

Decrements in auditory responses to a repeated conspecific song are long-lasting and require two periods of protein synthesis in the songbird forebrain

(avian/immediate-early gene/species recognition/natural stimulus/long-term memory)

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ABSTRACT Earlier work showed that playbacks of conspecific song induce expression of the immediate early gene ZENK in the caudo-medial neostriatum (NCM) of awake male zebra finches and that this response disappears with repeated presentations of the same stimulus. In the present study, we investigated whether repetitions of a song stimulus also elicited a decrement in the electrophysiological responses in the NCM neurons of these birds. Multiunit auditory responses in NCM were initially vigorous, but their amplitude decreased (habituated) rapidly to repeated stimulation, declining to about 40% of the initial response during the first 50 iterations. A similar time course of change was seen at the single unit level. This habituation occurred specifically for each song presented but did not occur when pure tones were used as a stimulus. Habituation to conspecific, but not heterospecific, song was retained for 20 h or longer. Injections of inhibitors of protein or RNA synthesis at the recording site did not affect the initial habituation to a novel stimulus, but these drugs blocked the long-term habituation when injected at 0.5–3 h and at 5.5–7 h after the first exposure to the stimulus. Thus, at least two waves of gene induction appear to be necessary for long-lasting habituation to a particular song.

Songbirds use their songs and calls to communicate with members of their own species. Male songbirds typically learn these vocalizations from adult conspecifics during a sensitive period in development. These learned songs and calls can differ markedly between individuals, though they also share species-typical features (1–3). Much is known about the brain pathways birds use for the production of learned vocalizations (4, 5), but our understanding of circuits that discriminate and store these signals is just beginning to develop (6, 7).

One forebrain area that may play a role in perceptual aspects of vocal communication is the caudo-medial neostriatum (NCM), where playback of conspecific song is more effective than heterospecific song or non-song sounds in inducing the expression of the immediate early gene ZENK (also known as zif-268, egr-1, NGFI-A, or Krox-24) (8, 9). The ZENK response in NCM disappears when the same song is played back repeatedly, but a full ZENK response is elicited again if a novel song is presented (10). ZENK is one of a group of immediate early genes that encode transcriptional regulators thought to mediate the long-term effects of depolarization on neural activity and thus to play a role in memory formation (11–14). These observations suggest that NCM is part of a system that processes and memorizes species-specific sounds.

In the present study we recorded the electrophysiological responses of NCM neurons to conspecific and heterospecific song in the presence or absence of protein or RNA synthesis inhibitors. We describe a long-lasting decrease in responsive-

ness of NCM neurons to repetitions of the same song stimulus and suggest that this may be a good system in which to study the cellular and molecular basis of perceptual learning.*

METHODS

Experimental Animals and Surgical Procedures. Forty adult male zebra finches (*Taeniopygia guttata*) obtained from our breeding colony or from a local supplier were used. Under anesthesia, birds were surgically prepared for recording. Twenty-four to 48 h later, the awake animals were restrained for recordings and microinjections. Recording sessions typically lasted 4–5 h, conforming to an approved animal use protocol. At the end of each experiment, small electrolytic lesions were made for use in locating the recording sites. The birds were then killed and the brains were processed for histology. For all procedural details, see ref. 15.

Auditory Stimulation. The songs of 50 male zebra finches and 5 canaries from our archives were digitized at 20 kHz (Signal, Engineering Design, Belmont, MA) to provide a set of conspecific and heterospecific songs that the birds had never heard before. The zebra finch songs were 1.2–2.0 s long and contained introductory notes and one or two motifs. The canary songs consisted of partial songs with the same duration as the zebra finch songs. Other stimuli included tone sequences (seven pure tones with frequencies from 0.5 to 4 kHz in an ascending–descending series of 1.5 s total duration), words from human speech, and white noise bursts. The stimuli (peak amplitude, 75 decibels) were played from a speaker placed 0.5 m from the bird in a soundproof chamber.

