Song Tutoring Triggers CaMKII Phosphorylation within a Specialized Portion of the Avian Basal Ganglia

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Received 25 May 2005; accepted 13 June 2005

ABSTRACT: **In several songbird species, a specialized anterior forebrain pathway (AFP) that includes part of the avian basal ganglia has been implicated specifically in song learning. To further elucidate cellular mechanisms and circuitry involved in vocal learning, we used quantitative immunoblot analysis to determine if early song tutoring promotes within the AFP phosphorylation of calcium/calmodulin-dependent kinase II (CaMKII), a multifunctional kinase whose phosphorylation at threonine 286 is critical for many forms of neural plasticity and behavioral learning. We report that in young male zebra finches likely to have begun the process of song acquisition, brief tutoring by a familiar conspecific adult promotes a dramatic increase in levels of phosphorylated CaMKII (pCaMKII) in Area X, the striatal/pallidal component of the AFP. In contrast, pCaMKII levels in this region were not elevated if 1) the tutor did not sing, 2) the tutor sang but was visually isolated from the pupil, or 3) the tutor was an unfamiliar adult. In young males that had not previously heard any conspecific song, first exposure to a song tutor produced a more modest, but significant rise in pCaMKII levels. Young females (who do not develop song behavior) did not exhibit any effect of tutoring on pCaMKII levels in that portion of the basal ganglia that corresponds to Area X in males. These data are consistent with the hypothesis that Area X participates in encoding and/or attaching reward value to a representation of tutor song that is accessed later to guide motor learning.**

 J Neurobiol 65: 179–191, 2005

Keywords: **CaMKII; birdsong; learning and memory; plasticity; phosphorylation; basal ganglia**

INTRODUCTION

Vocal learning has evolved in only a few vertebrate species, and perhaps our best understanding of the neural substrates underlying vocal imitation comes from research on avian song learning. Birdsong comprises a well-ordered sequence of vocal motor gestures whose development shares features of human speech acquisition (Doupe and Kuhl, 1999). For example, both exhibit striking stimulus selectivity and a sensitive period when learning is optimized, and both entail forming perceptual memories that, together with auditory feedback, guide later vocal production. During *sensory acquisition,* songbirds memorize external song templates. Then, during *sensorimotor learning,* they practice singing and use auditory feedback to gradually adjust their vocalizations to match the stored template(s). Importantly, sensory acquisition and sensorimotor learning in songbirds are separable processes. In some species they are distinctly non-overlapping (Marler and Peters, 1981, 1982), and in species where they do overlap, successful imitation can be achieved even

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Contract grant sponsor: National Institute of Mental Health; contract grant number: MH45906.

Contract grant sponsor: National Science Foundation; contract grant number: IBN-9983338.

Published online 19 August 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/neu.20198

when acquisition is deliberately offset from sensorimotor learning (Bohner, 1990; Funabiki and Konishi, 2003).

The neural circuits controlling song behavior have been described in great detail (Nottebohm et al., 1976; Reiner et al., 2004; Wild, 2004), but our understanding of how this system mediates song development is rudimentary. The circuitry includes a motor pathway that controls vocal production and as yet undefined aspects of vocal learning (Nottebohm et al., 1976; Simpson and Vicario, 1990; Wild, 2004), and a basal ganglia-thalamo-pallial loop (or ''anterior forebrain pathway,'' AFP) that is critical for normal song development, but not essential for the production of stable song in adulthood (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). The AFP consists of Area X (a specialized striatal-pallidal component of the avian basal ganglia), its efferent target DLM (dorsolateral medial nucleus of the thalamus), and the lMAN (lateral magnocellular nucleus of the anterior nidopallium), a principal target of DLM projections (Perkel, 2004). This circuit forms a closed loop via projections from lMAN back to Area X (Nixdorf-Bergweiler et al., 1995), but also provides a polysynaptic pathway that links two nuclei of the descending vocal motor pathway, HVC (acronym used as proper name) and RA (robust nucleus of the arcopallium). Area X receives a large projection from HVC that likely conveys both motor and auditory information to the AFP (Doupe and Konishi, 1991; Hessler and Doupe, 1999; Mooney et al., 2002), and lMAN provides the primary output of the AFP via projections to RA. Finally, Area X also receives a large dopaminergic input via projections from the ventral tegmental area (Lewis et al., 1981; Bottjer, 1993; Soha et al., 1996).

