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20. Control mice were 129. The mutant CD5$^{-/-}$ mouse strain was derived by intercrossing and subsequent inbreeding of heterozygous littermates born to C57BL/6 females, mated with the male chimeras, obtained by injecting CD5 gene targeted embryonic stem (ES) cells into C57BL/6 blastocysts (*8*). Splenic and peritoneal washout cells were obtained from 7- to 12-week old mice, and depleted of T cells by treatment with a cocktail of antibodies (anti-Thy 1.2, anti-CD8, and anti-CD4) followed by rabbit complement. Splenic and peritoneal B cells were depleted of macrophages by plastic adherence overnight at 37°C in 5% CO_2. B-1 cells were further purified by dual staining with Mac-1 and B220 and sorting of the double positive cells with a FACStar (Becton Dickinson) flow cytometer. Cells (2.5×10^5) in 0.2 ml of Iscove F-12 media, 5% fetal calf serum were cultured in flat bottom microtiter wells.
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22. Peritoneal B cells were pelleted and resuspended in 100 μl of Ho342 (5 μg/ml) (Molecular Probes). After a 30 min incubation at 37°C in the dark, the cells were pelleted and resuspended in 100 μl of a 0.4 μg/ml stock solution of MC540 (Molecular Probes). After a 20-min incubation at room temperature (in the dark), 1 μg of anti-B220 conjugated to fluorescein isothiocyanate (FITC) (Sigma) was added, and incubated on ice for 30 min. The cells were then pelleted, resuspended in 1 ml of PBS and 10,000 cells were analyzed immediately on a FACStar flow cytometer (Becton Dickinson). The gates to distinguish MC540 dull (R1, R2) and bright cells (R3, R4) were set by looking for a natural break in the staining profile of the resting or cycling B cells. The CD5$^{-/-}$ mice required different gates, presumably due to higher background staining of the untreated cells with MC540, which occurs whenever the responding population is activated (*10*). Data with peritoneal B cells from wild-type 129 mice were shown in Fig. 2 and similar results were obtained with peritoneal B cells from BALB/c mice.
23. Control and CD5$^{-/-}$ B-1 cells were purified as described (*20*). Cells (2×10^5) were incubated with F(ab')$_2$ GαmIgM (Cappel) or normal goat IgG (Sigma) for 60 min at 37°C in 5% CO_2. The cells were transferred onto microscope slides by cytocentrifugation, fixed with 4% paraformaldehyde, and permeabilized in 0.05% Triton X-100 (Bio-Rad). Cells were subsequently incubated with anti-NF-κB p65 (Santa Cruz Biotech) for 2 hours, then with biotinylated anti-rabbit-IgG (Vector) for 1 hour. Cells were finally incubated with avidin-FITC (Vector) for 30 min, and examined on a confocal laser scanning microscope (Molecular Dynamics, Sarastro 2000). To allow for quantitative comparisons of the relative fluorescence between the cells, the intensity of the laser beam and the sensitivity of the photodetector were held constant. The "Imagespace" software supplied by the manufacturer was used to obtain the values for average intensity of nuclear staining. Several fields of cells were examined on each slide.
24. B-1 cells (10^7) from wild-type or CD5$^{-/-}$ mice were stimulated with F(ab')$_2$ GαmIgM or goat-IgG for 1 hour at 37°C in 5% CO_2. Nuclear proteins were obtained as described (*25*). Protein concentrations were standardized (BCA, Pierce), and the electrophoretic mobility shift assay was done with a NF-κB binding protein assay kit (Gibco-BRL), following the procedure of the manufacturer.
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26. All animal procedures were conducted in accordance with the University of Kentucky "Guide for the Care and Use of Laboratory Animals."

27. We thank A. Kaplan and B. T. Spear for critical review of the manuscript, R. Hardy for a breeding pair of the CD5$^{-/-}$ mice, M. Howard for monoclonal antibody to CD40, M. Mattson for the use of the confocal laser microscope, J. Strange and R. Cross for flow cytometry, and R. S. Mattingly for technical help. Supported by NIH grants AI21490 and AG05731 to S.B.

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Quantal Duration of Auditory Memories

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Neuronal responses in the caudomedial neostriatum (NCM) of adult zebra finches (*Taeniopygia guttata*) decreased upon repeated, unreinforced presentations of conspecific song, calls, or other complex sounds. This "stimulus-specific habituation" is a form of learning, and its spontaneous loss, a form of "forgetting." Spontaneous forgetting occurred only at narrowly defined times (2 to 3, 6 to 7, 14 to 15, 17 to 18.5, 46 to 48, or 85 to 89 hours after first exposure to a stimulus), determined by stimulus class, number of presentations, and interval between presentations. The first five forgetting times coincided with periods when gene expression and protein synthesis in NCM were required for maintenance of the longer lasting (85 to 89 hours) habituation. The number of successive episodes of gene expression induced by a stimulus, but occurring long after stimulus presentation, appears to determine the quantal duration of auditory memories.

