MBP (25 μM) and assayed for MAP kinase activity or mutant catalytically inactive recombinant $p42^{mapk}$ (0.5 μg) was added and examined for in vitro phosphorylation. After a 20-min incubation at 37°C in the presence of [γ-^{32}P]ATP, the phosphorylated catalytically inactive $p42^{mapk}$ was immunoprecipitated for 2 hours at 4°C. The sedimented material was extensively washed, and the proteins were resolved by SDS-PAGE (10% gel), transferred to Immobilon-P, and detected by autoradiography or a phosphorimager.

18. Samples were analyzed for $p42^{mapk}$ and $p44^{mapk}$ by SDS-PAGE and by protein immunoblotting. The blots were probed with a monoclonal antibody to $p42^{mapk}$ or $p44^{mapk}$, a rabbit antibody to mouse IgG, and ^{125}I-labeled goat antibodies to rabbit F(ab)$_2$. Signals were detected and quantitated with a phosphorimager.
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Effects of cAMP Simulate a Late Stage of LTP in Hippocampal CA1 Neurons

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Hippocampal long-term potentiation (LTP) is thought to serve as an elementary mechanism for the establishment of certain forms of explicit memory in the mammalian brain. As is the case with behavioral memory, LTP in the CA1 region has stages: a short-term early potentiation lasting 1 to 3 hours, which is independent of protein synthesis, precedes a later, longer lasting stage (L-LTP), which requires protein synthesis. Inhibitors of cyclic adenosine monophosphate (cAMP)–dependent protein kinase (PKA) blocked L-LTP, and analogs of cAMP induced a potentiation that blocked naturally induced L-LTP. The action of the cAMP analog was blocked by inhibitors of protein synthesis. Thus, activation of PKA may be a component of the mechanism that generates L-LTP.

Behavioral and cellular studies of learning and memory in both invertebrates and vertebrates indicate that there are stages in memory storage. Long-term memory, lasting days or even weeks, can be distinguished from short-term memory, lasting minutes (or hours), with inhibitors of protein synthesis (*1–3*). Although LTP in the hippocampus is thought to be a candidate mechanism for explicit forms of memory (*4*), most cellular analyses of LTP have been done within hippocampal slices that typically survive only 1 to 2 hours. As a result, these analyses have focused only on an early phase of LTP. Hippocampal LTP in the CA1 region also has a later phase (*5–7*). The early phase (E-LTP) is initiated typically with a single train of high-frequency stimuli, starts immediately after the post-tetanic potentiation induced by the tetanus, and lasts about 1 to 3 hours. The late phase typically requires three or more trains of tetanic stimuli separated by 10 min, begins only slowly after the first 1 to 3 hours, and lasts for at least 10 hours. Only the late component of LTP (L-LTP) requires protein synthesis (*5, 6, 8*).

E-LTP is induced by activation of the *N*-methyl-D-aspartate (NMDA) receptor, which leads to Ca^{2+} influx, and requires the activation of serine-threonine and tyrosine protein kinases (*9–11*). Little is known about the second messengers necessary for the induction and maintenance of L-LTP. The only clues to a second messenger potentially important for L-LTP come from the finding that L-LTP is blocked by antagonists of dopamine receptors (*12, 13*), particularly by blockers of the D_1 type of dopamine receptors (*14*).

Because the D_1 receptor stimulates adenylyl cyclase, we explored the possibility that L-LTP is dependent on the cAMP-dependent protein kinase (PKA). We examined the effects on L-LTP of inhibiting PKA with Rp–cyclic adenosine 3′,5′-monophosphorothioate (Rp-cAMPS) (100 μM), a membrane-permeable cAMP analog and a competitive inhibitor of PKA. Application of Rp-cAMPS for 15 min before inducing LTP had no significant effect on normal synaptic transmission during the first 3 hours and only a small effect on E-LTP at 30 to 60 min, but the inhibitor completely blocked the late phase of LTP as evident in both the field excitatory postsynaptic potential (EPSP) and in the popula-