Given the established role of cortico-basal ganglia loops in motor sequence learning, it is not surprising that the AFP plays a key role in sensorimotor learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). For instance, lesioning either Area X or lMAN disrupts the normal trajectory of sensorimotor learning; the former manipulation prevents normal song crystallization, and the later promotes premature song stability (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). However, more recently this pathway also has been implicated in sensory acquisition. Neurons within lMAN and Area X respond selectively to *both* tutor song and the pupil's song when these are made to differ by peripheral manipulations (Solis and Doupe, 1999, 2000), suggesting that neurons within these regions encode, or at least have access to, the acquired song template. Furthermore, in young zebra finches, both lMAN and Area X neurons exhibit N-methyl-d-aspartate receptor (NMDAR) dependent long-term potentiation (LTP) (Boettiger and Doupe, 2001; Ding and Perkel, 2004), and song learning is impaired when an NMDAR antagonist is infused in the vicinity of lMAN just prior to tutoring sessions (Basham et al., 1996). Importantly, identical infusions offset from tutoring fail to disrupt song development. These results suggest that NMDAR-mediated processing or plasticity within the AFP contributes specifically to normal template encoding or storage.

One useful strategy for determining how specific brain regions contribute to song learning has been to search for molecular markers of neuronal plasticity in the context of specific aspects of vocal learning (Clayton, 1997). Here, we adopted this approach to investigate whether song tutoring promotes the phosphorylation of Ca^{2+}/calmodulin dependent protein kinase (CaMKII) in Area X of juvenile zebra finches, a species in which only males produce learned song. Influx of Ca^{2+} through NMDARs can activate CaMKII through the binding of Ca^{2+}/calmodulin, and activation of adjacent CaMKII subunits stimulates autophosphorylation at threonine 286 (Thr286) to render the kinase constitutively active. Because Thr286 phosphorylation of CaMKII is critical for NMDAR-mediated synaptic plasticity and many forms of learning (Lisman et al., 2002; Colbran and Brown, 2004), we hypothesized that song acquisition may invoke similar CaMKII-dependent plasticity within the AFP. Immunocytochemistry revealed relatively low levels of total CaMKII within most song nuclei, with the exception of Area X. Therefore, we focused on this component of the AFP to assess whether brief presentations of tutor song promote the phosphorylation of CaMKII. Using an antibody that specifically recognizes CaMKII phosphorylated at threonine 286 (pCaMKII), Western immunoblot analyses revealed a stimulus-specific, sexually dimorphic elevation of pCaMKII levels within Area X of the AFP that is consistent with the hypothesis that this specialized portion of the basal ganglia encodes and/or stores a representation of song that will be used to guide vocal motor learning.

METHODS

Subjects and Behavioral Methods

All male and female zebra finches *(Taeniopygia guttata)* were bred and raised in our laboratory on a 14:10 light: dark cycle. In this species, sensory acquisition is limited to an early sensitive period that extends from about posthatch

d25–65 (Immelmann, 1969; Price, 1979; Eales, 1985, 1987). In the first set of experiments, we used aviary-reared juveniles so that males had the opportunity to begin sensory acquisition before being assigned to an experimental group. Aviary-reared birds were raised until posthatch d30 in free flight aviaries containing six breeding pairs and their offspring. From d30–35, juveniles were housed in individual sound isolation chambers. In the second set of experiments, we used birds that had no prior exposure to song before being assigned to an experimental group. These ''early isolates'' were removed from the flight aviaries and placed in a cage with both parents when the oldest in the clutch reached d9. The following day, the father was removed and the chicks and mother were placed together in an individual sound isolation chamber. As juveniles reached d30, they were removed and individually housed in an isolation chamber until d35.