The songs and calls of songbirds have characteristics that can be used for species identification and individual recognition (*1*, *2*). In previous experiments, we used multi-unit activity (MUA) data to show (i) that auditory responses in populations of neurons in the zebra finch NCM habituate specifically to individual song stimuli; (ii) that this habituation can be long-lasting; and (iii) that the duration of habituation is longer for conspecific songs than for white noise, pure tones, or some exemplars of heterospecific sounds (*3*). We showed, too, that this habituation was anatomically circumscribed: It occurred in caudal, but not in rostral, NCM (*3*). NCM is one of the highest stations of the ascending auditory pathway (*4*). Here we describe habituation at the single-unit level and its relation to MUA habituation. We then use recordings of MUA to determine in a systematic manner how stimulus class, interstimulus interval (ISI), number of stimulus presentations, and manner of presentation affect the duration of stimulus-specific habituation in neurons of caudal NCM. Finally, we examine the relation between the duration of habituation and RNA and protein synthesis.

The firing rates of single neurons in caudal NCM were initially high and then decreased upon repeated presentations of the same conspecific song (Fig. 1) (*5*). After 100 presentations there was little if any further decrement in responsiveness. The reduction in firing rate was not selective for any one subset of the song's components but affected the whole song (Fig. 1C). The stimulus-specific habituation seen in multi-unit recordings does not appear to result from individual neurons "tuning in" to particular features of a stimulus and ceasing to respond to the rest of that stimulus, but rather from a general reduction in the responsiveness elicited by that stimulus.

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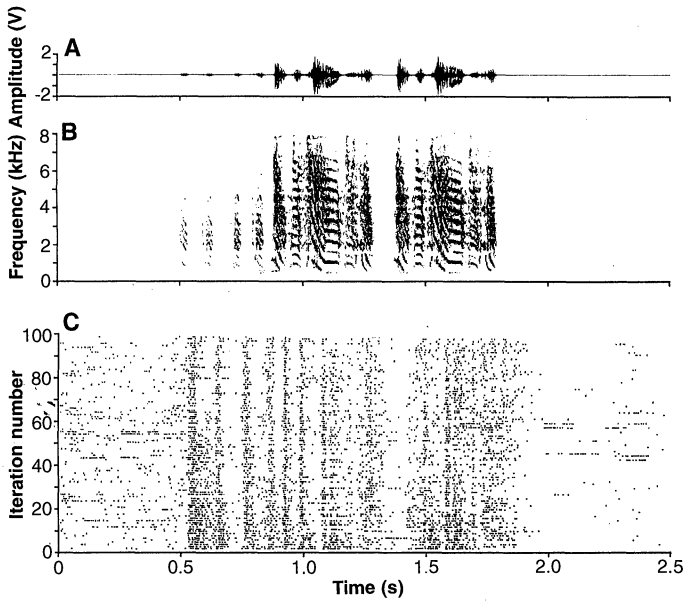

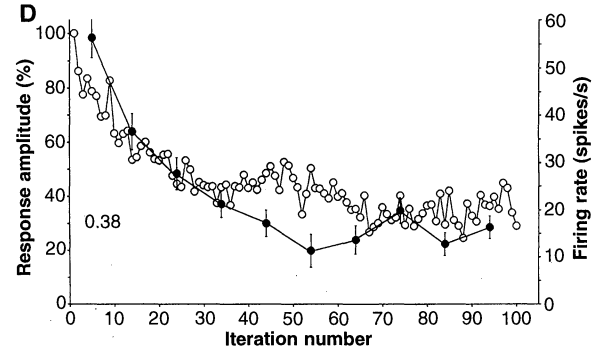

Fig. 1. Comparison of single-unit and multi-unit activity in caudal NCM in response to 100 iterations of a novel song. (**A**) Amplitude waveform of the conspecific song stimulus. (**B**) Sound spectrogram of the song in (A). (**C**) Raster of activity in a single unit (*5*); each dot represents the time of occurrence of an action potential, and each row represents one song presentation (sequence ordered from bottom to top). (**D**) Mean firing rate (spikes per second ± SE) (*5*) of the response elicited in the single unit shown in (C) over each group of 10 trials (filled circles) and normalized MUA amplitude (*8*) for each trial recorded at the same site (open circles). The MUA habituation rate at this site was 0.38 (Fig. 3) (*8*).

Single units exposed to 50 presentations of a song gave 21 ± 1 (mean ± SE) spikes per second when that song had not been heard before ("novel," 66 units), 12 ± 1 spikes per second when that song had been heard 6 to 40 hours earlier ("remembered," 99 units), and 21 ± 2 spikes per second when that song had been heard 50 to 100 hours earlier ("forgotten," 51 units) (*6*). The firing rates elicited by novel and forgotten songs were similar and significantly higher than those for remembered songs (unpaired t tests, $P < 0.0001$, two-tailed). These observations on stimulus-specific habituation at the single-unit level closely parallel observations reported earlier for MUA in NCM (*3*).