The auditory stimulation protocol began by “training” the birds with 200 repetitions of a novel stimulus, delivered with an interstimulus interval of 11 or 12 s. After a delay of 0–48 h, the animals were “tested” with exposure to 100 repetitions of the same stimulus. During the delay, most birds were trained and/or tested with one or more other stimuli. For delays >4 h, the birds were trained under freely moving conditions and were only restrained during recordings.

Physiological Data Analysis. Penetrations were stereotaxically targeted at NCM, using insulated tungsten microelectrodes (Microprobe, Clarksburg, MD). Noise bursts were used as a search stimulus to locate responsive sites. Physiological signals were filtered, amplified, and digitized at 20 kHz (Experimenter's Workbench, Datawave, Longmont, CO). Each presentation of an auditory stimulus constituted a trial with a duration of 2.5 s, including a 0.5-s control period preceding stimulus onset. These trials were then analyzed for single and multiunit activity (MUA). On individual trials, MUA response magnitudes were quantified by subtracting the root-mean-

Abbreviations: ZENK, an immediate early gene known as zif-268, egr-1, NGFI-A, or Krox-24; NCM, medial caudal neostriatum; MUA, multiunit activity; CYC, cycloheximide; ACT, actinomycin D.

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square (rms) value over the control period from the rms over the response period (from stimulus onset to offset plus 100 ms). These values were then normalized to the response magnitude on the first trial with each stimulus and plotted as a function of stimulus iteration number. This relationship, which showed systematic decreases in response magnitude (see *Results*), was quantified by computing the linear regression between iteration number and normalized magnitude for the first 100 iterations in either the training or testing condition. Although the relationship was often not strictly linear, the regression slope provided a conservative index of the average rate of response decrease. Since this rate was expressed in units of percent change per trial, independent of the absolute levels of activity in any given recording, it could be used to compare responses recorded at different sites and times. To study single neurons, the spike waveforms of single units were digitally discriminated and their spike trains were quantified by subtracting the average spike rate during the control period from the average rate during the response period. Responses were summed over groups of 10 trials to reduce variability and then plotted as a function of iteration number.

Drug Injections. In experiments in which the RNA synthesis inhibitor actinomycin D (ACT) or the protein synthesis inhibitor cycloheximide (CYC) were used, simultaneous recordings were made in NCM in both hemispheres. Forty nanoliters of ACT (50 μ M) or 40 nl of CYC (1 ng/nl) dissolved in saline was injected into the recording site on one side using a glass micropipette (tip o.d., 20 μ m). Saline vehicle alone was injected into the other side to serve as a control for the injection procedure itself. Comparing simultaneous recordings between sides also controlled for diffusion or generalized effects of the drug and for differences in the effectiveness of different sound stimuli. The side of drug injection was alternated in successive experiments to avoid potential effects

based on hemispheric differences. The effectiveness of CYC was tested by an immunocytochemical assay using an antibody to the amino terminus of a rodent homolog of the ZENK protein (kindly provided by David C. Hancock, Imperial Cancer Research Fund, U.K.) and a previously described protocol (16).

Statistics. Comparisons of mean habituation rates were made using the *t* test for independent samples. A criterion level of $P < 0.05$ (two-tailed) was used for statistical significance. Drug effects were assessed by comparing the mean habituation rates obtained on the injected and control sides at each time point.

RESULTS

Auditory Responses in NCM Are Initially Vigorous But Habituate Rapidly to Repeated Stimulation. Recordings were made in awake zebra finches at 36 sites verified histologically to be in NCM (Fig. 1A). The spontaneous activity in NCM was very low. Vigorous MUA in response to auditory stimulation was observed at all sites within 600 mm of the midline, with a mean latency of 16.5 ms (SE, 0.7 ms). These responses, which were seen with all stimuli presented, consisted of an increased rate of discharge during stimulus presentation, with phasic peaks that were time-locked to the onset of most individual syllables in the case of song stimuli (Fig. 1B) or onset and offset bursts in the case of simpler stimuli. The response patterns of single units typically showed bursts associated with these phasic peaks in the MUA. However, the majority of units showed responses that were more selective than those of the population at that site, as their bursts only occurred for one or a few of the phasic peaks (not shown). These units appear to be preferentially tuned to specific acoustic features of certain song syllables. Despite this tendency to selectivity by individual

