However, as the infection progresses and bacteria undergo unrestrained multiplication, the availability of nonmyelinating Schwann cells becomes a limiting factor. To avoid such a situation, leprosy bacilli may induce demyelination and axonal damage as an effective strategy to increase the number of nonmyelinating Schwann cells so that a sufficient intracellular niche is available for bacterial survival. Because myelinated Schwann cells do not serve as an intracellular niche for *M. leprae*, we propose that *M. leprae* propagates a nonmyelinating phenotype by inducing demyelination and nerve injury in myelinated Schwann cells in the early phase of infection, a novel bacterial survival strategy in the nervous system.

References and Notes

1. S. G. Waxman, *N. Engl. J. Med.* **338**, 323 (1998).
2. D. M. Wingerchuk, C. F. Lucchinetti, J. H. Noseworthy, *Lab Invest.* **81**, 263 (2001).
3. P.C. Johnson, in *Textbook of Neuropathology*, R. L. Davis and D. M. Robertson, Eds. (Williams and Wilkins, Baltimore, MD, 3rd ed., 1997), pp. 1233–1323.
4. G. L. Stoner, *Lancet* **10**, 994 (1979).
5. J. M. Jacobs, V. P. Shetty, N. H. Antia, *J. Neurol. Sci.* **79**, 301 (1987).
6. V. P. Shetty, L. N. Mehta, P. F. Irani, N. H. Antia, *Lep. India* **52**, 5 (1980).
7. V. P. Shetty, J. M. Jacobs, N. H. Antia, *J. Neurol. Sci.* **88**, 115 (1988).
8. C. K. Job, *Int. J. Lepr.* **57**, 532 (1989).
9. A. Rambukkana, *Curr. Opin. Microbiol.* **4**, 21 (2001).
10. S. Einheber, T. A. Milner, F. Giancotti, J. L. Salzer, *J. Cell Biol.* **123**, 1223 (1993).
11. P. Mombaerts *et al.*, *Cell* **68**, 869 (1992).
12. In vivo grown *M. leprae* were purified from armadillos and nude mice and provided by P. J. Brennan (Colorado State Univ., Fort Collins, CO) and J. Krahenbuhl (Hansen's Disease Center, Baton Rouge, LA), respectively.
13. A. Rambukkana, J. L. Salzer, P. D. Yurchenco, E. I. Tuomanen, *Cell* **88**, 811 (1997).
14. Details of the experimental procedures for in vitro infection (*14*) and evidence regarding lack of contamination are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5569/927/DC1.
15. Cells undergoing apoptosis in *M. leprae*–treated and control cultures were determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay kit according to the manufacturer's instructions (Roche Molecular Biochemicals).
16. A. Rambukkana, unpublished data.
17. Ramon y Cajal, *Degeneration and Regeneration of the Nervous System* (Oxford Univ. Press, Oxford, UK, 1928).
18. D. E. Pleasure, J. Towfighi, *Arch. Neurol.* **26**, 289 (1972).
19. S. W. Hunter, P. J. Brennan, *J. Bacteriol.* **147**, 728 (1981).
20. P. J. Brennan, *Rev. Infect. Dis.* **11** (Suppl. 2), S420 (1989).
21. V. Ng *et al.*, *Cell* **103**, 511 (2000).
22. Native PGL-1 was purified from armadillo tissue (*19*) and provided by P. J. Brennan.
23. C. D. Schmid *et al.*, *J. Neurosci.* **20**, 729 (2000).
24. Details of the experimental procedures for in vivo infection are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5569/927/DC1.
25. R. Curtiss III *et al.*, *Lepr. Rev.* **72**, 8 (2001).
26. V. P. Shetty, M. W. Uplekar, N. H. Antia, *Acta Neuropathol.* **88**, 300 (1994).
27. V. P. Shetty, *Int. J. Lepr.* **61**, 70 (1993).
28. Details of the experimental procedures for *M. leprae* invasion in vitro are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5569/927/DC1.
29. S. S. Scherer, J. L. Salzer, in *Glial Cell Development*, K. R. Jessen and W. D. Richardson, Eds. (Bios Scientific Publishers, London, 1996), pp. 165–196.
30. Details of the experimental procedures for Schwann cell proliferation are available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5569/927/DC1.