On d35, juveniles were placed for 2 h adjacent to a stimulus female. Aviary reared birds were housed in one of the following five conditions: 1) ''untutored controls'' were in a room that did not contain any potential song tutors; 2) ''father-tutored'' and 3) ''unfamiliar-tutored'' pupils were caged with an adult male tutor (their father or unfamiliar tutor, respectively) who sang during the tutoring session; 4) ''father: no song'' pupils were caged with their father who did not sing during the 2-h experimental session; and 5) ''father: hearing only'' pupils were separated by a visual barrier from their father who sang during the tutoring session. Early isolates were assigned to one of three conditions: 1) ''untutored controls,'' 2) ''unfamiliar-tutored,'' or 3) ''unfamiliar: no song.'' Males and females (both aviary reared and early isolates) were included in conditions 1 and 2; only males were run in the remaining conditions. Tutoring sessions began at the onset of the light cycle to ensure that juveniles had not sung during the hours immediately preceding the experimental session. Pupils and their tutor were housed behind a unidirectional microphone interfaced to a sound-activated recording system (Avisoft-Recorder, Avisoft Bioaccoustics, Berlin, Germany) with triggering configuration set to parameters previously confirmed to capture any subsong or tutor song produced. At the onset of each recording session, a human observer monitored the real-time spectrographic display to log when the first tutor song was produced (this set the clock for the 2-h tutoring session) and to ensure that the computer was accurately capturing and saving audio files. Tutors typically engaged in song behavior immediately after the lights were illuminated. Tutoring sessions were terminated 2 h after the onset of tutor song (or after placement into the experimental paradigm for those groups that were not tutored).

Song Bout Quantification

Adult zebra finch song consists of a sequence of notes (most with a clear harmonic structure) that are organized into a motif or phrase that is usually repeated several times. Songs usually, but not always, are initiated by a series of repeated introductory notes. In contrast, subsong is an immature song pattern that juveniles begin to produce around 30 days of age. Subsong is highly variable with individual notes lacking in clear harmonic structure and generally much longer than calls or song notes of adults. To quantify song behavior, all digitized files were analyzed manually using spectrographic analysis software (Avisoft-SASlab Pro). Because our sensitive configuration parameters resulted in many files that lacked song (triggered by calls or wing beats), files first were screened to discard files lacking song. During this screening, files were also carefully examined for episodes of subsong. No subsong occurred in these experimental conditions, although subsong was readily recorded in other experiments using these same recording parameters. Next, files were reviewed to count the total number of tutor song bouts in a recording session. For this purpose, a song bout was operationally defined as one or more song phrases followed by at least 1 s of baseline energy that did not contain any song notes or phrases. Finally, the time stamp of the last tutor song was recorded to determine the time lapsed between that song and the time of sacrifice.

Tissue Preparation and Western Blotting

Quantitative immunoblots were run on tissue punches collected from the dorsolateral portion of medial striatum that corresponds to Area X in males. Peroxidase-based immunocytochemistry run on tissue sections from tutored juveniles revealed intense CaMKII immunoreactivity (CaMKII-IR) within Area X but not in lMAN, HVC, or RA. Area X was rich in immunoreactive perikarya and neuropil and, in most animals, the boundaries of this nucleus were discernible because immunoreactivity was slightly higher in Area X than in the surrounding striatum (see Fig. 1). In contrast, lMAN, HVC, and RA all were clearly identifiable because of a rather abrupt transition from CaMKII-IR that was low within the nucleus to relatively high in the surrounding neuropil. Because these other song nuclei are relatively small and surrounded by areas rich in CaMKII-IR, it was not possible to obtain from them micropunches that were acceptably free of contamination from surrounding tissue.

