Virus-Assisted Mapping of Neural Inputs to a Feeding Center in the Hypothalamus
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Science 291, 2608 (2001);
DOI: 10.1126/science.1056602

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troops increased with time and eventually reached 25% of the dominant population after 400 days (Fig. 3). In mice inoculated with mutS" ancestors, the proportion of auxotrophs also increased, but only reached a maximum of 5%. Ninety percent of these auxotroph bacteria could be attributed to mutator subpopulations that had spontaneously emerged in some mice (Fig. 3). It appears that during intestinal colonization mutator bacteria lose robustness because of the accumulation of neutral mutations (20) that become deleterious in secondary environments. This may explain why mutator bacteria do not represent a larger fraction of natural isolates (1, 2).

To assess the relative importance of mutator-associated costs and benefits when bacterial experience both primary and secondary environments, we inoculated two mice: one with wild-type, the other one with mutator bacteria. Both were placed in the same cage with a third, initially germ-free, mouse. By summing the populations of mutator and wild-type strains subsequently colonizing the three mice (metapopulation), we monitored the combined effects on population sizes of competition in the gut and migration of bacteria between mice. In contrast to 1:1 competition, the competitive advantage of the mutator allele was not detectable in these metapopulations (Fig. 2D). When the same metapopulation experiment was done with mice inoculated with populations previously grown for 400 days in separate mice, the mutator metapopulation remained lower than the wild type (Fig. 2D), showing the prevailing effect of the costs associated with a mutator allele once the adaptive mutations have been acquired.

The mouse model showed that the advantage of mutator bacteria when colonizing new host is due to their capacity to generate adaptive mutations rapidly, allowing them to exploit the ecosystem resources more quickly than wild-type bacteria. This advantage is reduced to little or nothing once adaptation is achieved. Moreover, if the mutation rate is not reduced [as observed in some subpopulations (Fig. 1A)], it leads progressively to loss of functions that are dispensable in the current environment but compromise the long-term survival of mutator clones. Our experiments also showed that bacterial migration between hosts is a potent factor in reducing the benefits of enhanced mutation rate and should be taken into consideration for understanding the dynamics of mutator bacteria in natural populations. The heterogeneity of environmental conditions might be expected to favor variability in mutation rate, as we observed in some bacterial populations colonizing mice (Fig. 1A). This in vivo study shows that important variations of the mutation rate can happen within weeks. These results may account for the observation that some natural bacterial isolates, such as those of Pseudomonas aeruginosa found in the lungs of cystic fibrosis patients, have a strong mutator phenotype. It may also inspire studies on emerging pathogenicity and drug resistance in microorganisms (2, 4, 21, 22), as well as assisting studies on the somatic evolution of malignant mutator tumor cells (25).

References and Notes
13. Supplemental text describing detailed methods and results, Web figures 1 through 8, and Web table 1 are available at Science Online at www.sciencemag.org/cgi/content/full/291/5513/2606/DC1.
14. Given that rifampicin-resistant mutants are not selected for in the mouse gut, their frequency is an indicator of the overall population mutation rate (13).
15. The mutation rate of a clone from mouse m2 (Fig. 1) transformed with a plasmid carrying the mutS wild-type allele was reduced to its wild-type ancestor level. The mutation rate of a clone from mouse m1 (Fig. 1) was reduced to its wild-type ancestor level when transformed with a plasmid carrying mutS or mutH wild-type alleles. Results of the complementation are available at (13).
16. Switching the antibiotic resistance markers had no effect on competition. Detailed description of the strain construction is available at (13).
18. The fixation of neutral or deleterious mutations in populations with a high mutation rate undergoing strong bottlenecks is known as Muller’s ratchet [H. Muller, Mutat. Res. 1, 2 (1964); F. Funchain, et al., Genetics 154, 959 (2000)]. To model our experimental conditions, the accumulation of neutral mutations in large populations subjected to directional selection was simulated on the basis of the model published by Tenillon et al. (10). Even if the increase in the frequency of selective sweeps in mutant populations depended on the strength of selection, neutral mutations always accumulated 100-fold faster than in nonmutator populations (data not shown).
20. Different types of auxotrophs were isolated both between and within mice, thus suggesting that these mutations were not adaptive. See (13).
24. We are grateful to J. P. Coutanceau, A. M. Criné, and C. Dohet for their technical help; F. Marcille for her constructive discussion; and D. Brégeon, L. Le Chat, V. Colot, M. S. Fox, E. C. Friedberg, B. Godelle, A. Gomez, E. Stewart, and M. Vulic for comments on the manuscript. Funded by the Association de la Recherche contre le Cancer, the Association de la Recherche contre le Cancer, the Programme Environnement et Santé (MATE), the Fondation pour la recherche Médicale, and the Programme de Recherche Fondamentale en Microbiologie et Maladies Infectieuses et Parasitaires (MENRT).
known to receive inputs from other brain regions, the precise nature of these inputs has not been established. Identification of such neural inputs requires that one be able to trace neural connections across one or more synapses.