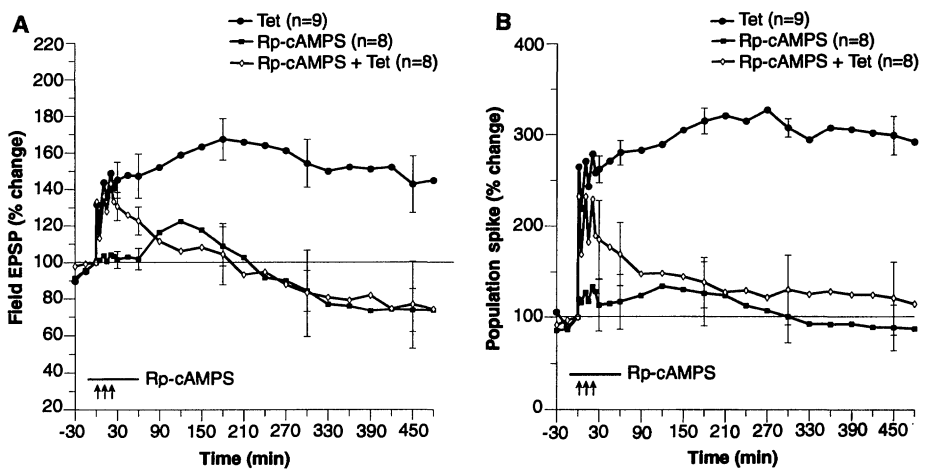

Fig. 1. Effect of Rp-cAMPS (100 μM) on LTP in hippocampal region CA1. (**A**) Time course of changes in the field EPSP measured as the slope function (SF). (**B**) Percentage change of the population spike amplitude (PS). Rp-cAMPS was applied 15 min before LTP induction and the drug was washed out 60 min after the first tetanization. LTP was induced (arrows) by giving three tetanic trains (Tet) (each train was 100 Hz for 1 s; 10-min intervals between trains)—a stimulation pattern that initiates stable L-LTP for at least 8 hours. The group treated with Rp-cAMPS was statistically significant different from control LTP at 45 min (PS) and 1.5 hours (SF) after LTP induction ($P < 0.05$). Hippocampal slices from 7-week-old male Sprague-Dawley rats were prepared for conventional electrophysiological recordings as described (*25*). Instead of a modified Krebs-Ringer solution, a medium was prepared containing 50% basal Eagle's medium (BME), 25% Hanks' balanced salt solution (HBSS), 25% horse serum, and glucose (6.5 mg/ml) (Specialty Media Inc. or Gibco). Under these conditions, the slices survived for at least 3 weeks in an O_2 incubator at 37°C. The superfused slices were incubated in a static bath (2.5 ml). The bath volume was exchanged every 2 to 3 hours with 7.5 to 10 ml of fresh medium. After incubation for 3 to 6 hours, a base line was recorded for at least 30 min. Four biphasic constant current pulses (0.2 Hz) were used for sampling at 10-min intervals in the first hour and at 30-min intervals in the following hours after LTP induction. The slope of the EPSP (mV/ms) was measured from the average waveform from four consecutive responses. Stainless steel electrodes were used (A-M Systems) for stimulation and recording. Error bars indicate ± SEM. The data were statistically evaluated with the two-tailed Mann Whitney U test.

tion spike (Fig. 1). The time course of this blockade was similar to that produced by inhibitors of protein synthesis (6).

PKA is constitutively active in CA1 neurons of the hippocampus (*15, 16*), and inhibiting the activity of PKA reduces the response of non-NMDA receptor channels to glutamate. Inhibition of a constitutive activity of PKA could account for the small reduction of the early phase of LTP produced by Rp-cAMPS and for the later reduction evident after 3 to 5 hours in the EPSP (Fig. 1). Alternatively, the later effect could reflect an action of this inhibitor unrelated to its effect on PKA.