31. We thank V. A. Fischetti and E. C. Gotschlich for continuing support and encouragement, P. J. Brennan and J. Krahenbuhl for providing *M. leprae* and its components through NIAID/NIH contract, and H. Shio for excellent electron microscopy analyses. This work was funded by R01 grants from the NIH/NIAID (to A.R.), NINDS (to J.L.S.), and the UNDP/World Bank/WHO Special Program for Research in Tropical Diseases (to A.R.).

11 November 2001; accepted 6 February 2002

Enhanced and Delayed Stress-Induced Alcohol Drinking in Mice Lacking Functional CRH1 Receptors

Inge Sillaber,[1]*† Gerhard Rammes,[1]* Stephan Zimmermann,[1] Beatrice Mahal,[1] Walter Zieglgänsberger,[1] Wolfgang Wurst,[1,2] Florian Holsboer,[1] Rainer Spanagel[1,3]

There is a relation between stress and alcohol drinking. We show that the corticotropin-releasing hormone (CRH) system that mediates endocrine and behavioral responses to stress plays a role in the control of long-term alcohol drinking. In mice lacking a functional CRH1 receptor, stress leads to enhanced and progressively increasing alcohol intake. The effect of repeated stress on alcohol drinking behavior appeared with a delay and persisted throughout life. It was associated with an up-regulation of the *N*-methyl-D-aspartate receptor subunit NR2B. Alterations in the CRH1 receptor gene and adaptional changes in NR2B subunits may constitute a genetic risk factor for stress-induced alcohol drinking and alcoholism.

Alcoholism is a multifactorial disorder in which the environment interacts with genetic predisposition to produce the final level of risk (*1*, *2*). Stressful life events and maladaptive responses to stress influence alcohol drinking and relapse behavior (*3*–*6*). Although the relation between stress and alcohol drinking in humans (*3*, *4*) and laboratory animals (*5*, *6*) is complex, it is known that in some individuals alcohol drinking is an attempt to cope with stress. Stress-induced alcohol drinking and relapse behavior apparently have a significant genetic component (*7*, *8*), but molecular and cellular mechanisms underlying stress-induced alcohol drinking and relapse behavior are still obscure. Recent studies have implicated the CRH system and the glutamatergic system in these processes (*9*, *10*).

CRH regulates endocrine responses to stress (*11*) and mediates stress-related behavioral responses by means of extrahypothalamic sites (*12*). The CRH signal is transmitted by two types of receptors, termed the CRH1 and CRH2 receptors, which differ in their pharmacology and expression pattern in the brain (*13*, *14*). Dysregulation in the CRH/CRH1-receptor system has been attributed to a variety of stress-related psychiatric disorders, including alcoholism (*9*). Mice lacking a functional CRH1 receptor (*Crhr1*$^{-/-}$) (*15*) represent a useful animal model to address the question of whether a dysfunctional CRH/CRH1-receptor system influences individual vulnerability for alcohol drinking, under basal and stress conditions, and whether long-term alcohol self-administration is influenced by this mutation.

