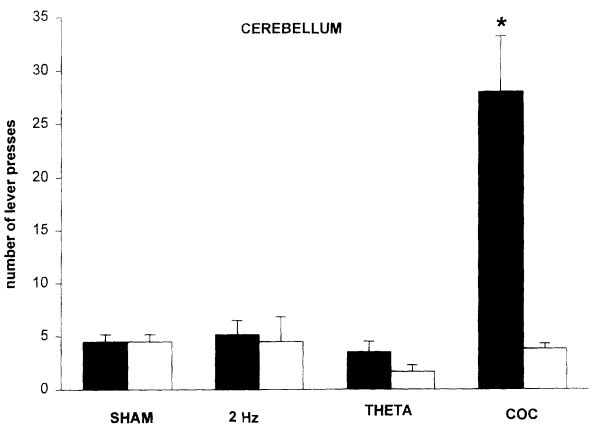

Fig. 3. Effect of different electrical stimulation patterns in a group of rats (n = 6) with electrodes in CBL. COC: cocaine prime (2.0 mg/kg i.v.). Legend as in Fig. 1B. The only significant effect occurred after i.v. cocaine prime (*P < 0.00001) (active lever presses).

An anatomical control group received CBL stimulation (*28*) (Fig. 3). The CBL supports electrical self-stimulation (*33–35*) and is activated after exposure to cues eliciting cocaine craving (*4*, *5*). Neither 2-Hz nor theta burst stimulation elicited reinstatement (Fig. 3). Reinstatement did occur after a noncontingent i.v. cocaine prime (2.0 mg/kg) (Fig. 3), indicating capability of reinstatement.

Another group received MFB stimulation (*28*) (Fig. 4, A and B). The MFB supports self-stimulation, which depends on NAC DA (*36*). MFB stimulation induces brief NAC DA release (*37–40*). Neither 2-Hz nor theta burst stimulation nor a stimulation pattern mimicking the intrinsic burst rhythm of DA neurons (*41–43*) elicited reinstatement. The cocaine prime control (2.0 mg/kg i.v.) induced reinstatement (Fig. 4B). The MFB electrode placements were physiologically relevant, because they supported self-stimulation (*41*) (Fig. 4C). MFB stimulation shown to increase running speed for MFB stimulation reward (*44*) also failed to elicit reinstatement (Fig. 4B).

The hippocampus subserves contextual learning (*17–19*). Reinstatement after VSUB stimulation may reflect the read-out of an encoded association between the context of the cocaine experience (i.e., the operant chamber) and (the previously available) cocaine (*45–47*). Reinstatement after VTA activation (Fig. 2) (*27*) concurs with the proposed function of VTA DA neurons as reward predictors (*48*, *49*). Thus, VSUB stimulation may harness a neural substrate involving the VTA that is predictive of cocaine reward.

VSUB burst stimulation elicited reinstatement and preceded lever pressing (Fig. 1, A

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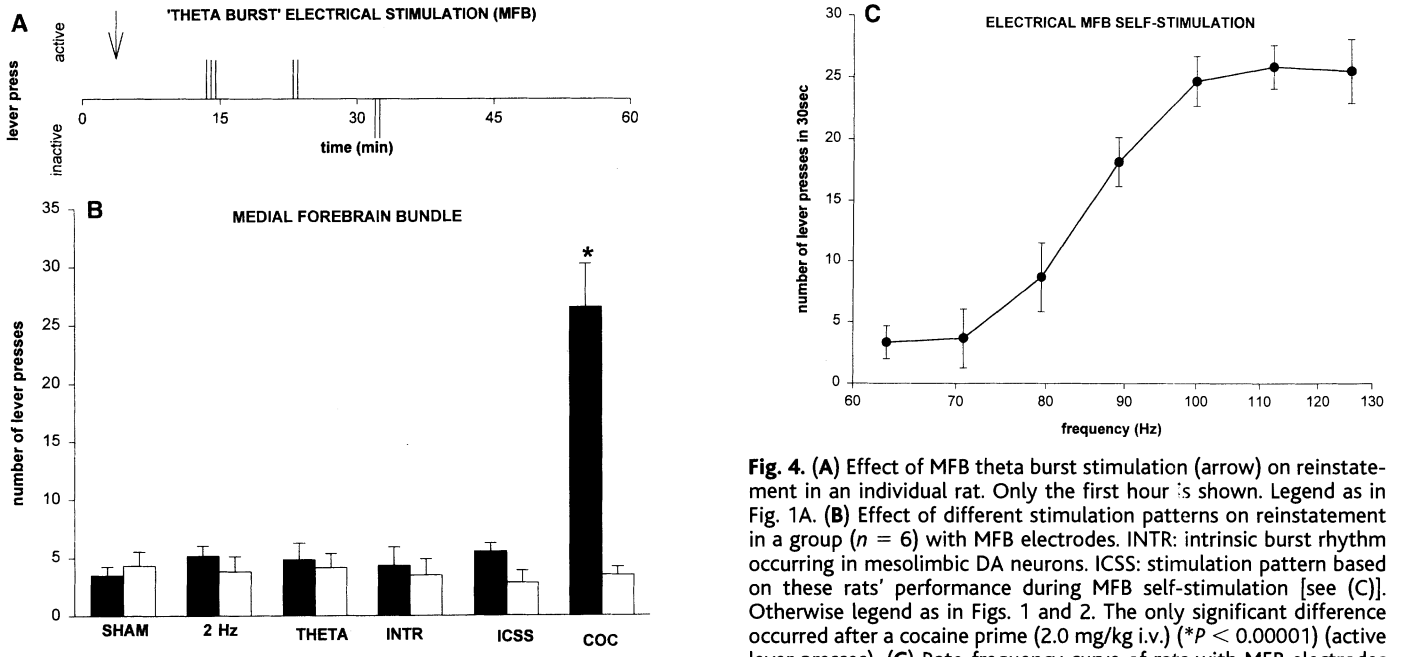