To obtain independent evidence that cAMP can actually mediate L-LTP, we applied a membrane-permeable analog of cAMP, Sp–cyclic adenosine 3′,5′-monophosphorothioate (Sp-cAMPS), that activates PKA to hippocampal slices (Figs. 2A and 3). Application of Sp-cAMPS (100 μM) resulted in an initial synaptic depression followed by a delayed and persistent facilitation similar to L-LTP. The facilitation reached its maximal amplitude in both the field EPSP and in the population spike after 90 to 120 min (Figs. 2A and 3) and lasted for at least 8 hours, the longest time tested (Fig. 3).

We next examined the relation between the late phase of LTP produced by tetanization and the synaptic potentiation evoked by Sp-cAMPS. We applied Sp-cAMPS to hippocampal slices either alone or after tetanus-induced LTP. In slices already exposed to tetanus, the late facilitation of the field EPSP produced by Sp-cAMPS was reduced (Fig. 2, A and B). This result suggests that the synaptic potentiation induced by Sp-cAMPS may share one or more steps in common with the mechanism that leads to tetanization-induced LTP. Conversely, pretreatment with Sp-cAMPS blocked the late phase of LTP induced by tetanization. Three high-frequency trains of stimuli, which normally induce L-LTP, only elicited a potentiation lasting about 100 min when applied 3 hours after the slices were treated with Sp-cAMPS (Fig. 2, C and D). Sp-cAMPS not only blocked L-LTP in slices in which the analog had induced stable potentiation before tetanization (Fig. 2D) but also blocked L-LTP in slices that were exposed to Sp-cAMPS but not exposed to sampling current pulses that would reveal potentiation (Fig. 2C). This suggested that the effect of Sp-cAMPS is likely to result from an activity-independent phosphorylation of the substrates of PKA.

If the delayed potentiation induced by cAMP corresponds to the protein synthesis–dependent late phase of electrically induced L-LTP, then it should be possible to inhibit it with an inhibitor of protein synthesis. We therefore applied anisomycin at a concentration (20 μM) that blocks about 85% of the incorporation of [^{14}C]leucine into proteins in hippocampal neurons (6) for 25 min before application of Sp-cAMPS and removed it 1 hour after adding Sp-cAMPS. Anisomycin inhibited the potentiation induced by Sp-cAMPS (Fig. 3) and revealed a synaptic depression. We do not know the cause of this depression because anisomycin by itself did not depress either the full EPSP or the population spike. This depression might represent an action of