Before studying alcohol drinking in *Crhr1*$^{-/-}$ mice, we further tested the functional impairment of the CRH1 receptor. *Crhr1*$^{-/-}$ mice that are lacking the G protein–coupling domain show a blunted hormonal stress response (*15*). Extracellular field potential measurements that are exemplary in the CA1 region of the hippocampus were used to document the dysfunctional signal transduction (*16*). In an in vitro prepara-

[1]Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany. [2]National Research Center for Environment and Health, Institute for Mammalian Genetics, Ingolstädter Landstrasse 1, 85764 Munich, Germany. [3]Central Institute of Mental Health, University of Heidelberg, J5, 68159 Mannheim, Germany.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: sillaber@mpipsykl.mpg.de

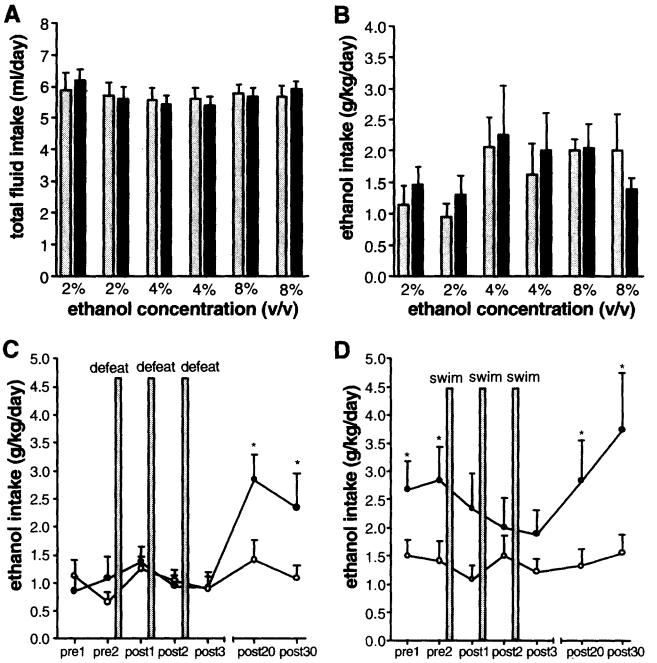

Fig. 1. Water consumption and initial preference for alcohol are the same in *Crhr1*$^{-/-}$ and wild-type mice. (**A**) Total fluid consumption and (**B**) ethanol intake of *Crhr1*$^{-/-}$ (black bar; $n = 9$) and wild-type (white bar; $n = 11$) mice during the acquisition phase in a two-bottle free-choice paradigm. In (**C**) and (**D**), the acute effects of stress on voluntary alcohol intake in *Crhr1*$^{-/-}$ (closed circles; $n = 9$) and wild-type (open circles; $n = 11$) mice are shown. Water and an ethanol solution (8% v/v) were presented in a two-bottle free-choice paradigm. Intake was measured 1 week (pre1) and 1 day (pre2) before the first exposure to the social defeat stressor (C) and forced swimming (D). Stress was elicited on 3 consecutive days, and fluid consumption was measured daily (post 1 to 30). *$P < 0.01$ (Newman-Keuls post hoc test: *Crhr1*$^{-/-}$ versus wild type).

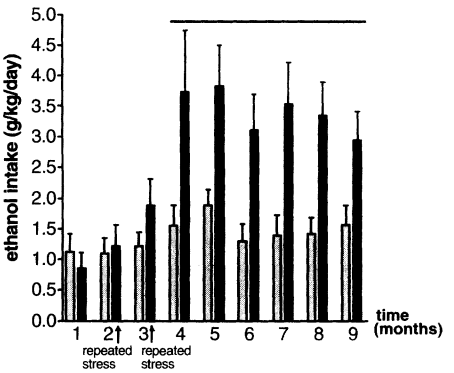

Fig. 2. Long-term voluntary alcohol intake of *Crhr1*$^{-/-}$ (black bar; $n = 9$) and wild-type (white bar; $n = 11$) mice. The animals were offered water and an ethanol solution (8% v/v) in a two-bottle free-choice paradigm. The data represent mean ethanol intake per month. Stress events are indicated by arrows. *$P <$ 0.01 (Newman-Keuls post hoc test: *Crhr1*$^{-/-}$ versus wild type).

tion, CRH increased the amplitude of population spikes in the CA1 region of the hippocampus (142.9 ± 4.7%) in wild-type mice. The population spike amplitudes in slices from *Crhr1*$^{-/-}$ mice were not affected by CRH, indicating an effective interruption of CRH signal transduction by the genetic manipulation [Web fig. 1 (*17*)].