Fig. 4. (**A**) Effect of MFB theta burst stimulation (arrow) on reinstatement in an individual rat. Only the first hour is shown. Legend as in Fig. 1A. (**B**) Effect of different stimulation patterns on reinstatement in a group (n = 6) with MFB electrodes. INTR: intrinsic burst rhythm occurring in mesolimbic DA neurons. ICSS: stimulation pattern based on these rats' performance during MFB self-stimulation [see (C)]. Otherwise legend as in Figs. 1 and 2. The only significant difference occurred after a cocaine prime (2.0 mg/kg i.v.) (*$P < 0.00001$) (active lever presses). (**C**) Rate-frequency curve of rats with MFB electrodes generated during MFB self-stimulation after finishing the experiment shown in (B) (n = 6, mean ± SEM). All rats showed stable self-stimulation patterns. The optimal self-administered current intensities varied between −251 and −158 μA.

and B). Thus, VSUB stimulation has predictive or incentive (*50–52*) properties that facilitate the initiation of lever-press responding. MFB stimulation had no such predictive or incentive properties, because it failed to initiate reinstatement (Fig. 4, A and B). In contrast, during MFB self-stimulation, electrical stimulation followed and positively reinforced (*53*, *54*) lever pressing (Fig. 4C). The hippocampus is much less effective at supporting self-stimulation than the MFB (*55–57*); i.e., VSUB (self-) stimulation is much less reinforcing than MFB stimulation. Therefore, separate neural systems may subserve positive reinforcing (self-stimulation: MFB) and incentive (reinstatement: VSUB) electrical brain stimulation. Separation of neural systems subserving positive reinforcing and incentive stimuli has been proposed previously (*58–60*).

The two neural systems presumably share the mesolimbic DA projection, because pharmacological stimulation of mesolimbic DA neurotransmission triggers reinstatement (Fig. 2) (*7*, *25–27*) and because VSUB stimulation enhances mesolimbic DA neurotransmission (*20–24*). Although both MFB and VSUB stimulation increase NAC DA, we propose that the long-lasting (about 30 min) DA release after VSUB stimulation (*22*)—as opposed to the brief (less than 5 s) release after MFB stimulation (*37–40*)—is critical for reinstatement. Our finding that reinstatement depends on VTA GLU agrees with neurochemical data (*23*).

Because the VSUB contains GLU (*61–63*) and because reinstatement could be blocked by KYN and elicited by NMDA in the VTA (Fig. 2), GLU seems to be involved in cocaine-seeking behavior (*27*, *64*). Therefore, GLU agents appear worthy of pursuit as potential pharmacotherapeutic candidates for cocaine addiction.