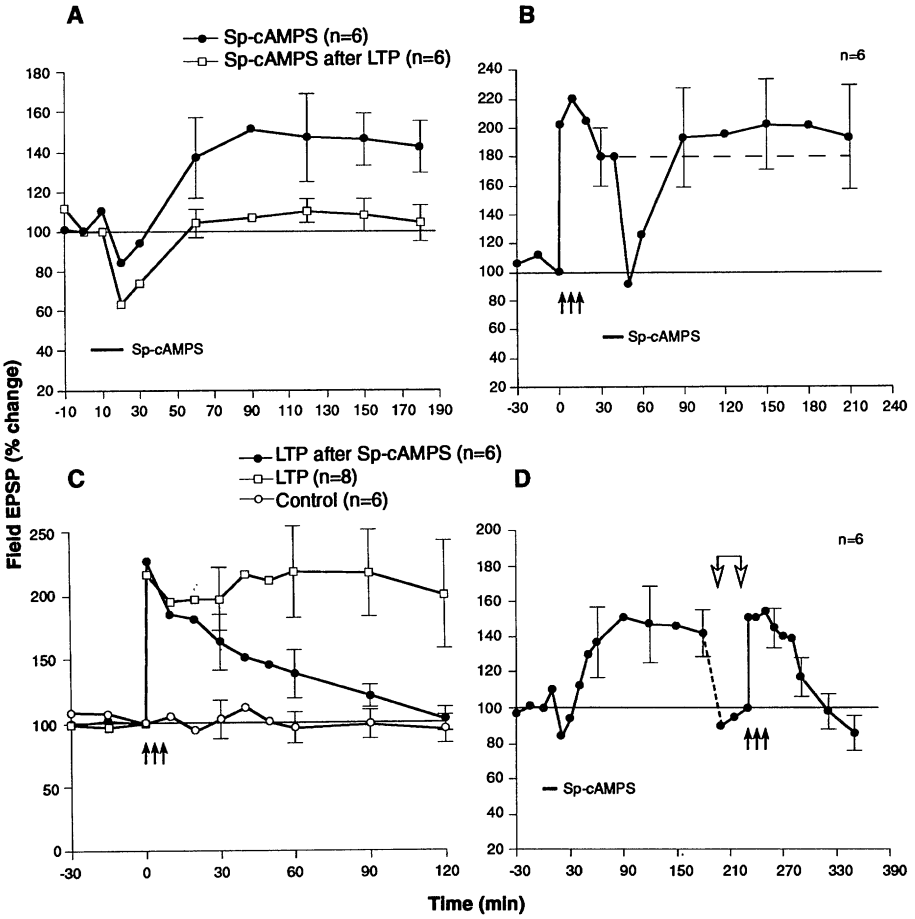

Fig. 2. Long-term synaptic potentiation induced by tetanization and by Sp-cAMPS occlude one another. (**A** and **B**) Preexposure to L-LTP induced by tetanization occluded the persistent facilitation produced by Sp-cAMPS. (A) Sp-cAMPS (100 μM for 15 min) produced a persistent facilitation after an initial transient inhibition (solid circles). (B) Tetanization, with three high-frequency trains (arrows), produced L-LTP. Thirty minutes after the induction of L-LTP, Sp-cAMPS was applied and produced a depression that was larger than that produced when Sp-cAMPS was given alone (A), but the synaptic facilitation that followed the depression was significantly reduced compared with a control without the preceding tetanus-induced L-LTP ($n = 6$, $P < 0.01$). The residual facilitation remaining after the occlusion by L-LTP is regraphed in (A) (open squares) to compare it with the facilitation produced by Sp-cAMPS alone (solid circles). (**C** and **D**) Preexposure to Sp-cAMPS occluded L-LTP induced by tetanization. (C) Three hours after Sp-cAMPS was applied (100 μM for 15 min), three high-frequency trains of stimuli were given. These trains produced an early facilitation that decayed in about 90 min. The residual potentiation present at 90 and 120 min was significantly reduced compared with L-LTP in the absence of Sp-cAMPS ($n = 6$, $P < 0.05$). (D) Three hours after Sp-cAMPS potentiation was induced (143 ± 13%), the stimulus intensity was reduced (open arrows) so as to achieve an EPSP slope value comparable to control (0.4 ± 0.03 mV/ms), and a new base line was taken. Three high-frequency stimuli (solid arrows) induced a transient facilitation (comparable to E-LTP) that decayed to base line in about 90 min. This residual facilitation, measured 90 and 120 min after tetanization, was significantly reduced compared with L-LTP produced in the absence of Sp-cAMPS ($n = 6$, $P < 0.01$). For these experiments, hippocampal slices were perfused at a flow rate of 1 ml/min with an oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid that contained 124 mM NaCl, 4.9 mM KCl, 1.3 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 25.6 mM $NAHCO_3$, and 10 mM D-glucose. The temperature in the chamber was maintained at 30°C.

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Sp-cAMPS that is not dependent on protein synthesis and is normally masked by the larger facilitation produced by Sp-cAMPS in the absence of anisomycin (*17*).

We also explored whether the stimulus protocol required to initiate L-LTP activated the formation of cAMP (*19*). We measured the concentration of cAMP in hippocampal slices after a single train of stimuli and after three high-frequency trains of stimuli (Fig. 4). A single stimulus (100 Hz for 1 s), which usually induces only E-LTP, produced no change in the concentration of cAMP 1 min after tetanization (Fig. 4A). Three trains of stimuli, which lead to L-LTP, produced a significant increase ($P < 0.05$) in the concentration of cAMP 1 min after the last tetanization (Fig. 4B). Consistent with the finding (Fig. 2) that the increase in the concentration of cAMP necessary to produce L-LTP need only be transient, we found that the tetanus-induced increase was not present 10 min later (Fig. 4E). The increase in cAMP after three tetani was blocked by the D1 receptor antagonist SCH 23390 (1 μM) and by DL-2-amino-5-phosphonovaleric acid (APV) (10 μM), an NMDA receptor antagonist (Fig. 4, C and D).

