Autophosphorylation at Thr^{286} of the α Calcium-Calmodulin Kinase II in LTP and Learning

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The calcium-calmodulin–dependent kinase II (CaMKII) is required for hippocampal long-term potentiation (LTP) and spatial learning. In addition to its calcium-calmodulin (CaM)–dependent activity, CaMKII can undergo autophosphorylation, resulting in CaM-independent activity. A point mutation was introduced into the αCaMKII gene that blocked the autophosphorylation of threonine at position 286 (Thr^{286}) of this kinase without affecting its CaM-dependent activity. The mutant mice had no N-methyl-D-aspartate receptor–dependent LTP in the hippocampal CA1 area and showed no spatial learning in the Morris water maze. Thus, the autophosphorylation of αCaMKII at Thr^{286} appears to be required for LTP and learning.

Long-lasting changes in synaptic strength (such as LTP) are thought to underlie learning and memory (1). Pharmacological and genetic lesions of CaMKII impair LTP and learning (2–4). Additionally, increasing the concentrations of constitutively active CaMKII affects LTP and learning (5, 6). A model has been proposed that suggests that the autophosphorylated CaM-independent (constitutively active) state of CaMKII is crucial for LTP and learning (7). Autophosphorylation at Thr^{286} endows αCaMKII with the ability to switch from a CaM-dependent to a CaM-independent state (8). Consistent with the model, LTP induction triggers a long-lasting increase in the autophosphorylated form of CaMKII (9, 10) and in its CaM-independent activity (11). These studies, however, do not demonstrate that the autophosphorylation of CaMKII is required for either LTP or learning.

To determine whether the autophosphorylation of αCaMKII at Thr^{286} is required for LTP and learning, we substituted Thr^{286} (T) for alanine (A) (T286A). The T286A mutation results in a kinase that is unable to switch to its CaM-independent state (8). We used a gene-targeting strategy that utilizes a replacement vector containing the point mutation and a *neo* gene flanked by loxP sites (the Pointlox procedure) (Fig. 1, A and B) (12). All of the homozygous mutants analyzed were F2 mice from a cross between the chimeras (contributing 129 background) and C57BL/6 mice (αCaMKII$^{T286A-129B6F2}$). Immunoblotting and immunocytochemical analyses (Fig. 1, C to E) determined that the point mutations and the loxP site did not alter the expression of the αCaMKII

gene (13). We confirmed that the αCaMKII$^{T286A-129B6F2}$ mutation decreased the total CaM-independent CaMKII activity in the mutants but did not affect their CaM-dependent activity (14). The residual CaM-independent activity in the mutants was presumably due to βCaMKII (13, 15).

Long-term potentiation was tested in the αCaMKII$^{T286A-129B6F2}$ mutants with extracellular field recordings in the stratum