References and Notes

1. A. I. Leshner, *Science* **278**, 45 (1997).
2. D. D. Simpson, G. W. Joe, B. W. Fletcher, R. L. Hubbard, M. D. Anglin, *Arch. Gen. Psychiatry* **56**, 507 (1999).
3. H. C. Breiter *et al.*, *Neuron* **19**, 591 (1997).
4. S. Grant *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12040 (1996).
5. G. J. Wang *et al.*, *Life Sci.* **64**, 775 (1999).
6. A. R. Childress *et al.*, *Am. J. Psychiatry* **156**, 11 (1999).
7. A. Markou *et al.*, *Psychopharmacology* **112**, 163 (1993).
8. D. W. Self, *Ann. Med.* **30**, 379 (1998).
9. Y. Shaham, S. Erb, J. Stewart, *Brain Res. Brain Res. Rev.* **33**, 13 (2000).
10. H. de Wit, J. Stewart, *Psychopharmacology* **75**, 134 (1981).
11. J. H. Jaffe, N. G. Cascella, K. M. Kumor, M. A. Sherer, *Psychopharmacology* **97**, 59 (1989).
12. S. Erb, Y. Shaham, J. Stewart, *Psychopharmacology* **128**, 408 (1996).
13. R. Sinha, D. Catapano, S. O'Malley, *Psychopharmacology* **142**, 343 (1999).
14. W. M. Meil, R. E. See, *Behav. Brain Res.* **87**, 139 (1997).
15. A. R. Childress *et al.*, *NIDA Res. Monogr. Ser.* **137**, 73 (1993).
16. U. Shalev, D. Highfield, J.Yap, Y. Shaham, *Psychopharmacology* **150**, 337 (2000).
17. J. J. Kim, M. S. Fanselow, *Science* **256**, 675 (1992).
18. N. R. Selden, B. J. Everitt, L. E. Jarrard, T. W. Robbins, *Neuroscience* **42**, 335 (1991).
19. R. G. Phillips, J. E. LeDoux, *Behav. Neurosci.* **106**, 274 (1992).
20. S. M. Brudzynski, C. J. Gibson, *Brain Res. Bull.* **42**, 303 (1997).
21. M. Legault, R. A.Wise, *Synapse* **31**, 241 (1999).
22. C. D. Blaha, C. R. Yang, S. B. Floresco, A. M. Barr, A. G. Phillips, *Eur. J. Neurosci.* **9**, 902 (1997).
23. M. Legault, P. P. Rompre, R. A. Wise, *J. Neurosci.* **20**, 1635 (2000).
24. C. L. Todd, A. A. Grace, *Ann. N. Y. Acad. Sci.* **877**, 688 (1999).
25. J. Stewart, *Pharmacol. Biochem. Behav.* **20**, 917 (1984).
26. J. Stewart, P. Vezina, *Brain Res.* **457**, 287 (1988).
27. J. L. Cornish, P. W. Kalivas, *J. Neurosci. (Online)* **20**, RC89 (2000).
28. Before surgery, rats were trained to self-administer food. A monopolar electrode was implanted in the MFB (AP +5.4, ML ±1.6, DV +1.6), VSUB (AP +2.7, ML ±5.2, DV +2.4), or CBL (AP −2.6, ML ±2.5, DV +5.0); coordinates relative to interaural line (*65*). Electrical stimulations were constant-current injections administered by pulse generator and stimulus isolation unit. Currents were square, cathodal, pulse width 0.4 ms, intensity 400 μA, 200 pulses per stimulation. Stimulation patterns were "sham": The rat was connected to the stimulation apparatus, but no actual electrical stimulation was delivered; 2-Hz repetitive; "theta burst": 40 trains of five pulses, frequency of trains 5 Hz, frequency of pulses within train 100 Hz. Means and variances of the number of lever presses of different treatment groups were compared with one-way analysis of variance and post-hoc Tukey tests.
29. O. S. Vinogradova, *Prog. Neurobiol.* **45**, 523 (1995).
30. Bilateral guide cannulae (22 gauge) were implanted into VTA: AP +4.2; ML ±1.0; DV +1.7. NMDA and KYN were dissolved in saline and the pH adjusted to 7.0 to 8.0 with NaOH. NMDA, KYN, and VEH were injected manually through inner cannulae (28 gauge) over 30 s in 0.5 μl; inner cannulae were left in place for 60 s after the injection. KYN or VEH were microinjected 15 min before electrical stimulation.
31. B. H. Westerink, H. F. Kwint, J. B. de Vries, *J. Neurosci.* **16**, 2605 (1996).
32. M. Karreman, B. H. Westerink, B. Moghaddam, *J. Neurochem.* **67**, 601 (1996).
33. A. Routtenberg, C. Malsbury, *J. Comp. Physiol. Psychol.* **68**, 22 (1969).
34. G. G. Ball, D. J. Micco Jr., G. G. Berntson, *Physiol. Behav.* **13**, 123 (1974).
35. D. Corbett, E. Fox, P. M. Milner, *Behav. Brain Res.* **6**, 167 (1982).
36. R. A. Wise, P. P. Rompre, *Annu. Rev. Psychol.* **40**, 191 (1989).
37. J. P. Ng, G. W. Hubert, J. B. Justice Jr., *J. Neurochem.* **56**, 1485 (1991).