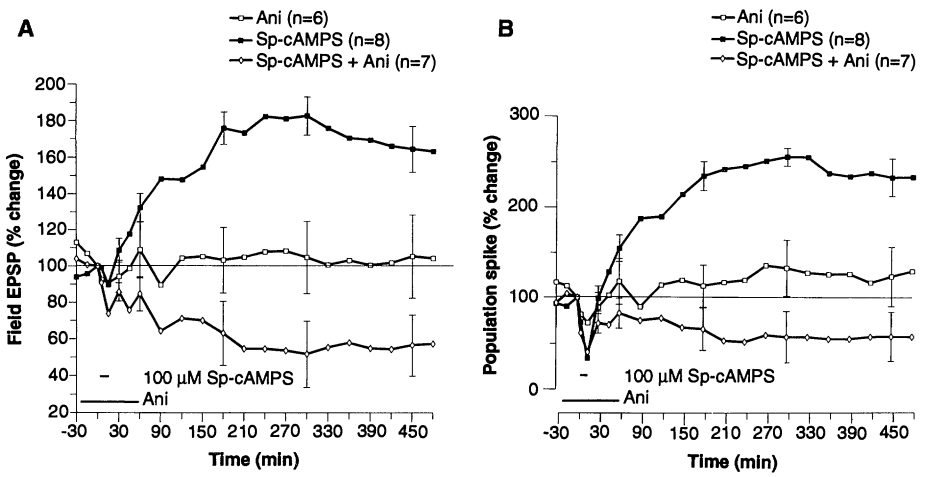

Fig. 3. Effect of Sp-cAMPS (100 μM) with and without anisomycin (Ani) (20 μM). (**A**) Time course of changes in the field EPSP measured as the slope function. (**B**) Percentage change of the population spike amplitude. Sp-cAMPS caused a statistically significant potentiation in both the field EPSP and the population spike 60 min after application of Sp-cAMPS, compared with controls to which incubation medium was applied, and at 90 min, compared with controls to which anisomycin was applied without Sp-cAMPS ($P < 0.05$). The depression of the field EPSP obtained after application of anisomycin with Sp-cAMPS was only statistically significant from that of controls 3 to 5 hours after the administration of the drugs. Compared with the slices treated with anisomycin alone, there was a statistically significant difference only after 4 to 5 hours by both field EPSP and population spike. Sp-cAMPS was applied for 10 min and washed out with fresh bath medium (10 ml). Anisomycin was added to the slices 25 min before the addition of Sp-cAMPS; it was washed out with fresh bath medium 1 hour after application of Sp-cAMPS.