radiatum of hippocampal slices (16). We focused our studies on the CA1 region because this region is important for learning (17). Long-term potentiation induced with a 100-Hz tetanus (1 s) was deficient in the αCaMKII$^{T286A-129B6F2}$ mutants (Fig. 2A). Sixty minutes after the tetanus, the mutants (seven mice, seven slices) showed 110.8 ± 6.2% potentiation, whereas wild-type mice (10 mice, 10 slices) showed 153.5 ± 7.5% potentiation. There was no overlap in the extent of potentiations in wild-type and mutant slices (Fig. 2B). We also determined that other stimulation protocols revealed similar LTP impairments in the αCaMKII$^{T286A-129B6F2}$ mutants (Fig. 2C). These LTP impairments were not caused by prepotentiation of synaptic transmission, because the relation between evoked fiber volleys and field excitatory postsynaptic potentials (fEPSPs) was indistinguishable between mutant (nine mice, nine slices) and wild-type mice (nine mice, nine slices) (Fig. 2D). This result also suggests that the αCaMKII$^{T286A-129B6F2}$ mutation did not affect synaptic connectivity in the CA1 region. Synaptic responses collected during the 10-Hz tetanus were similar in mutant and wild-type mice (18), indicating that the LTP deficit of the mutants was not due to decreased synaptic transmission during tetanic stimulation.
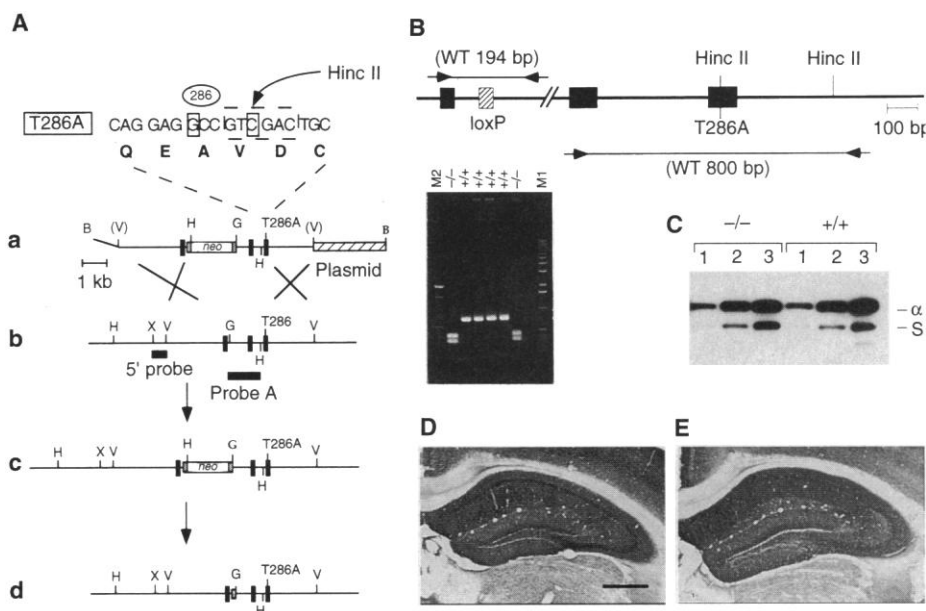

Fig. 1. Generation of the αCaMKII$^{T286A-129B6F2}$ mutants with the Pointlox procedure. (**A**) The targeting construct (a), a partial map of the αCaMKII gene (b), the resulting targeted allele (c), and the targeted allele after Cre recombination (d) are illustrated (11). B, Bam HI; G, Bgl II; H, Hind III; V, Pvu II; X, Xba I. (**B**) A first PCR detected the loxP site (12) determining the genotype. A second PCR was used to identify the point mutations (12). The gel shows the Hinc II–digested PCR products from homozygotes (−/−) and wild-type (+/+) mice. M1 and M2 refer to molecular weight marker lanes. (**C**) Immunoblot analysis (13) indicated normal expression of αCaMKII (α) and synaptophysin (S) in the mutants. Lane 1, 2.5 μg of protein; lane 2, 5 μg of protein; and lane 3, 10 μg of protein. (**D** and **E**) Immunocytochemistry of adult coronal hippocampal sections (13) showed expression of αCaMKII in the somata and dendrites of mutants (E) and wild-type mice (D). Calibration bar, 0.5 mm.