Juveniles used for biochemical experiments were immediately decapitated after the experimental session on d35. Brains were rapidly harvested and frozen on dry ice (within 1–2 min of decapitation), and stored at -70°C. They then were equilibrated to cryostat temperature (-10 to -15°C) and cut (90 μm) in the coronal plane. Starting with the first section after Area X became visible (approximately 1.8 mm from the anterior pole of the brain), bilateral punches (2 mm diameter) centered on Area X in males were collected from six consecutive sections while still positioned on the cryostat platform. Because Area X is not discernable in females, punches were taken in this sex from the comparable portion of the medial striatum in which Area X resides in males. For each animal, frozen punches were collected in an Eppendorf tube kept in the cryostat, and then stored at -70°C for later use. Tissue was kept frozen at all times to maintain the phosphorylation state of CaMKII. Sections from which punches were taken, along with several sections

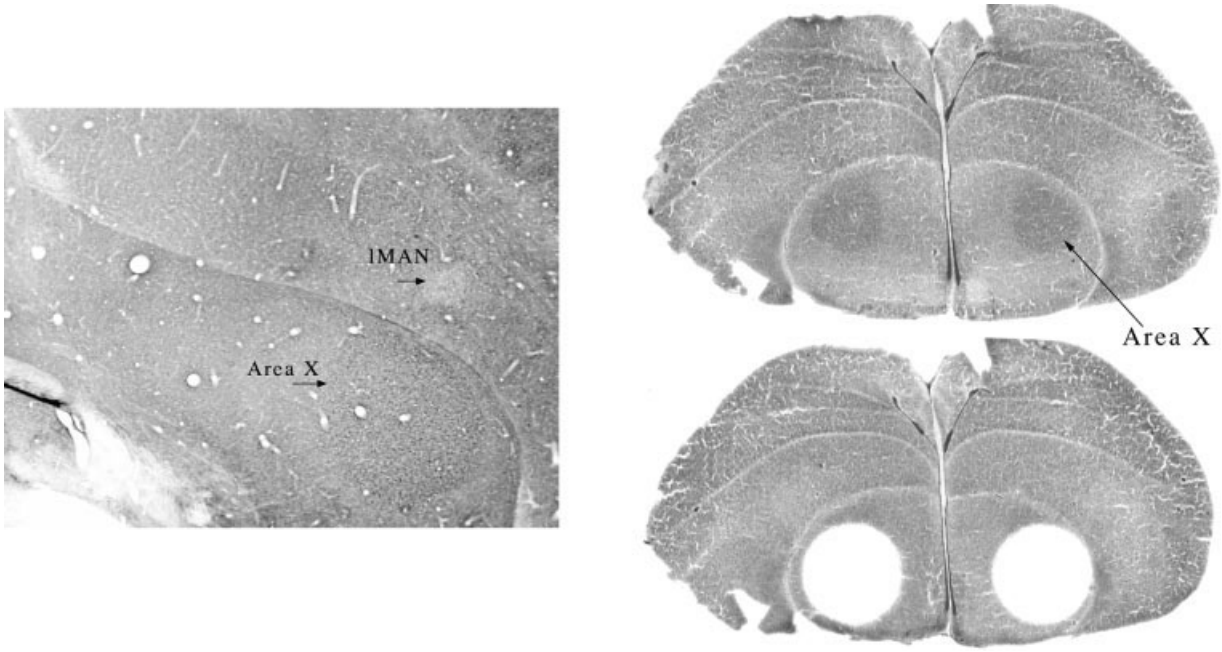

Figure 1 Sections through Area X of a juvenile male zebra finch. On the left (sagittal section), CaMKII immunocytochemistry shows significant levels of CaMKII expression within Area X, and a virtual lack of expression in lMAN. On the right (coronal sections), two adjacent Nissl stained sections show the location of Area X (top) and 2-mm micropunches (bottom) used to collect samples for immunoblot analysis.

both anterior and posterior to those punched, were mounted on Vectabond-coated slides, treated with a 4% formaldehyde solution and Nissl stained so that punch accuracy could be assessed. Animals used for immunoblot analysis included only those cases in which all punches included a substantial portion of the dorsolateral medial striatum (see Fig. 1) and did not extend dorsal or lateral to the surrounding pallial-subpallial lamina.