We next investigated the factors that determine multi-unit habituation (*7*). In our standard MUA experiment, a bird is exposed to playbacks of conspecific song at two times. During an initial training stage, birds (n = 49) heard 200 iterations of the same novel song. Then hours or days later, the same song was presented 100 times—the "testing" stage. The ISI was 11 s, during training and testing. MUA responses were recorded in caudal NCM with tungsten microelectrodes (*3*). The decrease in responsiveness during the first 100 iterations of a novel or familiar stimulus was used to calculate the habituation rate (*8*) (Figs. 1 and 2A). Novel songs—that is, songs with which a bird had not been trained earlier—induced a high habituation rate (range: 0.30 to 0.50); familiar songs induced low habituation rates (range: 0.01 to 0.25). In addition, the amplitude of the response to the first presentation was 14% lower for remembered than for novel songs when these recordings were made at the same site (time between training and testing with the

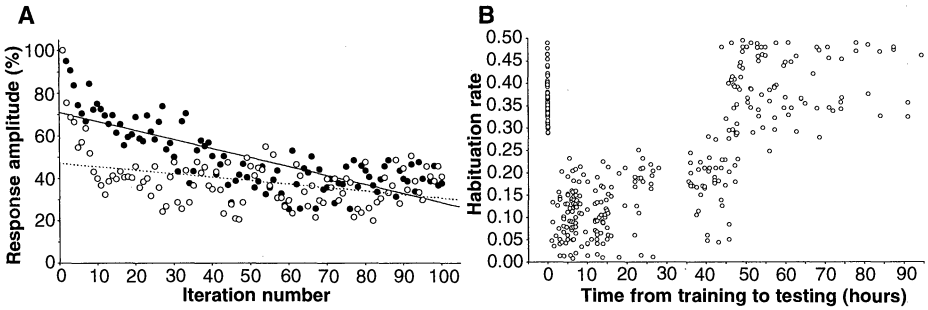

Fig. 2. (**A**) Examples of MUA responses to the first 100 presentations of a novel (solid symbols) and a familiar (open symbols) conspecific song at a single site in caudal NCM. MUA response amplitudes were normalized to the response on the first presentation of the novel or familiar song, respectively; the first response to the familiar song overlies that for the novel one. The bird had been trained with the familiar song 6 hours earlier. The habituation rates for the novel and familiar songs in this example were 0.42 and 0.16, respectively, and the corresponding best fit lines are plotted for each set of points (*8*). (**B**) Time course of spontaneous loss of habituation for conspecific song (200 iterations at an ISI of 11 s). Habituation rates (*8*) were measured at various training-to-testing intervals in 23 birds, with a total of 10 to 16 different songs per bird. The rates for novel songs are shown at time zero. The time from the onset of training until the time at which the habituation rates became not significantly different from the rates for novel song was used to define the duration of habituation (*10*).

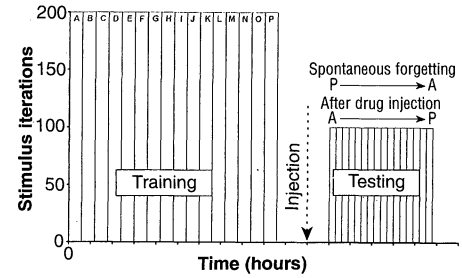

Fig. 3. The protocol for studying the time course of habituation rates in NCM in a single bird. Training consisted of presenting sequentially 200 iterations of each of up to 16 novel stimuli (A to P) at an ISI of 11 s (total time for each stimulus = 36.7 min). Testing consisted of recording MUA during 100 presentations of each of these now familiar stimuli at an ISI of 11 s (which remained constant even if the ISI used in training was, as in some experiments, shorter or longer). In experiments that assessed spontaneous forgetting, stimuli were tested in the reverse order (P to A) to create a wide range of training-to-testing intervals during a single recording session in each bird (*13*). In those experiments that tested for the importance of RNA and protein synthesis in memory retention, a single dose of CYC or ACT was injected (arrow) into NCM at various delays after onset of training. Testing began 1 hour after injection, starting with the first song presented during training (A to P). This protocol yielded 16 different training-to-injection intervals for each bird tested.

familiar song, 6 to 8 hours; $n = 42$ paired comparisons; paired t test, $P < 0.002$).

To examine habituation rates at many different intervals after training, we sequentially played multiple stimuli to each bird during training; then, during testing, we played the same stimuli again in reverse order (Fig. 3). Habituation rates to each familiar song remained significantly lower (9) than those elicited by novel songs until ~47 to 48 hours after onset of training, when there was a relatively abrupt transition to the higher habituation rates elicited by novel songs (Fig. 2B) (*10*). We defined duration of habituation as the time from initial exposure to a stimulus during training to the time when the habituation rate for that stimulus became statistically indistinguishable from the rates for novel songs.

The duration of habituation was affected by the type of sound presented (*11*); it was the same for all familiar exemplars of a given stimulus class and differed among stimulus classes (Fig. 4A). Human speech and canary (*Serinus canaria*) song produced memories lasting 3 hours; songs of the Bengalese finch (*Lonchura striata*), a member of the same Estrildid family as the zebra finch, 6.5 hours; all reversed conspecific vocalizations, 6.5 to 7 hours; conspecific male long calls (*2*, *12*), 18.5 hours; and conspecific male song and female long calls (*2*, *12*), 47 to 48 hours. These memory durations were the same whether our recording electrode was in the right or left NCM (*13*). Although gender features of the stimulus determined the memory duration for male and female calls, there were no differences in memory duration between male and female subjects for any type of stimulus (*13*).