38. P. A. Garris, R. M. Wightman, *J. Neurosci.* **14**, 442 (1994).
39. S. R. Jones, P. A. Garris, C. D. Kilts, R. M. Wightman, *J. Neurochem.* **64**, 2581 (1995).
40. M. Benoit-Marand, M. Jaber, F. Gonon, *Eur. J. Neurosci.* **12**, 2985 (2000).
41. "Intrinsic burst" stimulation: 67 trains of three pulses, frequency of trains 2 Hz, duration between first and second pulse within each train 60 ms, duration between second and third pulse 160 ms. Intracranial self-stimulation was done as described (*66*). During MFB self-stimulation, optimal stimulation intensity was determined for each rat and varied between −251 and −158 μA. Using this intensity, we applied "ICSS" priming stimulation: pulse width 0.1 ms, 20 trains of 64 pulses, interval between trains 1.0 s, interval between pulses within train 5.0 ms.
42. A. A. Grace, B. S. Bunney, *J. Neurosci.* **4**, 2877 (1984).
43. A. J. Bean, R. H. Roth, *J. Neurosci.* **11**, 2694 (1991).
44. C. R. Gallistel, *J. Comp. Physiol. Psychol.* **69**, 713 (1969).
45. R. Hirsh, *Behav. Biol.* **12**, 421 (1974).
46. P. C. Holland, M. E. Bouton, *Curr. Opin. Neurobiol.* **9**, 195 (1999).
47. L. L. Eldridge, B. J. Knowlton, C. S. Furmanski, S. Y. Bookheimer, S. A. Engel, *Nature Neurosci.* **3**, 1149 (2000).
48. J. Mirenowicz, W. Schultz, *Nature* **379**, 449 (1996).
49. W. Schultz, P. Dayan, P. R. Montague, *Science* **275**, 1593 (1997).
50. K. C. Walker, *J. Exp. Psychol.* **31**, 312 (1942).
51. W. K. Estes, *J. Exp. Psychol.* **32**, 150 (1943).
52. D. Bindra, *Psychol. Rev.* **75**, 1 (1968).
53. J. Olds, P. Milner, *J. Comp. Physiol. Psychol.* **47**, 419 (1954).
54. B. F. Skinner, *The Behavior of Organisms* (Appleton-Century-Crofts, New York, 1938).
55. R. Ursin, H. Ursin, J. Olds, *J. Comp. Physiol. Psychol.* **61**, 353 (1966).
56. D. van der Kooy, H. C. Fibiger, A. G. Phillips, *Brain Res.* **136**, 119 (1977).
57. K. L. Sweet, D. B. Neill, *Ann. N.Y. Acad. Sci.* **877**, 828 (1999).
58. S. Killcross, T. W. Robbins, B. J. Everitt, *Nature* **388**, 377 (1997).
59. J. W. Grimm, R. E. See, *Neuropsychopharmacology* **22**, 473 (2000).
60. P. Amorapanth, J. E. LeDoux, K. Nader, *Nature Neurosci.* **3**, 74 (2000).
61. R. Zaczek, J. C. Hedreen, J. T. Coyle, *Exp. Neurol.* **65**, 145 (1979).
62. I. Walaas, F. Fonnum, *Neuroscience* **5**, 1691 (1980).
63. M. J. Christie, R. J. Summers, J. A. Stephenson, C. J. Cook, P. M. Beart, *Neuroscience* **22**, 425 (1987).
64. J. L. Cornish, P. Duffy, P. W. Kalivas, *Neuroscience* **93**, 1359 (1999).
65. G. Paxinos, C. Watson, *The Rat Brain in Stereotaxic Co-ordinates* (Academic Press, New York, 1986).
66. M. Lepore, X. Liu, V. Savage, D. Matalon, E. L. Gardner, *Life Sci.* **58**, PL365 (1996).
67. We thank L. Brown, P. K. Stanton, and M. Makman for helpful suggestions; D. Colon for administrative assistance; and W. Paredes for technical support. Supported by the New York State Office of Mental Health, the New York State Office of Alcoholism and Substance Abuse Services, and the Julia Sullivan Medical Research Fund.