Pseudorabies virus (PRV) has been used previously to trace neural circuits across multiple synapses (4, 5). After injection of PRV into a number of peripheral sites, including the heart, gastrointestinal tract, and liver, viral antigens can be detected in precisely those brain regions known to innervate these organs. PRV has also been used to trace polysynaptic circuits after direct injection into the central nervous system (CNS) (6). However, because the virus infected many different neurons, no information could be deduced regarding inputs to specific classes of neurons, for example, those expressing a particular neurotransmitter, neuropeptide, or receptor. Here, we report the development of a recombinant PRV that is dependent on a Cre-mediated recombination event for replication and for expression of green fluorescent protein (GFP). This virus was used to map CNS inputs to hypothalamic neurons that play a role in regulating food intake.

The Bartha strain of PRV is an attenuated live vaccine strain that is propagated primarily in a retrograde fashion (opposite to the direction of impulse transmission) along chains of synaptically connected neurons. We constructed a PRV-Bartha strain that was conditional both for replication (in nonmitotic cells) and for expression of GFP.

Fig. 1. Structure and expression properties of PRV strain Ba2001. (A) A recombinant PRV that is dependent on a Cre-mediated recombination event for expression of TK and GFP was created. The parent virus, PRV-Bartha Blue (BaBlu) carries a deletion in the tk gene. The elements of the targeting construct pG2LHTK are shown below the region of recombination into the gG region of Bartha Blue. Flanking gG sequence is indicated by the red stippled boxes; lacZ open reading framed is indicated by the blue stippled box. Successful recombination of the targeting cassette replaces lacZ, resulting in a white plaque. (B) Conditional expression of Tau-GFP in tissue culture cells. PK15 cells or TE-Cre cells, a Cre-expressing cell line, were infected with Ba2001. GFP expression was observed only after infection of the Cre-expressing cells (right). (C) Cre-dependent GFP expression in Syn1-Cre mice. Ba2001 was injected intracranially into wild-type C57BL/6 (left panels, hypothalamus) or C57BL/6 Syn1-Cre transgenic mice (right panels, cortex) (30). Tau-GFP was detected by immunofluorescence from antibody to GFP (top panels), and the expression of viral late protein gC was detected using antibodies to gC (bottom panels) (31). Viral replication and expression of GFP were observed in the Syn1-Cre but not in the wild-type mice. Scale bars: left panel, 200 μm; right panel, 100 μm.

Fig. 2. Specificity of Cre recombination in NPY-expressing cells in vivo. The in vivo requirement of Cre for excision of the Lox-stop cassette was tested in mice that express Cre only in NPY-expressing neurons. A tk virus that was conditional only for GFP was used to infect a transgenic mouse that carried a 150-kb BAC in which Cre had replaced the Npy coding region. (A) Replication-defective virus Ba2000. This virus is identical to Ba2001 except that the expression cassette does not include the tk gene. As a consequence, this virus cannot replicate in nonmitotic cells but still requires Cre for expression of GFP. After infection of Cre-expressing neurons and deletion of the stop cassette, Ba2000 expresses GFP in all neurons, remains replication-defective and is unable to spread to higher order neurons. This strategy allows first-order neurons to be visualized exclusively. (B) A BAC transgenic mouse was developed in which the Npy gene was replaced by Cre. A nuclear localization sequence–Cre open reading frame (NLS-Cre)–polyadenylation sequence [poly(A')] was inserted 5' to the ATG in the first exon of the Npy gene. This BAC includes 30 kb of 5' upstream and 120 kb of downstream sequence and leads to expression of Cre in NPY neurons. (C) GFP expression is limited to NPY-expressing neurons. Ba2000 was injected into the somatosensory 2 cortex of Npy-Cre transgenic mice. Sections were stained for NPY or GFP. An overlay of the NPY and GFP immunofluorescence images (right panel) indicates that GFP expression is only observed in neurons that express NPY (yellow cells). Scale bars, 100 μm.
and requires a Cre-mediated recombination event. This construct was then used to replace the lacZ insert in the TK-BaBlu virus by homologous recombination. The recombinant virus, which we named Ba2001, was initially identified by the loss of β-galactosidase (β-gal) activity and further characterized by Southern blot analysis (11).