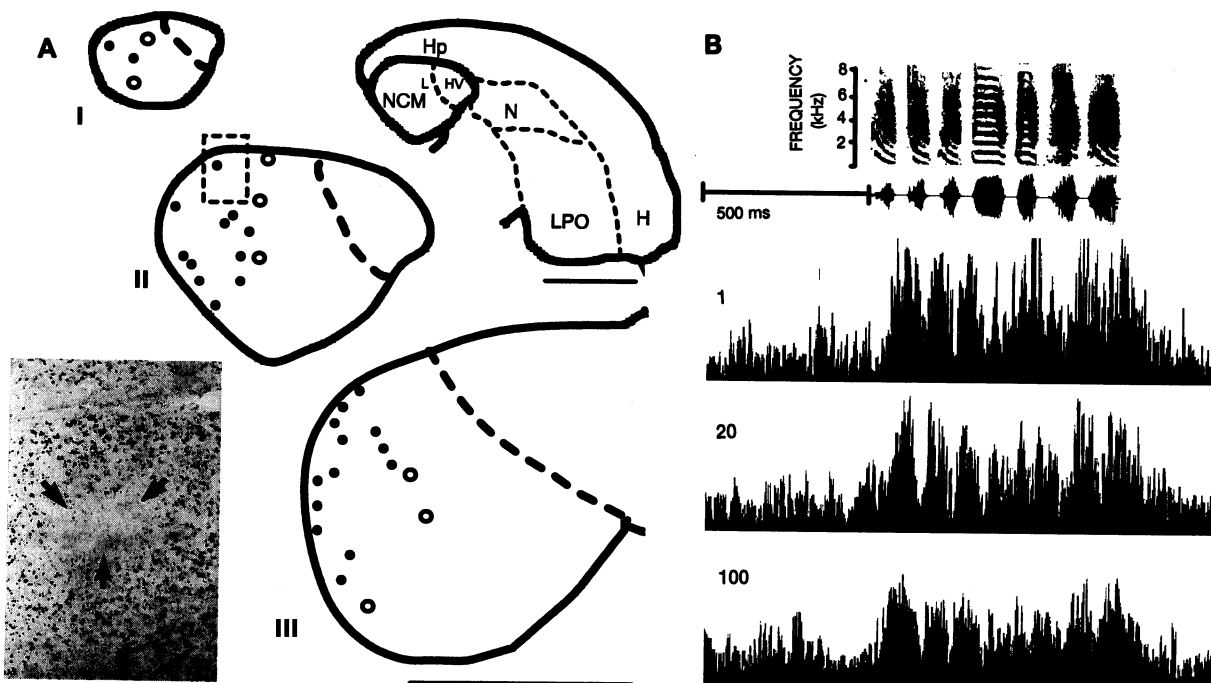


FIG. 1. (A) Location of recording sites in NCM that showed (●) or did not show (○) habituation to repeated song presentations. NCM is represented schematically at three parasagittal planes (distances from midline: I = 200 mm, II = 400 mm, III = 600 mm). Plane II corresponds to the level of the parasagittal section at the upper right. Dorsal is up and rostral is to the right. The photomicrograph (lower left) shows a section from NCM (corresponding to the hatched rectangle in II) of a metrazole-treated bird that had been reacted with an antibody to the ZENK protein. Nuclear staining is uniform throughout NCM, except at the site of a CYC injection (arrows). The unstained stripe at the top of the micrograph is the ventricular zone, which is known not to express ZENK (8). H, hyperstriatum; Hp, hippocampus; HV, hyperstriatum ventrale; LPO, lobus paraolfactorius; L, field L; N, neostriatum. (Bar = 2 mm.) (B) Multiunit responses decrease following repeated presentations of the same conspecific song. Traces from top to bottom show the sonogram of a representative zebra finch song, the amplitude envelope of the song, and the rectified multiunit recording on the 1st, 20th, and 100th song presentations.

units, strong MUA responses to all stimuli were recorded at virtually all sites, indicating that the response selectivity of the units at each site is probably heterogeneous.