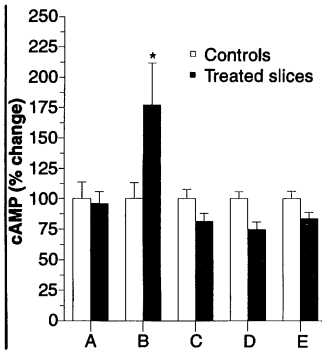

Fig. 4. Measurement of the amount of cAMP in hippocampal region CA1. Measurements were made (**A**) 1 min after single tetanization (control, $n = 8$; treated, $n = 11$); (**B**) 1 min after threefold tetanization ($n = 5$, $n = 8$); (**C**) 1 min after threefold tetanization and application of the D1 receptor antagonist SCH 23390 (1 μM) ($n = 8$, $n = 9$); (**D**) 1 min after threefold tetanization and DL-APV (10 μM) ($n = 4$, $n = 5$); and (**E**) 10 min after threefold tetanization ($n = 4$, $n = 6$). Tetanization procedure, 3 × 100 Hz/s with 10-min intervals. Hippocampal slices for each sample were prepared from the same animal for both the controls and treated slices. After a 2- to 3-hour incubation period and 15 to 20 min before tetanization, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 100 μM) was added. After the experimental procedure the slices were removed gently and put on dry ice. The CA1 region was dissected with a razor blade during freezing. CA1 regions from two hippocampal slices were pooled. After homogenization in ice-cold HBSS (60 μl), cold trichloracetic acid (TCA; 20%, 60 μl) was added to each sample. After centrifugation the supernatant was stored in liquid nitrogen; the sedimented material was dissolved in a solution containing 0.3% SDS (50 μl) and NaOH (10 mM), and the protein content was determined with the BCA protein assay reagent (Pierce Chemical Co.). The supernatant was extracted four times with ether and a radioimmunoassay (DuPont NEN Research Products) was used to determine the amount of cAMP per unit of protein. The data were statistically evaluated with the two-tailed Mann Whitney U test (*signifies $P < 0.05$).

Because a large proportion of the adenylyl cyclase in the rat brain is sensitive to calmodulin in the presence of Ca^{2+}, the Ca^{2+} influx caused by activation of the NMDA receptor could stimulate adenylyl cyclase directly (*18, 19*) or it could act synergistically to stimulate the adenylyl cyclase, perhaps together with dopamine or some other as yet unidentified transmitter. A similar increase in the intracellular concentration of cAMP occurs after tetanization in the dentate gyrus (*20*). In young rats an increase in the amount of cAMP has been found in the CA1 region after a single tetanus and after application of a high concentration of NMDA (*19*).

Our results demonstrate that PKA can stimulate a long-lasting potentiation and that this potentiation requires protein synthesis. Furthermore, our data suggest that the action of PKA and the synthesis of new proteins start within the first hour of the onset of LTP expression, while E-LTP is still in progress.

Although these studies are consistent with a role for PKA in the later stages of LTP, they also raise a number of questions. First, we do not understand the mechanisms that contribute to the inhibition of basal synaptic transmission produced by Rp-cAMPS or that produced by Sp-cAMPS when given together with anisomycin. Second, we do not know the natural ligand that might act alone or together with glutamate to initiate the putative cAMP-dependent late phase of LTP. Because D_1 receptor antagonists that block L-LTP (*12–14*) also block increases in the concentration of cAMP after tetanus, dopamine is one candidate. There are, in fact, dopaminergic fibers that course in the mesolimbic pathway to innervate the hippocampus (*21, 22*), and there is evidence in the CA1 pyramidal cells for the expression of D_5, a dopamine receptor related to D_1 that is coupled to adenylyl cyclase (*23*). However, although dopamine (50 μM) can induce L-LTP in a similar manner to Sp-cAMPS (*24*), other receptors that stimulate adenylyl cyclase could also be involved. We also have not explored the physiological mechanisms contributing to L-LTP. For example, we do not know whether L-LTP reflects an increase in excitatory transmission similar to that observed with E-LTP. Nevertheless, our data provide evidence that LTP has stages, that the later stages require protein synthesis, and that this protein synthesis–dependent later stage may be maintained by the generation of cAMP and activation of PKA, either alone or in conjunction with other second messenger pathways. The ability to apply a second messen-

ger analog (cAMP) or a transmitter (dopamine) to the whole slice to simulate L-LTP may allow study of the proteins and genes required for the induction and maintenance of L-LTP.

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TECHNICAL COMMENTS

Explaining Fruit Fly Longevity

Two recent reports have challenged the notion that death rates automatically increase with age. J. R. Carey *et al.* (*1*) studied a large, outbred population of the medfly, *Ceratitis capitata*, and report that mortality rate actually decreased in the older flies. J. W. Curtsinger *et al.* (*2*) examined smaller, inbred populations of *Drosophila melanogaster* and describe how mortality rate apparently leveled off at older ages. Although it has long been accepted that not all species undergo senescence (*3*), the idea that medflies and *Drosophila* might show constant or declining mortality at older ages was an unexpected and important result. Both these species might be expected to show progressive mortality rate increases with age.