The effectiveness of conspecific song in inducing long-lasting stimulus-specific habituation was affected by the ISI used during training (Fig. 4B). At an ISI of 3.5 s, immediate habituation to novel songs was weak (*14*). These "familiar" songs were subsequently regarded as novel even when tested 1 hour later. The duration of habituation for ISIs of 3.62 to 3.87 s was 6.5 to 7.5 hours; for ISIs of 4.0 to 4.5 s, 13.5 to 14 hours; for ISIs of 4.75 to 8 s, 17 to 18 hours; for ISIs of 9 to 30 s, 48 hours; and for ISIs of 35 to 54 s, 87 to 89 hours. Over this range of training ISIs (testing ISI held constant at 11 s), with corresponding increases in training time, changes in memory duration occurred in abrupt steps; there appeared to be different thresholds that the ISI had to exceed before the next quantum in memory duration occurred.

We also studied the effect of the number of stimulus iterations on the duration of habituation. As the number of iterations of conspecific song increased gradually during training from 30 to 1000, at a constant ISI of 11 s (training time ranged from 5.5 min to 3 hours), we again saw stepwise increases in memory duration (Fig. 4C). No lasting habituation was produced when only 30 to 40 iterations were presented. Habituation lasted 6.5 to 7 hours with 50 to 140 iterations, 17.5 to 18.5 hours with 150 to 185 iterations, and 47.5 hours with 185 to 200 iterations. There was no further increase in the duration of habituation when birds were trained with up to 1000 iterations. One thousand iterations at an ISI of 11 s required 3 hours for training, which is as long as it took to present 200 iterations at an ISI of 54 s, yet the habituation lasted 47.5 and 87 hours, respectively. Total training time

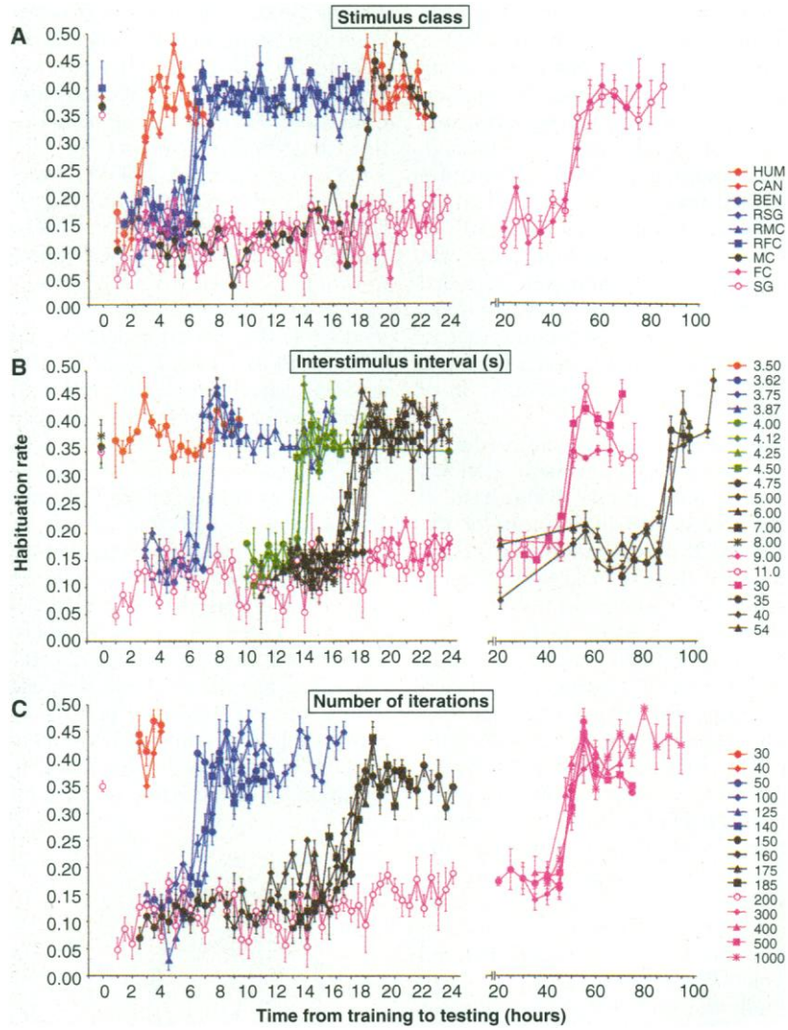

Fig. 4. (**A**) Duration of long-term habituation differed among stimulus classes. The habituation rates (±SE) are plotted as a function of the training-to-testing interval. The loss of long-term habituation to these familiar stimuli occurred at distinct times characteristic of the stimulus class, with all exemplars of that class being forgotten at the same time. The longest memory durations are seen for conspecific songs and female calls. In the following sentence, the number after each stimulus class indicates the number of exemplars tested for that class: HUM (words from human speech), 7; CAN (canary song), 5; BEN (Bengalese finch song), 3; MC (male zebra finch long call), 6; FC (female zebra finch long call), 6; SG (zebra finch song), 16; reversed conspecific vocalizations: RMC, 6; RFC, 6; RSG, 16. Forty-nine birds were tested with these stimuli. (**B**) Duration of long-term habituation depended on the ISI. ISI was systematically varied during training from 3.5 to 54 s, with 200 sequential iterations of each conspecific song stimulus in a total of 58 birds; training time for each song ranged from 11.6 min to 3 hours. Testing was always done at an ISI of 11 s. Small increments in ISI produced large, step-like increases in the duration of long-term habituation. (**C**). Duration of long-term habituation depended on the number of stimulus iterations. The number of sequential presentations of each conspecific song was systematically varied during training from 30 to 1000, with an ISI of 11 s in 52 birds. Small increases in the number of iterations produced large, step-like increases in the duration of long-term habituation. In all curves, at least four birds are represented per time point within 2 hours (graphs on left) or 5 hours (graphs on right) on either side of a step change leading from remembered to forgotten.

did not, by itself, determine the duration of the ensuing habituation.