Because TK is required for viral DNA synthesis in neurons, Ba2001 would not be expected to replicate in neurons that do not express Cre recombinase (12). Infection of a neuron that expresses Cre would be expected to lead to the removal of the stop signals thereby rendering the virus capable of replicating in other neurons in synaptic contact with the original neuron. These infected neurons should also express GFP. The removal of the stop signals is permanent, allowing the subsequent retrograde infection and labeling of any afferent neurons whether or not they express Cre. Conditional expression of GFP in Ba2001 was confirmed by infecting untransfected PK15 and Cre-expressing TE-Cre fibroblasts (13). GFP fluorescence was never observed in Ba2001-infected cells that did not express Cre but was readily detectable in the infected Cre-expressing cells (Fig. 1B).

Viral replication and GFP expression in vivo were also dependent on Cre-mediated recombination (Fig. 1C). Ba2001 was injected intracranially into wild-type C57BL/6 mice and C57BL/6 transgenic Syn1-Cre mice. Mice from the latter strain express Cre in all neurons because the Cre gene is under the control of a 4.4-kb neuron-specific rat synapsin promoter (14). Five days after infection, significant GFP fluorescence was observed in the cortex of Cre-expressing mice but not in the wild-type mice (Fig. 1C). The GFP-expressing neurons also stained for the gC protein, a late viral protein, expressed only after viral DNA replication (15).

To demonstrate that the stop cassette had been excised in vivo, we injected 2.4 × 10^6 plaque forming units (PFUs) of Ba2001 into the striatum of wild-type and Syn1-Cre mice and killed the animals 7 days later (16). Lysates were prepared from the contralateral striatum of each animal and used to infect PK15 cells in culture. Individual virus plaques were counted and scored for GFP fluorescence. We recovered 75 fluorescent plaques out of a total of 76 plaques per microliter from the lysates of infected Syn1-Cre mice and no fluorescent plaques (0/7) from infected wild-type mice. This indicates that the virus had undergone Cre-mediated recombinant in vivo. Southern blots confirmed that the Lox cassette had been removed in the fluorescent viruses (17).

We next used Ba2001 to trace the connections from neurons in which Cre expression was restricted to neurons that express the neuropeptide Y (Npy) gene. NPY is a 36–amino acid peptide that increases food intake and body weight after intracranial or intrahypothalamic injections (18, 19). NPY is abundantly expressed in neurons in a large number of brain regions including the cortex, medulla, olfactory bulb, and hypothalamus. Hypothalamic neurons that express NPY also coexpress the leptin receptor and are believed to play a key role in regulating feeding behavior (20). In these experiments, we used a bacterial artificial chromosome (BAC) transgenic mouse line that carries a modified 150-kb Npy BAC in which the Cre recombinase gene was inserted by homologous recombination into the ATG of the first exon of the Npy gene (Fig. 2B) (21). In the resulting BAC, 30 kb of 5′ upstream sequence and 90 kb of sequences 3′ to the Npy gene flank a Cre-poly(A−) cassette. To confirm that the recombination event occurred only in NPY-Cre–expressing cells, we constructed a tk− virus conditional only for GFP expression (Fig. 2A). Because