During training, when the initially novel song stimulus was repeatedly presented, there was a decrease in the response magnitude with each subsequent trial (Fig. 1B). The response decreased (habituated) to about 40% over 50 trials (Fig. 2A), followed by a slower decline toward an asymptote. The pattern of habituation during training was similar for all conspecific and heterospecific songs and for human speech. In contrast, when the tone sequence stimuli were used for training, no significant decrease was observed (not shown).

Of 15 single units analyzed (at 14 sites in 13 birds), 14 decreased their firing rates with a similar time course (Fig. 2B) to the habituation function for MUA (Fig. 2A). Thus, the habituation of MUA responses observed with training can be explained by changes in the firing rates of individual cells.

Recording sites where habituation occurred were located in the dorso-caudal part of medial NCM, in penetrations up to 600 mm from the midline (Fig. 1A). Although recording sites located more rostrally and ventrally also showed vigorous auditory responses, these responses did not change with training and were not further studied. Auditory responses were not seen in the hippocampal formation that overlies NCM.

Habituation Occurs Independently and Specifically for Each Song Presented. The habituation in response magnitude after repeated presentation of a stimulus is specific to that stimulus; presentation of a novel stimulus again produces a large response, which in turn begins to habituate as this stimulus is repeated (Fig. 3). Although exposure to other songs occurred during the delay between training and testing for any particular song in this figure, the habituation level achieved during training was retained (dotted lines). Fig. 3 also shows that, at a single site, the magnitude of the initial response to different songs exhibits considerable variation.

Different Habituation Rates Distinguish Responses to Novel vs. Familiar Songs. In contrast to the rapid habituation observed with novel songs, the magnitude of responses to a stimulus that had become familiar through repeated presentations decreased very little with additional repetitions of that stimulus (e.g., Fig. 3, song A). To quantify this effect during testing, we used the linear regression slope between normalized response magnitude and iteration number to define a habituation rate. The resulting rates had different distributions for novel and familiar songs (Fig. 4, histograms). The mean

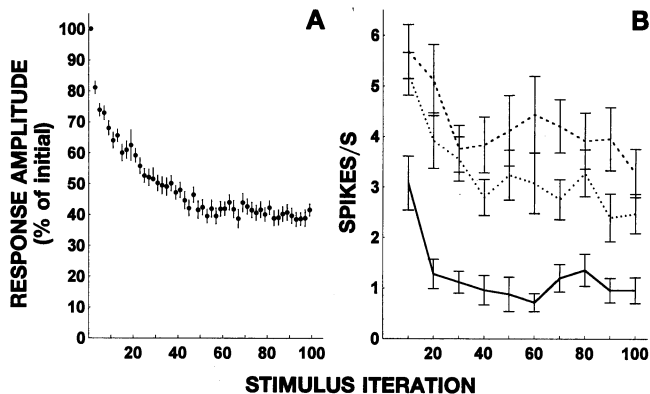


FIG. 2. (A) Mean responses (\pm SE) to repeated presentations of 50 novel conspecific songs (in 25 birds) as a function of iteration number. The MUA response to each stimulus presentation was normalized to the response on the first presentation for each song and then averaged with the normalized responses to the other songs and plotted as a function of iteration number. (B) Responses of three single units discriminated at a single recording site to repeated stimulation with one song. The mean firing rates (\pm SE) over every 10 iterations are plotted for each unit.