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Fig. 2. Long-term potentiation impairments in the αCaMKII[T286A-129B6F2] mutant mice. (**A**) Long-term potentiation induced by a 100-Hz (1 s) tetanus (16) was impaired in the mutants. Representative traces before and 30 min after the tetanus are shown for mutant (T286A) (■) and wild-type (WT) (O) mice. Calibration bars, 1 mV, 10 ms. The arrow indicates when the tetanus was delivered. (**B**) A cumulative histogram for the 100-Hz tetanus-induced LTP (measured 30 min after the tetanus) is presented for mutant (dashed line) and wild-type (solid line) mice. (**C**) A variety of protocols detected the LTP impairment of the mutants 30 min after tetanization: 2 theta bursts (2TB) (five wild-type mice, six slices and six mutant mice, six slices), 10-Hz tetanus for 10 s (10Hz) (five wild-type mice, five slices and seven mutant mice, seven slices); and 100-Hz tetanus for 1 s (100Hz). In contrast, for other protocols, no difference was detected: 1-Hz tetanus for 100 s (1Hz) or for 900 s (34) and 100-Hz tetanus for 1 s in the presence of D-AP5 (AP5). (**D**) The plot indicates normal basal synaptic transmission in the mutants.
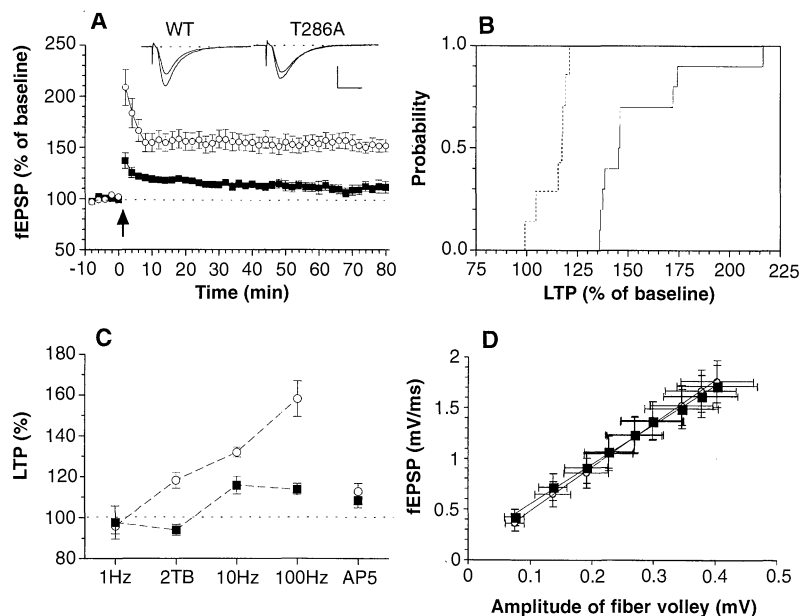
Because it has been proposed that αCaMKII regulates the frequency-response function of hippocampal synapses (6), we also investigated field synaptic responses after low-frequency stimulation (1 Hz for 100 s or 900 s). This stimulation,

however, did not induce a stable synaptic depression in either αCaMKII[T286A-129B6F2] mutants or wild-type controls (Fig. 2C).

We confirmed the LTP deficits of the αCaMKII[T286A-129B6F2] mutants with whole-cell recordings in CA1 neurons (Fig. 3, A

and B) (19). Long-term potentiation was induced with a pairing protocol: Postsynaptic depolarization (up to +10 mV), sufficient to reverse the excitatory postsynaptic currents (EPSCs), was paired with synaptic stimulation (2 Hz for 50 s) (Fig. 3A). At 3.5 to 8.5 min after this pairing protocol, robust LTP was obtained in wild-type mice (277 ± 21%, 16 neurons; nine mice). As observed with field recordings, a much smaller potentiation was observed in mutant mice (132 ± 8%, 17 neurons; nine mice) (Fig. 3A). There was no overlap in the extent of potentiations in wild-type and mutant slices (Fig. 3B). Because γ-aminobutyric acid$_A$ (GABA$_A$) receptors were blocked with picrotoxin (PTX) during these recordings, the LTP impairments observed are not due to abnormalities in inhibition (20).