Fig. 3. Ba2001 infection of NPY-Cre mice after injection into the arcuate nucleus. Npy-Cre transgenic mice were killed 4 days after injection of 3.1 × 10^6 PFUs of Ba2001 into the arcuate nucleus. (A) Viral spread within the posterior hypothalamus. GFP was expressed in the following hypothalamic structures as indicated: ARC, arcuate nucleus; DMH, dorsomedial nucleus; LH, lateral hypothalamus; VMH, ventromedial hypothalamus; SOX, supraoptic decussation; 3V, third ventricle. Scale bar, 100 μm. (B) Viral spread to the medial dorsal thalamus (MD). Arrows indicate locations of GFP fluorescent neurons. Scale bar, 25 μm. (C) Viral spread to the dentate gyrus (DG). Scale bar, 25 μm. (D) GFP expression in the piriform cortex (PC) and ventral basolateral amygdala (BLV) 5 days postsinfection. Scale bar, 25 μm.
this virus is defective for DNA replication, the expression of Tau-GFP should be restricted to those neurons that express NPY (and Cre). After cortical injections of the virus into NPY-Cre mice, large numbers of NPY-expressing cell bodies (scored using a specific antibody against NPY) were observed proximal to the injection site (Fig. 2C, left). GFP fluorescence was evident only in a subset of these neurons (Fig. 2C, middle). Superimposition of the NPY antibody and GFP images revealed yellow and red, but not green, cells (Fig. 2C, right). If GFP expression were independent of Cre, green cells would have been observed. These data confirmed that, in these mice, GFP expression was restricted to Cre- and NPY-expressing neurons.

To map neural inputs to NPY-expressing cells that play a role in regulating feeding behavior, we injected Ba2001 into the arcuate nucleus of the NPY-Cre mice stereotaxically. The arcuate nucleus is the hypothalamic site of the NPY-Cre mice stereotaxically. The arcuate nucleus of the NPY-Cre mice and the arcuate nucleus of the ObR-Cre mice and the arcuate nucleus of embryonic stem (ES) cell-derived mice that express Cre from an IRES element inserted into the 3′ untranslated region of the ObR gene. Animals were killed after infection at the times indicated. Brains were sectioned and GFP expression was visualized by immunofluorescence from antibody against GFP (B to D). Low power (10×) and high power (40×) images are shown on the left and right panels, respectively. (A) ObRb-IRES-Cre targeting construct. A cassette containing an IRES-NLS-Cre and neo gene, flanked by frt sites, was inserted immediately 3′ to the stop codon in the last exon of ObRb. This cassette was then used for recombination in ES cells, and positive recombinant ES clones were used to generate the ObRb “knock-in” mouse. (B) Expression of GFP in the hypothalamus 3 days postinfection. At 3 days, GFP was expressed in the arcuate, DMH, LH, and VMH. Higher magnification of the DMH (right panel) revealed a possible connection between two DMH neurons (white arrow). Scale bars: left, 200 μm; right, 50 μm. (C) GFP expression in the limbic and cortical brain regions. GFP expression in the BLA, Pir, and LEnt 5 days postinfection (left). Population of GFP-expressing neurons in the BLA (right). Scale bars: left, 200 μm; right, 25 μm. (D) GFP expression in the retrosplenial cortex 7 days postinfection. GFP-expressing neurons in the granular retrosplenial and agranular cortex. Right panel illustrates cells with the morphology of pyramidal cells (red arrows) and interneurons (white arrow). Scale bars: left, 200 μm; right, 50 μm.
between neurons, a possibility that can be confirmed using electron microscopy (Fig. 4C, right). Labeled neurons were also observed in the agranular and granular retrosplenial cortex (Fig. 4D). In this region, the morphology of the GFP-expressing cells suggested that both pyramidal neurons (red arrows) and interneurons (white arrow) were infected. Labeled neurons in these brain regions are most likely second-order or higher because they appear only at later stages of infection. Fluorescence was restricted to small, discrete numbers of neurons in each of these nuclei and revealed that the infected cells are not contiguous. These data are consistent with previous results indicating that the virus spreads predominately in a transneuronal fashion rather than laterally (25).