We then offered *Crhr1*$^{-/-}$ and wild-type mice a free choice between water and an alcohol solution, at increasing concentrations, as drinking fluids (*18*). We found no difference in total fluid consumption (ml/day) between *Crhr1*$^{-/-}$ and their wild-type littermates (Fig. 1A), independent of the co-offered ethanol concentration ($P > 0.7$). The genotypes did not differ in the daily intake of alcohol (g/kg/day) at concentrations of 2, 4, and 8% ethanol (v/v) (Fig. 1B) ($P > 0.7$). After a habituation period of 8 weeks to 8% ethanol versus water, we monitored the voluntary alcohol consumption of the mice during and after repeated stress. All of the mice were repeatedly exposed to a social defeat stress as a psychological and severe stressor (*19*) for 3 consecutive days. We found no difference in alcohol intake during repeated social defeat stress as compared to baseline drinking in either the wild-type ($P > 0.5$) or the *Crhr1*$^{-/-}$ mice ($P > 0.8$) (Fig. 1C). However, 3 weeks after repeated social defeat stress, the voluntary alcohol intake in the *Crhr1*$^{-/-}$ mice was markedly increased (versus baseline, $P < 0.001$), whereas it remained unaltered in the wild-type mice (versus baseline, $P > 0.2$). During this poststress phase, the *Crhr1*$^{-/-}$ mice consumed much larger amounts of alcohol than did their wild-type littermates (post 20, $P < 0.001$) (Fig. 1C). During a second period of repeated stress, the mice were forced to swim on three consecutive days. Forced swimming is predominantly a physical stressor, but it also has an emotional component (*20*). Monitoring of behavioral parameters during the forced swim test (i.e., time spent struggling, swimming, or floating) did not reveal any significant difference between the two groups [Web fig. 2 (*17*)]. Although *Crhr1*$^{-/-}$ mice slightly decreased alcohol intake during the three swim stress days (Fig. 1D), this decrease was statistically not significant as compared to baseline drinking, neither in the wild-type ($P > 0.7$) nor in the *Crhr1*$^{-/-}$ mice ($P > 0.2$) (Fig. 1D). However, after about 3 weeks, the *Crhr1*$^{-/-}$ mice started again to progressively increase their alcohol intake. This poststress alcohol intake of *Crhr1*$^{-/-}$ mice was significantly higher than that of the wild-type mice (post 20, $P < 0.002$) (Fig. 1D). Enhanced alcohol intake in *Crhr1*$^{-/-}$ mice was long lasting and was still present 6 months after the second set of stressors (Fig. 2). Thus, alcohol intake developed differentially in the two groups (genotype × time interaction: $P < 0.001$), and the mutation had a significant effect on alcohol intake ($P < 0.01$). In comparison, *Crhr1*$^{-/-}$ mice that had continuously free access to alcohol over 3 months without receiving the two sets of stressors showed no changes in alcohol intake over time [Web fig. 3 (*17*)].

To exclude the possibility that the mutation of the *Crhr1* gene affected the pharmacokinetics of alcohol, we determined blood alcohol concentrations in separate groups of mice before and after alcohol drinking (*21*). No differences in alcohol metabolism were found between *Crhr1*$^{-/-}$ and wild-type mice, in terms of blood alcohol concentration. Thus, acute clearance of ethanol did not differ between genotypes (*Crhr1*$^{-/-}$ mice cleared 0.43 ± 0.04 mg ethanol/ml blood/hour, and control mice cleared 0.41 ± 0.03 mg ethanol/ml blood/hour) ($P > 0.4$) [Web fig. 4 (*17*)].