Spaced training has been shown to produce a longer behavioral memory than massed training in *Drosophila* (*15*). We examined whether the same held true for neuronal memory in NCM by comparing the duration of habituation in a paradigm where iterations of each stimulus were either presented in a single group (massed) or in several smaller groups separated in time (spaced) in a balanced design (*16*). When we compared these two paradigms, using 200 iterations for each song, habituation was lost by 48 hours for the songs presented in the massed-training manner, as expected, but spaced training produced habituation that persisted for 89 hours (*17*). When we used a spaced-training paradigm (*18*) to present canary song and human speech, these heterospecific stimuli were regarded as novel when testing started at 4 to 5 hours (training took 3 hours), suggesting that in this instance the spaced training paradigm had added little if any to the duration of habituation.

We also investigated the mechanism of quantal memory. Earlier work had shown that when stimulus-specific habituation of the auditory responses in neurons of the zebra finch NCM was long lasting, its persistence in awake animals could be interrupted by blocking RNA and protein synthesis in NCM during two sensitive periods, 1 to 3 hours and 6 to 7 hours, respectively, after onset of exposure to a particular stimulus, but not during the intervening time (*3*). This earlier observation was compatible with the possibility that the second wave of gene expression was, as suggested also for other systems (*19*), part of a two-step mechanism of memory consolidation. However, the quantal nature of the memory durations just described was so robust that we wondered whether long-term habituation consisted of consecutive memory segments, each induced by a specific molecular process.

To study the role of RNA and protein synthesis in the maintenance of long-term habituation in NCM, we trained animals with one of three protocols: (1) massed training at an ISI of 11 s (n = 28), (2) massed training at an ISI of 40 s (n = 14), or (3) spaced training at an ISI of 11 s (n = 4) (*16*). At various intervals after the end of training, but well within the time when the habituation induced would still have been present, each bird was injected into the right or left NCM with either the RNA synthesis inhibitor actinomycin-D (ACT, for protocols 1 to 3) or the protein synthesis inhibitor cycloheximide (CYC, for protocols 1 and 2) (Fig. 3); saline was injected into the contralateral NCM as a control (*20*). We identified the sensitive periods during which injection of CYC or ACT blocked long-term habituation by varying the time elapsed between training and injection while using the same intervals between injection and testing (Fig. 3).

After CYC injections (protocols 1 and 2) (Fig. 5), the habituation rates for familiar songs were similar to those induced by novel songs when injections occurred 0.5 to 3.0, 6.5 to 7.0, 14.0 to 15.0, 17.5 to 18.5, 33.0 to 38.0, or 44.0 to 48.6 hours after onset of training with the song tested (t = −0.11 to −1.47, $P > 0.05$). At these times, the rates were also significantly different from those obtained for the same songs on the saline-injected side (t = −2.34 to −8.45, P = 0.0063 to 0.035). After ACT injections (protocols 1 and 2) (Fig. 5), the habituation rates to familiar songs were similar to those induced by novel songs when injections occurred 0.5 to 1.5, 6.0 to 6.5, 14.0 to 14.5, 17.5 to 18.5, 32.5 to 36.5, and 44.0 to 47.0 hours after onset of training (t = −0.14 to −1.32, $P > 0.05$). The rates at these times were significantly different from those for the same songs recorded from the control side (t = −3.64 to −8.27, P = 0.0004 to 0.04).

Injections of CYC or ACT at times other than the six sensitive periods defined above did not affect the retention of long-term habituation for the familiar stimulus (Fig. 5). We infer that long-term habituation that lasted for up to 80 hours after training with conspecific song depended on RNA and protein synthesis that occurred during the six sensitive periods defined above (*21*). Very similar periods of sensitivity to CYC and ACT were found for the retention of habituation to exemplars of human speech, canary song, and male and female zebra finch long calls, when habituation to any of these stimuli lasted long enough (Fig. 4A) to encompass any of these periods.

Each of the characteristic times for the spontaneous forgetting of a familiar sound corresponded closely to one of the sensitive periods when blockage of RNA or protein synthesis resulted in a loss of habituation (Fig. 6). However, none of the stimulus types or training paradigms used produced spontaneous forgetting that corresponded to the 33- to 38-hour time window during which RNA synthesis and protein synthesis was required for habituation to be maintained.

Those are the facts that must be evaluated. The term "habituation" has been used in the past mainly to refer to a situation in which an animal ceases to give behavioral responses to repeated presentations of an unreinforced stimulus (*22*). The same term has also been used to denote a decrement in neuronal responsiveness under conditions in which this decrement was known to be related to behavior (*23*). We do not know whether the single-unit and multiple-unit habituation that we saw in caudal NCM were related to changes in behavior, but it is apparent that the changes in neuronal responsiveness were related to experience.