We have examined the results of these reports and believe that there are important aspects of the interpretation of these data that have not received due attention. First, there is the problem of heterogeneity. Carey *et al.* point out that genetic heterogeneity at the level of the cohort might at least partly explain their finding, because in a mixed population the frail individuals die earlier, leaving the hardiest to survive to the oldest ages. However, they do not explore this possibility further. We have calculated the theoretical survival curve of a population in which we assumed individuals varied in the rate parameter of a Gompertz mortality model (Fig. 1). We found that the population mortality rate declined in the same way the medlfly rate declined even though in our model every individual fly has an exponentially increasing risk of dying (*4*).

Second, there is the problem of sample size. As noted by Carey *et al.* (*1*), a large sample size is essential to avoid large statistical fluctuations in mortality rate estimates for later ages. Even so, there comes an age for any sample when the population has dwindled to the last few survivors. In (*2*), the largest sample was only 5751 flies. The mortality rates plotted in (*2*) would not be inconsistent with a model of increasing mortality that allowed for this statistical variation. We therefore ran many simulations of populations of 5751 flies assuming a Gompertz mortality model (Fig. 2). As can be seen from our results, an apparent flattening of the mortality curve in old flies is consistent with chance.

The interpretations of Carey *et al.* and Curtsinger *et al.* now seem questionable. Where genetic heterogeneity exists as in (*1*), the mortality pattern for the population as a whole contains little information about the mortality pattern for individual genotypes. Heterogeneity can also be non-genetic, so there may be implications for the results of (*2*). When sample sizes are small, as in (*2*), caution must be exercised in drawing conclusions. Some commentators on these reports have noted these problems, but also noted that Carey *et al.* (*1*) addressed the sample size issue, while Curtsinger *et al.* (*2*) addressed the heterogeneity issue. However, both problems need to be addressed together.

A third problem that received little comment in either report is the effect of age-related behavior changes on mortality risk. If old flies lead quieter lives (for example, crawling rather than flying), mortality may level off or even decline in spite of the fact that the animal gets progressively more frail. Data on age-related behavior changes are essential to complete the picture. Crowding is another factor. Carey *et al.* mention that flies in their large population (experiment 3 in their figure 2) were held in groups of 7200 and "subject to conditions that increase mortality risk—large cage size for flying, mating, some egg-laying, mechanical wear, and considerable stress due to crowding . . . ." This population was the

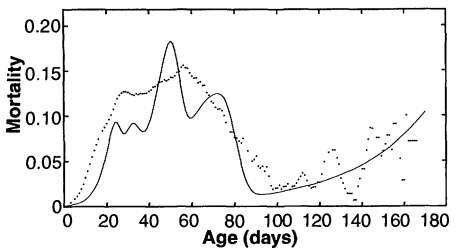

Fig. 1. Smoothed age-specific mortality rates (dots) taken from table 2 of (*1*). Age-specific mortality rates for a theoretical population comprising eight genotypes, each having a Gompertz mortality function (continuous curve) [see (*4*) for details].

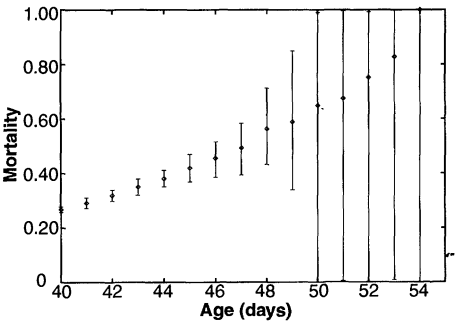

Fig. 2. Medians and interquartile ranges of age-specific mortality rates from 1000 random simulations of a population of 5751 flies, with the assumption of a Gompertz mortality function with intercept parameter 0.008 and slope parameter 0.09.