Under basal conditions, up-regulation of CRH (*15*) and vasopressin (*22*) is observed as adaptational changes due to CRH1 receptor deficiency; however, other molecules such as proopiomelanocortin-derived adrenocorticotropic hormone and melanocyte-stimulating hormone remain unchanged in *Crhr1*$^{-/-}$ mice (*15*). A dysfunctional CRH system is expected to induce adaptational changes within functionally related systems, such as the glutamatergic system. We thus performed protein profiling for various glutamate receptors (*23*). We analyzed selected brain regions for the protein expression levels of the *N*-methyl-D-aspartate (NMDA)–receptor subunits (*24*), subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, and the kainate receptor in *Crhr1*$^{-/-}$ and wild-type mice. Western blot analysis revealed a highly selective up-regulation in the NR2B subunit of the NMDA receptor (Fig. 3). No other ionotropic glutamate–receptor subtype was altered (*23*). The expression levels of NR2B were enhanced in *Crhr1*$^{-/-}$ as compared to wild-type mice in the hippocampus (*Crhr1*$^{-/-}$: to 122.5 ± 7.4% ; $P < 0.01$) and in the nucleus accumbens (*Crhr1*$^{-/-}$: to 128.1 ± 8.3%; $P < 0.05$). In the amygdala, the expression levels of NR2B were not significantly different between the two groups (100 ± 6.3 for wild types, and 109.8 ± 3.5 for *Crhr1*$^{-/-}$, $P > 0.1$). CRH1 receptor deficiency induced a

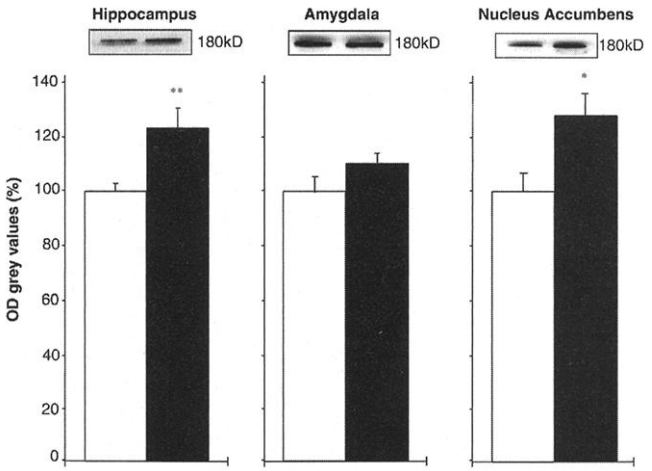

Fig. 3. Quantification of NR2B protein levels in the hippocampus, amygdala, and nucleus accumbens of wild-type (white bars, $n = 6$) and *Crhr1*$^{-/-}$ (black bars, $n = 6$) mice by Western blot analysis. The data are presented as normalized optical-density grey values with wild-type values set as 100%. *$P < 0.05$ (Student t test). **$P < 0.01$.

selective up-regulation of the NMDA receptor subunit NR2B, particularly in the nucleus accumbens and hippocampus. Why might this adaptational increase in the expression of NR2B subunits be associated with the observed stress-induced alcohol drinking behavior? Mesencephalic neurons implicated in the mediation of the reinforcing effects of alcohol (*25*) bear predominantly NMDA receptors with ethanol-sensitive NR2B subunits (*26*, *27*), and it has been shown that NR2B subunits are increased by stress (*28*). Interestingly, a gene variant of the NR2B subunit has recently been found to be associated with alcoholism (*29*). Alternatively, enhanced NR2B subunit expression within the hippocampus and the nucleus accumbens could lead to altered learning abilities, as has been shown recently in transgenic mice (*30*). However, this possibility seems unlikely, because *Crhr1*$^{-/-}$ mice show no differences as compared to wild-type animals in various learning tasks (*31*).