Frozen punches were suspended in sample buffer consisting of a 1:1 ratio of lysis buffer (100 m*M* Tris-HCl (pH 6.8) with 4% sodium dodecyl sulfate (SDS)) and cold suspension buffer containing a battery of protease and phosphatase inhibitors [100 m*M* NaCl, 10mM Tris-HCl (pH 7.6), 1 m*M* ethylenediminetetraacetic acid (EDTA), 2 m*M* 1,4-Dithiothreitol (DTT), 100 μg/ml phenylmethylsulfonyl sulfide (PMSF), 2 μg/ml Aprotinin, and 1% phosphatase inhibitor cocktail I (Sigma, St. Louis, MO)]. Samples were immediately sonicated briefly on ice, vortexed, and incubated at 55°C for 15 min to dissolve the protein. Protein concentrations were determined using the DC Protein Assay (Bio-Rad, Hercules, CA). For Western blots, 12 μg total protein of each sample was brought to a volume of 15 μl by adding sample buffer, and then 15 μl of 2× Laemmli buffer (100 m*M* Tris-HCl (pH 6.8), 20% glycerol, 6.6% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) was added. Samples were heated in a boiling water bath for 5 min, and then resolved by discontinuous SDS-polyacrylamide gel electrophoresis on 10% pre-cast gel (Bio-Rad, Ready Gel) at 100–150 V for 1 h. Samples from four control (untutored) and four experimental birds were always run on each gel, and groups were matched for the storage age of the tissue. Separated proteins were transferred onto PVDF membranes (Bio-Rad, Immuno-Blot) at 100 V for 1.5 h in chilled transfer buffer (25 m*M* Tris, 192 m*M* glycine, 15% methanol; pH 8.3). Following transfer, PVDF membranes were briefly washed in 0.1 *M* Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and blocked for 2 h at room temp in 5% non-fat dry milk (Bio-Rad) dissolved in TBST with slight agitation.

For pCaMKII detection, membranes were incubated overnight at 4°C in blocking solution with anti-ACTIVE CaMKII rabbit polyclonal antibody (1:5000; Promega, Madison, WI), which recognizes the multifunctional CaMKII phosphorylated on Thr286, and does not bind to the non-phosphorylated form of CaMKII. After incubation in primary antibody, membranes were washed in TBST (4 × 10 min) and then incubated in donkey anti-rabbit IgG HRP-linked secondary antibody (1:15000; Amersham Biosciences UK Limited, Buckinghamshire, UK) for 2 h at room temp with slight agitation. Membranes then were washed in TBST (3 × 10 min) and in TBS (2 × 5 min). Proteins were visualized using chemiluminescence (Amersham Biosciences: ECL plus). After film exposure, membranes were briefly rinsed in water, stripped for 15 min with 0.2 N NaOH, and rinsed briefly again with water. The incubation and detection processes then were repeated, first using Anti-CaMKII Kinase IIα rabbit polyclonal antibody (1:15000; Sigma) which recognizes both phosphorylated and unphosphorylated CaMKIIα and then with Anti-Actin

rabbit polyclonal antibody (1:2000; Sigma). Titration experiments using serial dilutions of protein identified appropriate antibody dilutions and verified that signals for each protein were within the linear range of measurement.