Exposure to conspecific vocalizations elicited longer lasting habituation than any of our other auditory stimuli. A species' own set of signals may often have the longest claim to memory duration because of the relevance of the information it conveys. If so, conspecific signals have special advantages for studying the mechanisms that determine memory duration. Had we used

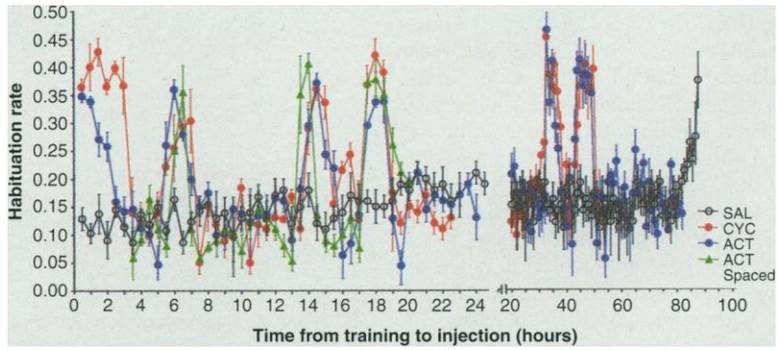

Fig. 5. Sensitive periods when injections of CYC and ACT into NCM resulted in loss of stimulus-specific habituation. The habituation rates (*3*, *8*) shown (mean ± SE) were plotted as a function of the interval between the onset of training with a particular song and the time of injection. CYC curve (red): data from 0.5 to 19.8 hours were obtained with protocol 1 (ISI = 11 s); data from 20 to 51 hours, with protocol 2 (ISI = 40 s) (see text). ACT curve (blue): data from 0.5 to 19 hours were obtained with protocols 1 and 2; data from 20 to 87 hours, with protocol 2. ACT spaced curve (green): data were obtained with protocol 3. Each point represents data from 3 to 10 birds, with a mean of four birds per point in each training protocol. Abbreviations: SAL, saline; CYC, massed training, CYC injection; ACT, massed training, ACT injection; ACT Spaced, spaced training (five songs, each played 200 times in four groups of 50 iterations; ISI = 11 s), ACT injection. See text for other details.

only white noise or tones as probes, we would not have discovered that habituation occurs for various, fixed durations of time determined by stimulus class and manner of presentation.

The most counterintuitive result from our experiments is the observation that habituation lasted for fixed periods of time—2 to 3, 6 to 7, 14 to 15, 17 to 18.5, 46 to 48, or 85 to 89 hours after onset of training—and that even when some stimulus parameters were altered linearly—for example, ISI and number of iterations—the resulting duration of habituation did not fall along a continuum. Moreover, the same fixed periods emerged when we used different classes of sound, changed the ISI, or varied the number of iterations. In all instances, the duration of habituation increased by fixed, quantal amounts.

The times for forgetting corresponded closely to the sensitive periods during which RNA or protein synthesis was required for maintenance of long-lasting habituation (Fig. 6). Previous studies have reported that ACT blocks long-term memory (*24*, *25*) through interference with genetic transcription (*23*). Similarly, CYC blocks protein synthesis necessary for learning—an effect that is reversible (*15*, *20*, *25*, *26*). In both instances, effectiveness depends on the time relation between training and drug exposure. By extending the interpretation of these earlier studies to our present results, we infer that the maintenance of long-term habituation to a familiar sound required multiple episodes of gene expression and protein synthesis in NCM. Moreover, we suggest that the duration of habituation was determined by the number of successive sensitive periods during which mnemogenic RNA and protein synthesis occurred.

If the times for the prolongation of habituation are fixed, then a reference "time zero" must be set. Because the times for forgetting and for sensitivity to CYC and ACT corresponded so well across a diversity of training protocols (Figs. 5 and 6), although some of these protocols took much longer than others (the duration of training with any one stimulus ranged from 36 min to 2.4 hours), we suggest that time zero was set during the first half-hour of stimulation (the level of resolution provided by our methods), and possibly even during the first few presentations of a stimulus. An extraordinary implication of this hypothesis is that each new stimulus starts its own molecular clock, and that therefore a very large number of such clocks must be running all the time in the brains of awake animals immersed in a sea of sensory stimulation.

It is tempting to explain our observations on memory duration by a specific mechanism, but the best we can do is to suggest features that such a mechanism should have: (i) It must be stimulus-specific. (ii) It must have a time zero from which subsequent durations are determined. (iii) It must be able to encode memory duration in fixed quanta, such that memories can last 3, 7, or 14 hours or longer without expressing durations that fall in-between. (A mechanism that activates each quantal duration when the input reaches a threshold comes to mind.) (iv) It must be able to integrate input over varying periods of stimulation, so that repeated instances of a particular stimulus can be added to an ongoing record, even when they occur at varying intervals and intermingled with other stimuli. (v) It must be able to induce segments of memory duration that occur always in the same order, such that an initial duration of 3 hours is followed by another one of 3 to 4 hours, which in turn is followed by one of 6 to 7 hours, and so forth. (vi) There must be a dependence among successive memory segments, because blockage of RNA and protein synthesis during the sensitive period that initiates a segment causes a memory loss that persists into at least what would have been the next memory segment (*27*).