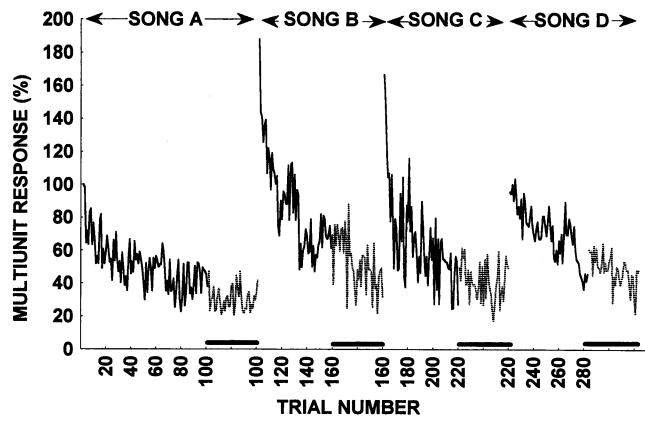


FIG. 3. MUA responses to four different songs presented sequentially (solid lines) at a single site. All responses have been normalized to the initial response to song A. Different songs elicited different initial responses but all habituated during training. Immediately after training, songs A–D were tested on trials 281–320, 321–360, 361–400, and 401–440, respectively. The responses collected during the testing period (dotted lines, dark bars on x axis) are plotted consecutively with responses collected during training for each song to show that the habituated response level achieved for a given song was retained even after training with other songs.

habituation rate for novel songs (mean, 0.350; SE, 0.008; $n = 100$ songs) was significantly greater ($t = -16.272, P < 0.0001$) than that for familiar songs tested after delays of ≤ 10 h (mean, 0.114; SE, 0.008; $n = 114$ songs). These habituation rates provided a neural correlate of whether a bird had heard a given song during the recent past. The habituation rates to novel heterospecific songs (mean, 0.380; SE, 0.04; $n = 4$ birds) and human speech (mean, 0.370; SE, 0.04; $n = 4$ birds) were similar to the rates for novel conspecific song. As noted above, responses to the tone sequence stimuli did not decrease and thus had a low habituation rate (mean, 0.061; SE, 0.05; $n = 9$ birds) not significantly different from zero ($t = 0.779, P = 0.440$).

Habituation to Conspecific Songs Is Retained for at Least 20 h. To determine how long the habituation to novel conspecific songs lasted (i.e., for how long these songs were recognized as familiar), birds were tested at various delays after initial training, and habituation rates were plotted as a function of the time between the first training presentation and the first testing presentation of each stimulus (Fig. 4). Habit-

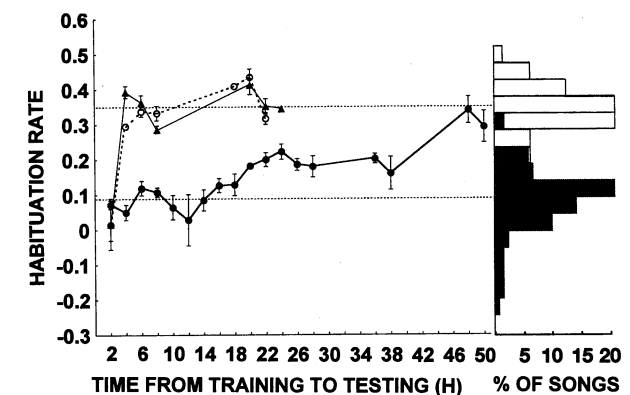


FIG. 4. The habituation rates (\pm SE) for conspecific (\bullet) and heterospecific (\circ) songs and for human speech (\blacktriangle) at various delays from training to testing are plotted. The histograms show the percent frequency distributions of habituation rates for novel songs (open bars) and familiar songs tested after ≤ 10 h (solid bars). The small area of overlap of these distributions is represented by shaded bars. The dashed horizontal lines indicate the mean habituation rates for novel (upper line) and familiar (lower line) songs.

uation rates were low when the birds were tested after delays of up to 12 h and then increased gradually. The rate tested at 10–20 h was not significantly different from the rate for familiar songs (tested at ≤ 10 h) but was significantly different at 22 h ($t = 3.084$, $P = 0.0028$) and all longer delays. The rate was significantly different from the rate for novel songs until 48 h after training, when it was no longer different, suggesting that by that time, familiar songs were being treated as novel.