To investigate whether N-methyl-D-aspartate receptor (NMDAR)–dependent LTP was absent in the αCaMKII[T286A-129B6F2] mutants, we used field recordings to compare LTP (100 Hz, 1 s) induced in the presence or absence of D-2-amino-5-phosphonopentanoate (D-AP5) (50 μM), an NMDAR antagonist (Fig. 2C). In the presence of D-AP5, LTP was severely decreased in wild-type hippocampal slices (30 min after the tetanus: 112.8 ± 3.9%; seven mice, seven slices) compared with control slices (30 min after the tetanus: 158.1 ± 8.7%; 10 mice, 10 slices). In contrast, the potentiation in mutant slices was D-AP5–insensitive (30 min after the tetanus; without D-AP5: 113.9 ± 2.8%; seven mice, seven slices; with D-AP5: 108.4 ± 3.5%; six mice, six slices) (21). Two seconds after a 2 theta burst tetanus (21), the potentiation in mutants (108.8 ± 2.6%; six mice, 11 slices) was indistinguishable from that in wild-type mice (113.7 ± 2.0%; 12
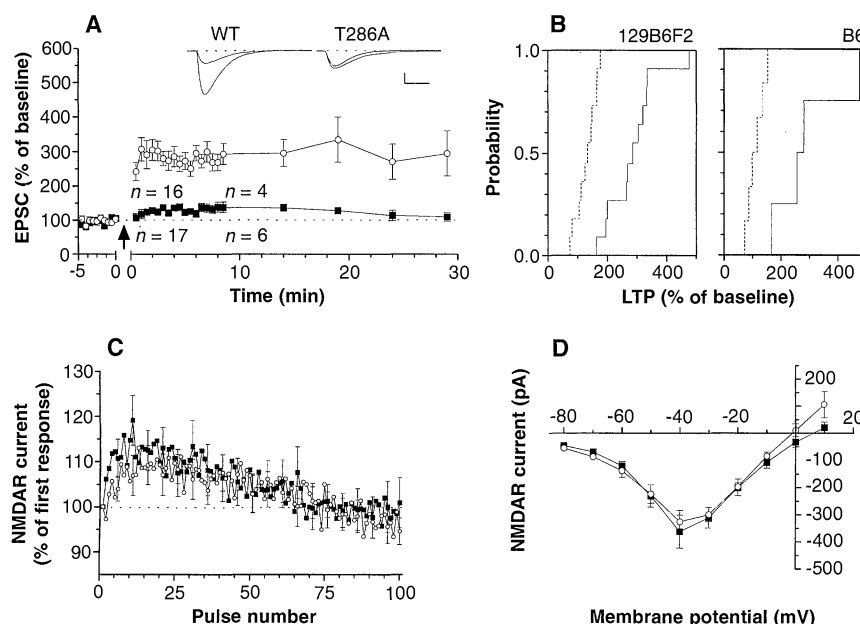

Fig. 3. Pairing-induced LTP deficits in the αCaMKII[T286A-129B6F2] mutant mice. (**A**) Long-term potentiation was induced by pairing postsynaptic depolarization with low-frequency stimulation (19) (arrow). Averaged traces (n = 10) from a representative experiment before and 6 to 8 min after pairing are shown for mutant (T286A) (■) and wild-type (WT) (O) mice. In an independent experiment, the potentiation of six mutant (having four crosses into C57BL/6) and four wild-type neurons was recorded up to 30 min after LTP induction. Because both LTP experiments gave the same results, the data were combined. Calibration bars, 50 pA, 10 ms. (**B**) A cumulative histogram for the pairing-induced LTP in the 129B6F2 background (left) (measured 200 to 500 s after pairing) and in the B6 background (right) (four crosses into C57BL/6, measured 25 to 30 min after pairing) is presented for mutant (dashed line) and wild-type (solid line) mice. (**C**) The average amplitude of NMDAR currents is plotted for each pulse in the pairing protocol. (**D**) The current-voltage relation of NMDAR currents was indistinguishable between mutant and wild-type mice.

mice, 15 slices), and this early potentiation was not NMDAR-dependent (wild type with D-AP5: 120.0 ± 1.2%; four mice, eight slices).

The autophosphorylation of αCaMKII at Thr^{286} leads to trapping of CaM (22), a molecule that can reduce the opening probability of NMDARs (23). Consequently, in the αCaMKII$^{T286A-129B6F2}$ mutants, an abnormal reduction of NMDAR currents by CaM could block the induction of LTP. With whole-cell recordings in the presence of PTX (100 μM) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (25 μM), we analyzed NMDAR function during the pairing protocol previously used to induce LTP (Fig. 3, C and D). The amplitude of NMDAR currents was normal in the mutants (eight neurons from five mutant mice and nine neurons from five wild-type mice) (Fig. 3C). Additionally, the voltage dependence of the NMDAR currents was also normal in the mutants (nine neurons from five mutant mice and seven neurons from five wild-type mice) (Fig. 3D) (24). Thus, the LTP impairments in the αCaMKII$^{T286A-129B6F2}$ mutants were not due to abnormal NMDAR function.

To test whether the autophosphorylation of αCaMKII was required for spatial learning, we tested the mutants ($n = 10$) and wild-type mice ($n = 11$) in the hidden-platform version of the Morris water maze (25), a hippocampus-dependent task (26).