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Hemichannel-Mediated Inhibition in the Outer Retina

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An essential feature of the first synapse in the retina is a negative feedback pathway from horizontal cells to cones. Here we show that at this synapse, connexin26 forms hemichannels on horizontal cell dendrites near the glutamate release site of the cones. Blocking these hemichannels hyperpolarizes horizontal cells, modulates the Ca^{2+} channels of the cones, and abolishes all feedback-mediated responses. We propose a feedback mechanism in which the activity of the Ca^{2+} channels and the subsequent glutamate release of the cones are modulated by a current through these hemichannels. Because the current through the hemichannels depends on the polarization of the horizontal cells, their activity modulates the output of the cones.

In all vertebrate retinas, photoreceptors project to horizontal cells (HCs) and bipolar cells (BCs). The synaptic complex of this interaction reveals a peculiar and conserved ultrastructure. The cone pedicles are characterized by a presynaptic ribbon (where neurotransmitter release takes place), centrally positioned BC dendrites, and laterally positioned HC dendrites (Fig. 1A). These lateral contacts are thought to be the origin of negative feedback from HCs to cones. In goldfish, this feedback modulates the Ca^{2+} current of the cones. Hyperpolarization of HCs shifts the Ca^{2+} current to more negative potentials, which increases the Ca^{2+} influx and subsequently leads to an increase in glutamate release. Various neurotransmitters have been proposed for this pathway, but this retrograde neurotransmitter has not yet been unequivocally identified (*1*).

In the carp retina, connexin26 (Cx26) immunolabel (*2*, *3*) was restricted to the membrane of the lateral processes of the HCs close to the voltage-dependent Ca^{2+} channels on the opposing cone membrane (Fig. 1, B and C) (*4*). Septilaminar structures indicative of gap junctions between the cones and the HC are not discernible at this site nor have such structures been reported by physiological studies, suggesting that the immunolabel reflects the presence of hemichannels. That functional hemichannels are present on HCs and do not compromise cell viability has been shown in dissociated HCs (*5*, *6*).

The location of Cx26 immunolabel suggested that such hemichannels might be involved in the synaptic interactions between HCs and cones. Thus, we studied the effect of carbenoxolone, a blocker of gap-junctional channels on this feedback pathway (*7–9*). Figure 2A, left, shows the feedback-induced responses of a cone clamped at various membrane potentials (*10*). The feedback-mediated responses in cones can be measured most effectively when the cone response is saturated with a white 20-μm-diameter spot and the retina is stimulated with a full-field white light stimulus (*1*). Such a stimulus induces a shift of the Ca^{2+} current in the cones to more negative potentials, which will be seen as an inward current in a voltage-clamped cone. In the presence of 100 μM carbenoxolone, these feedback-induced responses disappeared (Fig. 2A, right) (n = 13). The effect of carbenoxolone could be washed out within 15 min (Fig. 2B). During the application of carbenoxolone cones hyperpolarized by −4.6 mV ± 1.7 mV (n = 5), whereas their light response amplitude was unaffected (Fig. 2C).

Because blocking the hemichannels led to the disappearance of the feedback-induced responses in cones, the feedback-mediated responses in HCs should also disappear. Carbenoxolone hyperpolarized HCs strongly and reduced their light responses (Fig. 2D). Because of this large hyperpolarization, the effect of carbenoxolone on the feedback-mediated responses was studied before the HCs had hyperpolarized more than about 25% of their maximal hyperpolarization (Fig. 2D, ③). HC light responses show a characteristic transient component (arrow Fig. 2E, left), mainly attributable to negative feedback from HCs to cones (*11*, *12*). In monophasic HCs (MHCs), which hyperpolarize to light of all wavelengths, this pronounced transient component was blocked by carbenoxolone (Fig. 2E, right) (n = 8). Biphasic HCs (BHCs) hyperpolarize to full-field green light stimulation but depolarize upon red light stimulation (Fig. 2F, left). The depolarizing responses are thought to originate from negative feedback from MHCs to middle-wavelength sensitive cones (*1*, *12–15*). Carbenoxolone blocked these depolarizing responses, whereas the hyperpolarizing responses were almost unaffected (Fig. 2F, right) (n = 5).

Is the block of the hemichannels or the hyperpolarization of the HCs responsible for

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