In the case of heterospecific song and human speech, the rates were characteristic of novel songs as early as 4 h after training and were significantly different from the low rates for conspecific songs at that point ($t = -5.268$, $P < 0.00001$, for canary songs; $t = -6.418$, $P < 0.00001$ for human speech). The habituation rate function appears to document a process of “forgetting” by neurons in NCM and suggests that conspecific songs were quite well “remembered” for 20 h, whereas the other types of stimuli were “forgotten” as early as 4 h.

Long-Term Habituation Depends on RNA and Protein Synthesis During Two Specific Periods After Training. To examine the role of protein synthesis in long-term habituation, CYC was injected at the recording site at various times after the onset of training with novel song stimuli. Testing occurred after an average delay of 10 h (a time when songs were normally remembered; see Fig. 4). When CYC was injected at times from 0.5 to 3 h and from 5.5 to 7 h after training with particular songs, responses to these familiar songs showed high habituation rates at testing that were comparable to those for novel songs (Fig. 5). For these injection times, the rates were significantly different from the ones measured on simultaneous recordings from the contralateral NCM, which received only a saline injection (see legend to Fig. 5 for statistics); for other time points, the rates did not differ from the contralateral side. CYC only affected long-term habituation; when it was injected before or at the onset of training, auditory responses recorded at the injection site and the initial habituation rates for novel songs were indistinguishable from the normal case (not shown).

Similar results were obtained when the RNA synthesis inhibitor ACT was injected at various times after training, as evidenced by habituation rates typical of novel song (Fig. 5): ACT prevented long-term habituation when injected at 0.5–2 h and again at 5.5–6.5 h after onset of training (see legend to Fig. 5 for statistics). These periods appear to be shorter than the periods observed for CYC and terminate 0.5–1 h before the

end of the corresponding time windows during which protein synthesis was required.

Four birds were injected with CYC or ACT into one NCM and saline vehicle into the other and then were either exposed to song playback or injected with metrazole, as previously described (8, 17). To determine the effective area of protein synthesis blockade in NCM, the tissues were allowed to react with the antibody to the ZENK protein. Small electrolytic lesions placed in the pipette insertion track helped to localize the injection sites (not shown). On the saline-injected side, labeled nuclei were seen throughout NCM. On the drug-injected side, labeling was not seen in clear zones of about 400 μm in diameter centered at the site of injection (Fig. 1A *Inset*).

DISCUSSION

There is a significant decrement in the electrophysiological responsiveness of NCM neurons to repeated presentation of the same song stimulus. The habituation rate measured for each individual song stimulus depended on the animal's previous experience with that stimulus and was not affected by exposure to other stimuli. Once habituation to a familiar conspecific song set in, the relative responsiveness to that song, compared to a novel song stimulus, remained low for many hours. These findings are similar in several respects to “stimulus-specific adaptation,” a well-documented form of visual memory in the primate inferior temporal cortex (18–21). In those studies, cells showed a long-lasting and specific decline in responsiveness to repeated presentations of a complex visual stimulus.

Habituation was seen in both single and multiple unit recordings, so we infer that it occurred in the majority of the NCM neurons that responded to any particular song. However, this raises the question of how individual units, each of which responds to just a subset of the acoustic features present in any one song (22, 23), are nonetheless able to habituate only to the whole song that has been repeatedly presented. We suggest that the habituation of the NCM responses to a particular song resulted from an interaction between NCM neurons that responded to the various acoustic features of that song. For example, such an interaction would allow NCM neurons to show decreased responsiveness to a particular acoustic feature when it occurred in a familiar song but not when it occurred in a novel song. As an abstraction, we call the interactive neurons that habituate to a particular song an “ensemble”; individual neurons could be part of more than one ensemble. The high degree of interconnectivity that has been described for neurons in the caudal neostriatum (24, 25) could link the members of each NCM ensemble, though membership in these ensembles need not be restricted to NCM.