Although the mutants needed more time than wild-type mice did to locate the platform, during the first three training blocks, they were normal (Fig. 4A). Thus, the spatial learning impairments of the mutants were not due to initial performance deficits. During a transfer test given at the end of training, the wild-type mice searched selectively for the platform, whereas the mutant mice did not (Fig. 4, B and C).

Nine αCaMKII$^{T286A-129B6F2}$ mutants and ten wild-type mice were also tested in the visible-platform version of the Morris water maze (25), a hippocampus-independent task (Fig. 4D) (26). Despite an impairment in blocks 2 and 3, the mutants were able to learn this task (Fig. 4D), indicating that they had the vision, motivation, and motor coordination required for learning in the water maze. A hidden-platform test after this task confirmed the spatial learning impairments of these mutants (25) (Fig. 4, E to G).

Here, we have provided direct evidence that the autophosphorylation of αCaMKII at Thr^{286} is required for NMDAR-dependent LTP in the hippocampal CA1 region. Like the αCaMKII^{null} mutants (3, 4), the αCaMKII$^{T286A-129B6F2}$ mice showed impaired NMDAR-dependent LTP and spatial learning. The observed LTP deficits in the αCaMKII$^{T286A-129B6F2}$ mutants were not due to abnormalities in GABA$_A$ inhibition, NMDAR currents, or synaptic function before and during the tetanus, suggesting that the deficits act downstream of Ca^{2+} influx through NMDARs. These

findings are consistent with the observation that, after LTP induction, the autophosphorylated form of αCaMKII phosphorylates glutamate receptor subunits that may be required for LTP (9). In accordance with a model implicating the CaM-independent state of CaMKII in learning and memory (7), we showed that the autophosphorylation of αCaMKII at Thr^{286} is required for spatial learning. Thus, the autophosphorylation of αCaMKII at Thr^{286} is crucial for hippocampal LTP and hippocampus-dependent learning.

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12. Two point mutations (ACCGTGGAC was changed to GCCGTCGAC) were introduced by polymerase chain reaction (PCR) mutagenesis into the 866–base pair Hind III fragment containing the exon encoding Thr^{286} (27). The wild-type Hind III fragment of the 6.1-kb Pvu II genomic clone (4) was substituted by the mutagenized Hind III fragment. A PGKneo cassette flanked by loxP sites was inserted into the Bgl II site. After transfection of R1 embryonic stem cells (28) and selection with G418, targeted clones were identified by Southern (DNA) blot analyses. Because the PGK-neo cassette could interfere with the expression of neighboring genes (29), it was removed by transient transfection with pBS185 (30), a plasmid containing Cre recombinase DNA. Chimeras, generated by injection of proper cells into blastocysts, were mated with C57BL/6J mice, and crosses of F1 heterozygotes yielded a Mendelian distribution of F2 offspring. The mutants were identified by PCR (primers: 5'-CTG-TACCAGCAGATCAAAGC-3' and 5'-ATCACTAG-CACCATGTGGTC-3'). Polymerase chain reaction (primers: 5'-GATGCTGACCATCAACCCAT-3' and