The neuronal habituation that we have studied is a form of learning that occurs in the absence of reinforcement (*22*, *28*). Its properties may differ in important ways from other kinds of memory, for example, associative learning. Yet even associative learning relies on stimulus recognition, and so the differences may be minor. Our observations raise three hypotheses that may apply to learning in general: (i) memory comes in quantal durations; (ii) these quantal durations are determined by successive episodes of RNA and protein synthesis, with longer durations resulting from the sequential action of several such episodes; and (iii) the duration of long-term memory is determined by mechanisms that are an integral part of learning.

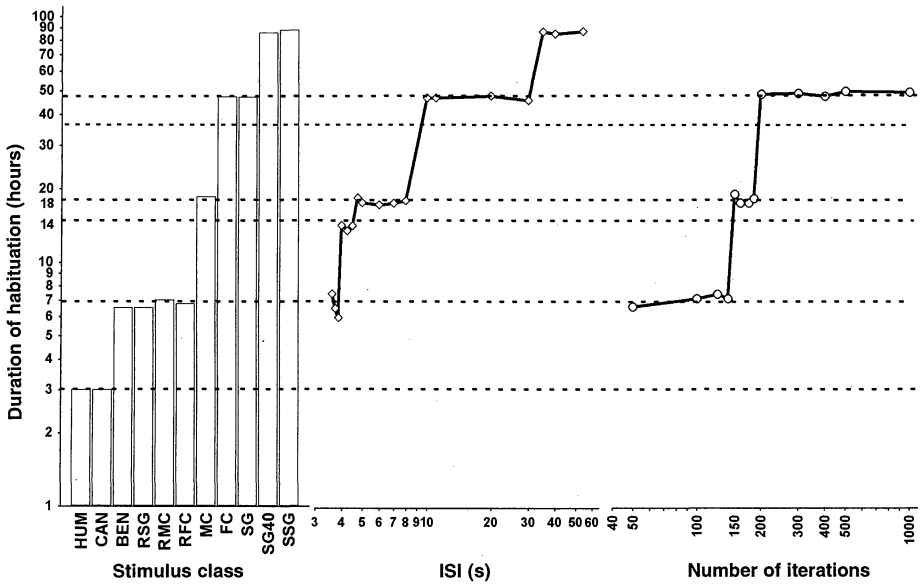

Fig. 6. Temporal correspondence between times when spontaneous forgetting occurred (Fig. 4) and times when RNA and protein synthesis were required for maintaining long-term habituation in different stimulation paradigms (Fig. 5). Duration of habituation is plotted for different classes of stimuli (left graph), different ISIs (middle), and numbers of iterations (right); all numeric scales are logarithmic. In each type of experiment, spontaneous forgetting occurred only at three to five fixed times. The dashed horizontal lines indicate the ends of periods when macromolecular synthesis was required for habituation to be maintained, as determined by injecting CYC and ACT. Abbreviations are as in Fig. 4A. All stimulus classes were trained with 200 repetitions at an ISI of 11 s, with the exception of SG40 (conspecific song, ISI = 40 s) and SSG (spaced training with four groups of 50 iterations of conspecific songs).

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5. Zebra finches (97 male and 96 female) obtained from our breeding colony were prepared for long-term recording as described (*3*). Auditory stimuli (*11*) were played from a speaker placed 0.5 m from the bird in a soundproof experimental chamber (*3*, *12*). During training the bird was freely moving; electrophysiological recording was carried out in awake, restrained birds. Insulated tungsten microelectrodes were used to record physiological activity in the right or left caudal NCM at sites that exhibited habituation to novel songs (*3*). Recording sessions lasted ~5 hours. All procedures conformed to an animal use protocol approved by the Rockefeller University Animal Care

and Use Committee. The auditory stimulus and microelectrode data were digitized at 20 kHz. Single-unit action potential waveforms were digitally discriminated and displayed as rasters (Experimenters Workbench, Datawave). Single-unit responses were quantified by averaging the firing rate during the stimulus period (plus the ensuing 100 ms) and then subtracting the firing rate during the control period (500 ms preceding stimulus onset). This mean firing rate per presentation was used to produce averages, for example, of 10 successive presentations (Fig. 1D) or of the first 50 presentations.