Habituation to novel conspecific and heterospecific songs was comparable, but habituation to conspecific songs lasted much longer. Tone stimuli elicited physiological responses that did not habituate. Interestingly, conspecific song is more effective in eliciting an increase in ZENK expression in NCM than heterospecific song, and pure tones do not induce ZENK at all (8). In addition, repeated presentations of the same song lead to an extinction of the ZENK gene response in NCM, but presentation of a novel song fully reactivates ZENK expression (10). Thus, the stimuli that are most effective in evoking ZENK expression in NCM also lead to the longest-lasting habituation. These results point to a correlation between ZENK expression and long-term modification of neuronal responsiveness to song.

Similarly to other systems where long-lasting neuronal plasticity has been studied in relation to learning (26), such as hippocampal long-term potentiation (27), the long-term habituation to conspecific song depended on RNA and protein synthesis during a defined period that began at the onset of training. In our paradigm, we have also demonstrated a second

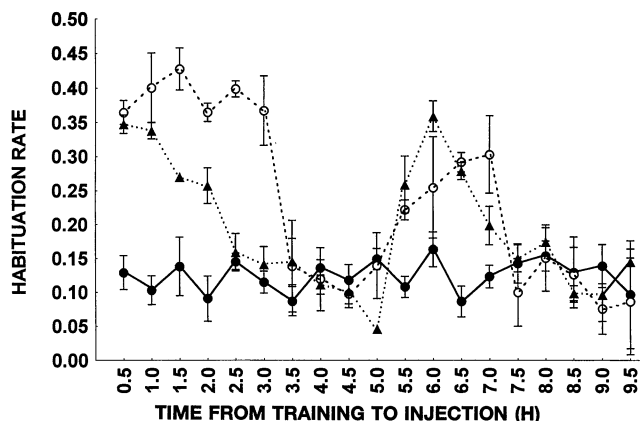


FIG. 5. Effect of local injections of CYC (○), ACT (▲), or saline (●) on habituation in NCM. The habituation rates (\pm SEM) are plotted as a function of the interval from onset of training to injection. For CYC injections at 0.5–3.0 h, 5.5 h, and 6.5–7.0 h, the rate was significantly different ($t = -2.68$ to -9.66 , $P = 0.023$ – 0.0004 , two-tailed) from the rate for control saline injections. For ACT injections at 0.5–2 h and 5.5–6.5 h, the rate was significantly different ($t = -2.74$ to -6.58 , $P = 0.030$ – 0.00008 , two-tailed) from the rate for control injections.

distinct period where blocking agents disrupt long-term habituation. This implies that there are two waves of necessary RNA and protein synthesis, separated by a gap during which blockers are ineffective. The occurrence of two waves of protein synthesis has also been suggested in another study of learning phenomena (28).

It is possible that the short-term habituation to song occurred primarily elsewhere and that the responses in NCM merely recorded those changes. This can be tested by determining whether neurons that project to NCM (9, 24, 25) also habituate to a repeated song. Nevertheless, our data showed that local injections of CYC and ACT affected the long-term habituation in NCM. We suggest that some of the molecular and biochemical machinery necessary for long-term habituation to a particular conspecific song resides within cells in NCM itself and could regulate the long-term interactions between members of a putative ensemble. ZENK may be one of the genes necessary for this form of long-term habituation.

In summary, we have shown that the electrophysiological response of NCM neurons to a complex species-specific stimulus habituates with repetition and that the long-term habituation depends on local protein and RNA synthesis during two discrete time windows after training with a novel stimulus. In addition, there is a correlation between the classes of stimuli that produce habituation and those that induce ZENK expression in NCM neurons. These observations strongly suggest that NCM plays an essential role in the establishment of long-lasting memories for conspecific vocal signals and that the expression of specific genes, such as ZENK, is an important component of this process. These results, coupled with our knowledge of song behavior, make this a powerful system for studying the cellular and genomic basis of perceptual phenomena involved in vocal communication.

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