Fig. 4. Spatial learning deficits of the αCaMKII$^{T286A-129B6F2}$ mutants. (A) The mutants were impaired in locating a hidden platform (25) ($P < 0.001$), but they were normal during the first three training blocks ($P > 0.30$); $\bigcirc$, wild-type mice; $\blacksquare$, mutants. (B) During a transfer test after training, the mutants did not search selectively in the target quadrant (TQ) ($P > 0.75$) and showed no swimming speed deficits ($P = 0.21$). AR, AL, and OP refer to the platform sites adjacent right, adjacent left, and opposite to the training site, respectively. Open bars, wild-type mice; solid bars, mutants. (C) The mutants also did not selectively cross the TQ site ($P > 0.75$). (D) The mutants were only impaired during blocks 2 and 3 in locating a visible platform in a fixed position (25) ($P < 0.05$ for both). (E) The visible platform was replaced by a hidden platform (25), and the mutants were impaired during training ($P < 0.01$). (F) The mutants did not search selectively in a transfer test after training ($P > 0.25$). (G) The mutants also did not preferentially cross the training site ($P > 0.75$). Wild-type mice searched selectively in all transfer test measurements (B, C, F, and G).
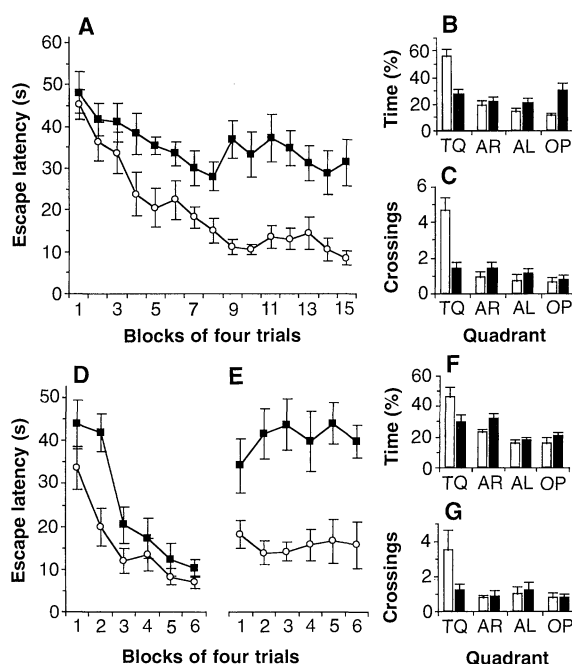
5'-CCCATTGTGACTCTACACCT-3') followed by a Hinc II restriction indicated the presence of the point mutations in the homozygous mutants.

13. The immunoblot analysis was done with protein from adult brain with monoclonal antibodies to αCaMKII and synaptophysin (Boehringer). The secondary antibody was labeled with ^{125}I. Blots analyzed with an antibody to βCaMKII (Zymed Laboratories) showed that βCaMKII is expressed at normal amounts in adult brain of the αCaMKII$^{T286A-129B6F2}$ mutants. For the immunocytochemical analysis, coronal sections of the adult brain were incubated with the monoclonal antibody to αCaMKII. Antigen-antibody complexes were visualized with the glucose oxidase–diaminobenzidine-nickel method (31).

14. The kinase activity was determined from hippocampal slices with autocamtide-3 as substrate as previously described (11, 32). For wild-type mice (n = 3), the total activity was 5.07 ± 0.91 pmol^{-1} µg^{-1} min, and the CaM-independent activity was 1.09 ± 0.12 pmol^{-1} µg^{-1} min ($22.1 \pm 1.8\%$). For the homozygous mutants (n = 3), the total activity was 4.74 ± 1.12 pmol^{-1} µg^{-1} min, and the CaM-independent activity was 0.40 ± 0.06 pmol^{-1} µg^{-1} min ($9.2 \pm 1.9\%$).

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16. Transverse hippocampal slices (400 µm) from 5- to 10-month-old mice were placed in a submerged recording chamber perfused continuously with artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2 at 31°C. Extracellular fEPSPs were recorded with an electrode filled with ACSF in CA1 stratum radiatum, and the Schaffer collaterals were stimulated. A second pathway was used to control the stability of the recordings. The stimulus duration was 100 µs. The ACSF contained 120 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl$_2$, 1.3 mM MgSO$_4$, 1.25 mM NaH$_2$PO$_4$, 26 mM NaHCO$_3$, and 10 mM D-glucose.

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18. Synaptic transmission during the 10-Hz tetanus undergoes biphasic (increase minus decrease) changes. For wild-type mice (n = 6), the maximal increase was $140.3 \pm 3.2\%$, and for mutants, it was $146.7 \pm 4.4\%$ (n = 5). The maximal decrease (at the end of the tetanus) was $79.2 \pm 6.9\%$ for wild-type mice and $91.6 \pm 8.9\%$ for mutants.