In summary, *Crhr1*$^{-/-}$ mice do not differ from wild-type mice in alcohol intake and preference under stress-free housing conditions. However, after repeated stress, *Crhr1*$^{-/-}$ mice step up their alcohol consumption, which persists at an elevated level throughout their life. This behavior in knockout mice is associated with enhanced protein levels of the NR2B subunit, which is an ethanol-sensitive site and is also influenced by stress. It is feasible that alterations in the CRH1 receptor gene and adaptational changes in NR2B subunits might constitute a genetic risk factor for alcoholism, in particular when associated with stressful life events.

References and Notes

1. M. A. Schuckit, *JAMA* **254**, 2614 (1985).
2. ———, *J. Stud. Alcohol* **59**, 485 (1998).
3. L. A. Pohorecky, *Alcohol. Clin. Exp. Res.* **15**, 438 (1991).
4. S. S. O'Malley, S. Krishnan-Sarin, C. Farren, R. Sinha, M. J. Kreek, *Psychopharmacology* **160**, 19 (2002).
5. L. A. Pohorecky, *Alcohol Alcohol.* **25**, 263 (1990).
6. M. J. Kreek, G. F. Koob, *Drug Alcohol Depend.* **51**, 23 (1998).
7. K. S. LaForge, V. Yuferov, M. J. Kreek, *Eur. J. Pharmacol.* **410**, 249 (2000).
8. G. S. Wand *et al.*, *Neuropsychopharmacology* **26**, 106 (2002).
9. Z. Sarnyai, Y. Shaham, S. C. Heinrichs, *Pharmacol. Rev.* **53**, 209 (2001).
10. R. Spanagel, P. Bienkowski, in *Therapeutic Potential of Ionotropic Glutamate Receptor Antagonists and Modulators*, D. Lodge, W. Danysz, C. G. Parsons, Eds. (Graham Publishing Co., Mountain Home, TN, 2002, chap. 12).
11. W. Vale, J. Spiess, C. Rivier, J. Rivier, *Science* **213**, 1394 (1981).
12. S. C. Heinrichs, F. Menzaghi, P. E. Merlo, K. T. Britton, G. F. Koob, *Ann. N.Y. Acad. Sci.* **771**, 92 (1995).
13. E. B. De Souza, *Psychoneuroendocrinology* **20**, 789 (1995).
14. D. T. Chalmers, T. W. Lovenberg, E. B. De Souza, *J. Neurosci.* **15**, 6340 (1995).
15. P. Timpl *et al.*, *Nature Genet.* **19**, 162 (1998).
16. The experiments were performed on male F2 hybrids (129/Ola × CD1) and wild-type littermates (129/Ola × CD1) generated by F1 (129/Ola × CD1) intercrosses. The knockout was made in E14.1 embryonic stem cells (129/Ola), and the chimera were bred to CD1 females [for a detailed description, see (*15*)]. Hippocampal slices (300 μM) were obtained from 1- to 2-month-old *Crhr1*$^{-/-}$ and wild-type mice. Extracellular recordings were made from the dendritic region of CA1, and population spikes (PS's) were evoked by test stimuli (0.066 Hz, 4 to 5 V, 20 μs) delivered through a bipolar electrode positioned in the Schaffer collateral-commissural pathway. The PS amplitudes were measured from the negative to the positive maximum values within a 3- to 18-ms interval after the stimulus.
17. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/296/5569/931/DC1.
18. Single-housed male mice (age of 4 to 5 months) were offered two water-filled bottles, and after 1 week of habituation, one of the bottles contained ascending concentrations (2, 4, and 8% v/v) of ethanol in tap water. The bottles were weighed every 3 days, and the concentration was increased every 6 days.