6. Birds that had heard the testing stimulus earlier had been exposed to 200 repetitions thereof at an ISI of 11 s; testing also occurred at an ISI of 11 s.
7. Single units are difficult to "hold" with a recording electrode for periods of more than 30 min. Therefore, most of this study was done with MUA data.
8. The multi-unit response amplitude to each stimulus presentation was calculated by subtracting the root-mean-square (rms) value over the 500-ms preceding stimulus onset from the rms over the period from stimulus onset to offset plus 100 ms. The difference between the two root-mean-square values measures the net response per unit time and corrects for differences in duration between stimuli. Each amplitude was then normalized to the response amplitude on the first presentation (typically the largest) and plotted as a function of stimulus iteration. We used the least-squares method to determine the slope of the straight line that best fitted each set of 100 normalized responses to a stimulus that the bird had or had not heard during an earlier training session. The habituation rate was defined as the absolute value of the slope of the best-fit line (Fig. 2A). Habituation rates were independent of the absolute response level at any given site and so could be used to compare different sites, in different birds, recorded at different intervals after training. We have previously shown that the distributions of habituation rates to novel and familiar songs differ significantly (3). Figure 2A demonstrates, too, that habituation to the familiar song occurred, typically, during the first few repetitions of the stimulus, after which responses stayed at a lower level; it took longer for this lower level to be reached by responses to a novel song.
9. All statistical comparisons were done with the Student's t test ($P < 0.05$, two-tailed).
10. The distribution of entries in Fig. 2B suggests that, even though there was a relatively abrupt change in habituation rate between 46 and 48 hours, a less marked drift toward higher habituation rates occurred between 15 and 46 hours after onset of training. Our stringent criterion for forgetting—habituation rates similar to those induced by presentations of a novel song—did not recognize these changes, which may, however, represent an early forgetting stage that deserves further study.
11. These sounds, which provided a set of conspecific and heterospecific stimuli that the birds had not previously heard, were digitized at 20 kHz (Signal, Engineering Design). The songs and words from human speech were 1.2 to 2.0 s long, and calls were 100 to 400 ms long.
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16. Seventeen birds were trained with 200 iterations (massed) each of two songs, followed by 50 iterations of each of five other songs, and this latter spaced protocol was repeated four times. At an ISI of 11 s, the spaced-training part of this protocol took 3 hours. Another two songs were then played with the massed-training paradigm. The total duration of this protocol was 5.5 hours. In a second version of this protocol, birds ($n = 8$) were trained with 50 iterations (massed training: ISI = 11 s, total training time = 9.2 min) of each of two novel songs; this was then followed by 10 iterations of each of a group of five other novel songs, and the latter sequence was repeated five times (spaced training: ISI = 11 s, total training time = 46 min). We then played another three songs using the massed-training paradigm. Thus, in 1.5 hours, we exposed the bird to 50 iterations of five songs presented in the massed-training paradigm, and 50 iterations of five songs presented in the spaced-training paradigm.
17. When a total of 50 iterations of each song were used, habituation was lost after only 7 hours for songs presented in the massed-training manner (Fig. 4C), but spaced training (*16*) produced habituation that persisted for at least 43 hours (*14*).
18. Five zebra finches that were trained with canary song presented in a spaced paradigm heard four groups of 50 iterations for each of five stimuli, for a total of 200 iterations for each stimulus; this training was compared with 200 massed iterations for a same canary song.
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20. ACT (40 nl, 50 μM) or CYC (40 nl, 1 ng/nl), dissolved in saline, was injected into 46 adult male or female zebra finches into a right- or left-hemisphere NCM recording site that exhibited habituation to novel songs. These injections were made with a glass micropipette (tip outer diameter, 20 μm). As a control, saline vehicle alone was injected into the other hemisphere. The effective sphere of the ACT or CYC injections, as determined by immunocytochemistry, was limited to a subregion of NCM (3). Such injections did not affect auditory responses or the immediate habituation of NCM neurons to playbacks of a novel song. The physiological effect of these RNA and protein synthesis blockers in NCM was reversed in <1 hour, as determined by the loss of long-term habituation for stimuli presented 0.5 hour, but not 1 hour, after injection [S. J. Chew, thesis, Rockefeller University (1966)], allowing for a fairly accurate pinpointing of the time when gene expression or protein synthesis is necessary for the maintenance of long-term habituation. The side of drug injection was alternated in successive experiments to eliminate side-to-side biases in injection or recording technique. Starting 1 hour after drug injection, insulated tungsten microelectrodes were used to record physiological activity at the injection sites. Comparison of simultaneous recordings in the two sides controlled for nonspecific effects or drug diffusion and for the particular songs used in any given experiment. The two sides were previously shown not to differ in habituation rates (*13*). Test stimuli were presented in the same order as during training, starting 1 hour after injection.
21. The presence and duration of the first sensitive period was not established with protocols 2 and 3 because training with a single song with either of these protocols lasted 2.2 and 2.4 hours. The second, third, and fourth sensitive periods during which injections of ACT or CYC in NCM blocked long-term habituation were very similar, regardless of whether we used protocols 1, 2, or 3 . In addition, protocols 2 and 3 revealed a fifth and sixth sensitive period of mnemogenic RNA and protein synthesis, while retaining those that had occurred earlier (Fig. 5).
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Ethylene as a Signal Mediating the Wound Response of Tomato Plants

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Plants respond to physical injury, such as that caused by foraging insects, by synthesizing proteins that function in general defense and tissue repair. In tomato plants, one class of wound-responsive genes encodes proteinase inhibitor (pin) proteins shown to block insect feeding. Application of many different factors will induce or inhibit *pin* gene expression. Ethylene is required in the transduction pathway leading from injury, and ethylene and jasmonates act together to regulate *pin* gene expression during the wound response.

The wound response of tomato plants has been studied for some 25 years and represents a model system for the analysis of cell signaling pathways in plants (*1*). Proteinase inhibitor (*pin*) genes are up-regulated throughout aerial tissues in response to wounding (*2*). *Pin* genes are also responsive to compounds applied experimentally through the transpiration stream of excised leaves and the use of this bioassay has identified a range of positive and negative regulators. Positive regulators (elicitors) include oligogalacturonide fragments of pectin polysaccharides (OGAs) (*3*), an 18mer

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