19. Excitatory postsynaptic currents were recorded from CA1 pyramidal neurons from 6- to 12-month-old mice with a patch electrode (7 to 10 Mohm) in the whole-cell voltage-clamp mode. The pipette solution contained 122.5 mM cesium methanosulphonate, 17.5 mM CsCl, 10 mM Hepes buffer, 0.1 mM EGTA, 8 mM NaCl, 2 mM Mg–adenosine triphosphate, and 0.3 mM Na$_3$–guanosine triphosphate (pH 7.25, 290 to 300 mosm). A second pathway was used to control for the stability of the recordings. Picrotoxin (100 µM) was present in all experiments.

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21. Similar results were obtained 10 s after a 2 theta burst (two high-frequency bursts of four stimuli at 100 Hz, with 200 ms separating the onset of each burst) tetanus (wild-type mice without D-AP5: $131.3 \pm 5.6\%$; six mice, 12 slices; wild-type mice with D-AP5: $106.3 \pm 2.0\%$; four mice, eight slices; mutants without D-AP5: $112.4 \pm 3.3\%$; five mice, five slices).

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24. Extracellular field recordings (in the presence of CNQX and PTX, with a stimulation strength of 50 µA) indicated that the NMDAR potentials did not differ between mutant (0.159 ± 0.053 mV; three mice, four slices) and wild-type mice (0.200 ± 0.020 mV; three mice, five slices).

25. Two- to five-month-old mice were studied in the spatial version of the water maze for 5 days (12 trials per day; blocks of four trials), as previously described (33). Transfer tests were given at the end of days 3 and 5. In another experiment, the mice were tested for 2 days with a visible platform in a fixed location (12 trials per day); then the visible platform was replaced by a hidden platform, and the mice were tested for another 2 days (12 trials per day). A transfer test was given at the end of training. Data were studied with one-way and two-way analysis of variance.

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Conjugative Transfer by the Virulence System of *Legionella pneumophila*

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Legionella pneumophila, the causative agent of Legionnaires' pneumonia, replicates within alveolar macrophages by preventing phagosome-lysosome fusion. Here, a large number of mutants called *dot* (defective for organelle trafficking) that were unable to replicate intracellularly because of an inability of the bacteria to alter the endocytic pathway of macrophages were isolated. The *dot* virulence genes encoded a large putative membrane complex that functioned as a secretion system that was able to transfer plasmid DNA from one cell to another.

A number of intracellular bacterial pathogens, such as *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and *Legionella pneumophila*, grow within membrane-bound compartments diverted from the normal endocytic pathway of host cells (1). *Legionella pneumophila* replicates within alveolar macrophages by preventing acidification of the nascent phagosome and subsequent fusion with lysosomes (2). Several *L. pneumophila* genes (*dotA* and *icmWXYZ*) that are required for growth in macrophages have been identified (3). Mutations in these genes allow bacteria to be internalized into compartments that fuse with lysosomal components (3, 4).

To understand how this organism prevents phagosome-lysosome fusion, we isolated a large collection of additional mutants that were defective for intracellular growth. Twenty-six spontaneous mutants were isolated on the basis of the observation that *L. pneumophila* strains resistant to low amounts of sodium chloride are often unable to replicate in macrophages (5, 6). We independently isolated six additional mutants by screening mutagenized *L. pneumophila* for a lack of intracellular growth (7). Complementation of these mutants revealed two 20-kb regions on the *L. pneumophila* chromosome that contain a large number of genes required for growth in macrophages (Fig. 1). Region I contains three genes, *dotDCB*, located about 10 kb from the previously identified *dotA-icmWXYZ* locus. Region II contains 11 genes in three potential operons (*dotML*, *dotKJIHGFE*, and *dotNO*). The majority of the *dot* and *icm* genes identified to date, 14 of 19, are predicted to encode proteins that are membrane-associated. Although most of these proteins are not homologous to any known open reading frames (ORFs), four Dot proteins have limited homology to components of bacterial conjugation systems (Fig. 1). The COOH-terminus of DotG is homologous to Trb I, a protein required for conjugation of the IncP plasmid RP4 (23% identity over the COOH-terminal 442 amino acids of DotG) (Fig. 1) (8). DotM and DotL have homology to TrbA and TrbC, respectively, from the Inc I plasmid R64 (23% identity for DotM and TrbA and 26% identity for DotL and TrbC)

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