In addition to the sites shown in Fig. 4, labeling was also observed in several other sites, particularly at later times postinfection (Table 1). Five days after infection, labeling was observed in the perihinial, entorhinal, and somatosensory cortex 2. At 7 days, GFP-labeled neurons were visible in the somatosensory 1 and retrosplenial cortex, as well as in the CA1 region of the hippocampus and brainstem nuclei. Although neurons that were first observed at 5 days or later are most likely third-order or higher, further analyses will be necessary to determine the precise number of synapses between these sites and the hypothalamic nuclei.

Table 1. Summary of Ba2001 infections in NPY-Cre and ObRb-Cre mice. Data from all of the experiments were compiled and the CNS sites that expressed GFP after infections by Ba2001 are shown. Column 1 lists the anatomic sites where GFP expression was observed in the infected NPY-Cre and/or ObRb-Cre mice. Column 2 shows the time postinfection, in days, when GFP labeling was first observed. Columns 3 and 4 show results from Ba2001 infection time course in NPY-Cre transgenic mice. Columns 5 and 6 show results from Ba2001 infection time course in ObRb-IRECre mice. Frequency, the fraction of mice out of the total that expressed GFP at that site. ND, not done. Intensity rating, ++ indicates uniform site labeling intensity among all mice examined; +++, site labeled in some but not all mice; --, no labeling observed in any mice.

- **Arcuate nucleus**
  - 1.5 days
  - Time postinfection: 1.5 days
  - Frequency: 2/2
  - Intensity: ++
  - NPY-Cre: 4/4
  - Intensity: ++
  - ObRb-IRECre: 3/5
  - Intensity: ++

- **Ventromedial hypothalamus**
  - 3 days
  - Frequency: 3/3
  - Intensity: +++
  - NPY-Cre: 5/5
  - Intensity: +++
  - ObRb-IRECre: 5/5
  - Intensity: +++

- **Lateral hypothalamus**
  - 3 days
  - Frequency: 3/3
  - Intensity: ++
  - NPY-Cre: 5/5
  - Intensity: ++
  - ObRb-IRECre: 5/5
  - Intensity: ++

- **Paraventricular nucleus**
  - 2/3
  - Frequency: 0/2
  - Intensity: +
  - NPY-Cre: 3/5
  - Intensity: +
  - ObRb-IRECre: 3/5
  - Intensity: +

- **Amygdala**
  - 2/2
  - Frequency: 1/2
  - Intensity: +
  - NPY-Cre: 4/4
  - Intensity: +
  - ObRb-IRECre: 4/4
  - Intensity: +

- **Bed of stria terminalis**
  - 1/2 (5 days)
  - Frequency: 1/2
  - Intensity: +
  - NPY-Cre: 0/4
  - Intensity: --
  - ObRb-IRECre: 0/4
  - Intensity: --

- **Thalamus**
  - 4 days
  - Frequency: 1/2
  - Intensity: +
  - NPY-Cre: 5/5
  - Intensity: +
  - ObRb-IRECre: 5/5
  - Intensity: +

- **Lateral entorhinal cortex**
  - 5 days
  - Frequency: ND
  - Intensity: +
  - NPY-Cre: 5/5
  - Intensity: +
  - ObRb-IRECre: 5/5
  - Intensity: +

- **Perihinal cortex**
  - 5 days
  - Frequency: ND
  - Intensity: +
  - NPY-Cre: 5/5
  - Intensity: +
  - ObRb-IRECre: 5/5
  - Intensity: +

- **Somatosensory cortex 2**
  - 5 days
  - Frequency: ND
  - Intensity: +
  - NPY-Cre: 5/5
  - Intensity: +
  - ObRb-IRECre: 5/5
  - Intensity: +

- **Mesencephalic trigeminal nucleus**
  - 5 days
  - Frequency: ND
  - Intensity: +
  - NPY-Cre: 5/5
  - Intensity: +
  - ObRb-IRECre: 5/5
  - Intensity: +

- **Somatosensory cortex 1**
  - 7 days
  - Frequency: 0/2
  - Intensity: +
  - NPY-Cre: 3/3
  - Intensity: +
  - ObRb-IRECre: 3/3
  - Intensity: +

- **Retrospinal cortex**
  - 7 days
  - Frequency: 0/2
  - Intensity: +
  - NPY-Cre: 1/3
  - Intensity: +
  - ObRb-IRECre: 1/3
  - Intensity: +