19. For social defeat stress, either *Crhr1*$^{-/-}$ or wild-type mice were confronted with an unfamiliar male mouse (resident) in a special cage (20 × 12 × 20 cm. The resident (6- to 7-month-old Swiss mice) had been housed in this cage for 2 days. In this paradigm, the resident attacked the intruder within 1 min. Immediately after the first attack, the two mice were separated by a wire mesh screen, and the intruder was left in the smaller section of the cage (7 × 12 × 20 cm) for another 15 min. The intruder was then returned to its home cage, where it had free access to water and alcohol (*32*).
20. Mice were placed in a water-filled glass cylinder (25 cm high, 14 cm wide) for 5 min (the water temperature was 21°C). Afterward, the mice were gently dried and moved back to their home cages with free access to water and alcohol (*33*).
21. Alcohol-naive mice (wild type and *Crhr1*$^{-/-}$, $n = 5$ per group, 5 months old) were intraperitoneally injected with 3.5 g/kg ethanol (20% ethanol solution, v/v). According to (*34*), blood alcohol levels were measured in blood samples (25 to 30 μl) drawn from the tip of the tail at various time points after injection (30, 90, 150, 210, and 340 min). Blood-alcohol content was determined by the reduced form of nicotinamide adenine dinucleotide enzyme spectophotometric method (Sigma).
22. M. B. Müller *et al.*, *Endocrinology* **141**, 4262 (2000).
23. *Crhr1*$^{-/-}$ and littermate wild-type mice ($n = 5$ per group, 6 to 8 weeks old) were decapitated, and their brains were removed. The brains were rapidly dissected into the hippocampus, nucleus accumbens, and amygdala. The following antibodies were used for Western blot analysis: NMDAR1 (NMDAR1-1/1-2; CHE Ab1516, Biozol, Eching, Germany), NMDAR1 alternative C-terminus (NMDAR1-3/1-4; UB 06-314, Biozol), NMDAR2A (CHE Ab1555, Biozol), NMDAR2B (CHE Ab1557P, Biozol), NMDAR2C (Ab: M-266, RBI, Natick, Munich, Germany), NMDAR2D, GluR1 (Ab06-306, Upstate, Buckingham, UK), GluR2 (Ab60686N, Pharmingen, Heidelberg, Germany), GluR4 (Ab06-308, Upstate), GluR6/7 (Ab06-309, Upstate), KA2 (Ab06-315, Upstate). For methodological details see (*35*).
24. A. Winkler, B. Mahal, K. Kiianmaa, W. Zieglgänsberger, R. Spanagel, *Mol. Brain Res.* **72**, 166 (1999)
25. R. Spanagel, F. Weiss, *Trends Neurosci.* **22**, 521 (1999).
26. B. Chu, V. Anantharam, S. N. Treistman, *J. Neurochem.* **65**, 140 (1995).
27. C. Allgeier, P. Scheibler, D. Müller, T. J. Feuerstein, P. Illes, *Br. J. Pharmacol.* **126**, 121 (1999).
28. V. Bartanusz *et al.*, *Neuroscience* **66**, 247 (1995).
29. G. Schumann *et al.*, *Mol. Psychiatry*, in press.
30. Y. P. Tang *et al.*, *Nature* **401**, 63 (1999).
31. M. Sauvage, T. Steckler, unpublished observations.
32. J. M. Koolhaas, S. F. De Boer, A. J. De Ruiter, P. Meerlo, A. Sgoifo, *Acta Physiol. Scand.* **640**, 69 (1997).
33. E. L. Abel, *Physiol. Behav.* **56**, 795 (1994).
34. J. B. Crabbe *et al.*, *Nature Genet.* **14**, 98 (1996).
35. G. Rammes *et al.*, *Neuropharmacology* **40**, 749 (2001).

36. We thank B. Hauger and C. Hilf for excellent technical assistance. Partially supported by grants from the Bundesministerium für Bildung und Forschung 01GS0117 (R.S.) and the Volkswagenstiftung (F.H. and W.W.).

15 January 2002; accepted 26 March 2002