- **CA1/hippocampus**
  - 7 days
  - Frequency: 1/2 (4 days)
  - Intensity: +
  - NPY-Cre: 1/3
  - Intensity: +
  - ObRb-IRECre: 1/3
  - Intensity: +

- **Lat. paragigantocellular nucleus**
  - 7 days
  - Frequency: 0/2
  - Intensity: +
  - NPY-Cre: 1/3
  - Intensity: +
  - ObRb-IRECre: 1/3
  - Intensity: +

- **Raphe magnus**
  - 7 days
  - Frequency: 0/2
  - Intensity: +
  - NPY-Cre: 1/3
  - Intensity: +
  - ObRb-IRECre: 1/3
  - Intensity: +
analyzed tissue samples from brain. Brain tissue was minced, transferred to a 1.5-mL microcentrifuge tube, and washed twice with sterile phosphate-buffered saline (PBS). Tissue chunks were resuspended in 1.0 mL Dulbecco’s MM with 2% serum. The tissue was then homogenized and subjected to three freeze-thaw cycles. Particulate matter was pelleted and the supernatant was used for plaque assays.

17. J. DeFalco, unpublished observations.
21. BAC clones containing the Npy gene were identified by hybridization to a mouse BAC library filter array using a mouse Npy cDNA fragment. A pSV1 shuttle vector subclone containing pSV1-Npy was degenerate generated to give pSV1-Npy. Using PCR, we mutated the first ATG to AT T and generated the putative AT G PCR. The CAT gene was then transplanted into the first ATG to AT T and introduced the Pac I site of pSV1-Npy. Recombination into the BAC clone was performed as in X. W. Yang et al. [Nature Biotechnol. 15, 859 (1997)].
30. Intracerebral virus injections were performed as in J. P. Card et al. [J. Comp. Neurol. 407, 438 (1999)] with two exceptions: a 32-gauge cannula was used to minimize tissue trauma and fluorescent microspheres (Molecular Probes, Eugene, OR) were co-injected to mark injection site. Stratal injection coordinates were +0.38 mm bregma, 1.5 mm lateral of sagittal suture, and −3.5 mm dorsal-ventral. For cortex injections, coordinates were −1.2 mm bregma, 3.65 mm lateral and −2.65 dorsal-ventral. Coordinates for stratum injections were −1.9 mm bregma, 0.15 mm lateral, and −5.5 mm dorsal-ventral. A total of 100 nl of virus was injected at a rate of 10 nl/min. The needle was removed after 10 min.
31. Animals were killed by pentobarbital overdose and periodically thaw cycles. Particulate matter was pelleted and the supernatant was used for plaque assays. To study the effect of ACC on fatty acid oxidation and synthesis, we generated ACC2−/− mice. ACC2−/− mice had 10- and 30-fold lower levels of malonyl-CoA in heart and muscle, respectively. The fatty acid oxidation rate in the soleus muscle of the ACC2−/− mice was 30% higher than that of wild-type mice and was not affected by addition of insulin; however, addition of insulin to the wild-type muscle increased fatty acid oxidation by 45%. The mutant mice accumulated 50% less fat in their adipose tissue than did wild-type mice. These results raise the possibility that pharmacological manipulation of ACC2 may lead to loss of body fat in the context of normal caloric intake.

Malonyl-coenzyme A (malonyl-CoA), generated by acetyl-CoA carboxylases ACC1 and ACC2, is a key metabolite in the regulation of energy homeostasis. Here, we show that ACC2−/− mutant mice have a normal life span, a higher fatty acid oxidation rate, and lower amounts of fat. In comparison to the wild type, ACC2-deficient mice had 10- and 30-fold lower levels of malonyl-CoA in heart and muscle, respectively. The fatty acid oxidation rate in the soleus muscle of the ACC2−/− mice was 30% higher than that of wild-type mice and was not affected by addition of insulin; however, addition of insulin to the wild-type muscle increased fatty acid oxidation by 45%. The mutant mice accumulated 50% less fat in their adipose tissue than did wild-type mice. These results raise the possibility that pharmacological manipulation of ACC2 may lead to loss of body fat in the context of normal caloric intake.

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