In vitro autophosphorylation experiments with whole forebrain homogenates from mouse (positive control) and zebra finch were conducted to test the phospho-specificity of the anti-ACTIVE CaMKII antibody. Forebrain tissue was homogenized in 25 m*M* benzamidine, 20 m*M* Tris-HCL, 10 m*M* sodium phosphate, 2 m*M* EGTA, 2 m*M* EDTA, 2 m*M* DTT, 1 m*M* PMSF, 25 μg/ml soybean trypsin inhibitor, 10μg/μl Aprotinin, and 5μg/μl leupeptin. Samples were centrifuged (2500 rpm, 8 min), and supernatant was collected, assayed for protein concentration, and then frozen (-80°C). To promote autophosphorylation, samples were placed in a phosphorylation buffer (final concentration: 50 m*M* HEPES, 10 m*M* $MgCl_2$, 1.0 m*M* DTT, 1.0 m*M* $CaCl_2$, 5 μM ATP, 0.5 μg/ml calmodulin, and 0.5% phosphatase inhibitor cocktail I) and kept on ice for 15 min. Additional samples were placed in a control buffer (final concentration: 50 m*M* HEPES, 10 m*M* $MgCl_2$, 2.0 m*M* EGTA, 1.0 m*M* DTT, 0.5% phosphatase inhibitor cocktail I) and either kept on ice for 15 min or kept at room temp for 15 min to augment dephosphorylation. For each sample, 50 μg (12.5 μl) total protein then was diluted with 20.5 μl of water and 67 μl of Laemmli buffer, boiled for 5 min, chilled, and 30 μl aliquots were run on SDS gels. Proteins were transferred and membranes were processed as described above.

Densitometric analysis of signal intensity was performed using NIH Image (http://rsb.info.nih.gov/nih-image/). To control for slight differences in evenness of protein loading across individual lanes, both pCaMKII and total CaMKII (tCaMKII) values were normalized to actin. Also, for each animal, the ratio of pCaMKII:tCaMKII was calculated. To control for differences between experiments that could affect the absolute intensity of the protein signals analyzed (e.g., optimal exposure time), each measurement from a given gel was expressed as a percentage of the average of the control animals run on that same gel. Furthermore, statistical comparisons were made only between groups that were run directly against one another within a gel. Non-homogeneity of variance, and the presence of outliers with potential biological significance (e.g., learning under these tutoring conditions is itself variable) dictated the use of Mann–Whitney *U*-tests for comparison between means.

RESULTS

In zebra finch brain, the Promega Anti-ACTIVE CaMKII polyclonal antibody detected a ~52 kDa protein (α-pCaMKII subunit) with a substantially weaker signal at ~58 kDa (β-pCaMKII). As shown in Figure 2, *in vitro* phosphorylation experiments confirmed that immunoreactivity to this phospho-specific antibody was absent in both zebra finch and mouse brain homogenates allowed to dephosphorylate at room temperature (lane 1), low in unstimulated homogenates kept on ice (lanes 2 and 6), and high in homogenates in which autophosphorylation was enabled through the addition of ATP, calcium, and calmodulin (lanes 3–5 and 7–8). Specificity of the Sigma anti-actin and anti-CaMKII-α antibodies also was confirmed: each detected in zebra finch brain homogenates a single protein band of the expected size (~42 kDa, and ~52 kDa, respectively; data not shown).

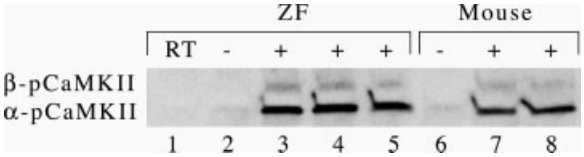

Figure 2 Immunoblots from phosphorylation/dephosphorylation experiments using zebra finch (ZF) and mouse brain homogenate probed with an antibody that targets CaMKII phosphorylated on Thr286. Condition is indicated at the top and lane number is indicated at the bottom. Lane 1: Homogenate dephosphorylated at room temperature (RT). Lanes 2 and 6: Unstimulated (−) homogenate kept on ice. Lanes 3–5 and 7–8: Homogenates kept on ice and stimulated (+) with ATP and calcium/calmodulin.

pCaMKII Signaling in Aviary-Reared Juveniles

In aviary-reared males, pCaMKII levels in Area X were nearly 10-fold greater in birds tutored for 2 h by their father than in untutored controls ($p < 0.02$, U = 5.0; see Fig. 3). Importantly, none of the juveniles produced subsong during the tutoring session, thus the observed group difference in pCaMKII protein levels did not relate to vocal practice. Variability in pCaMKII levels also was significantly greater in father-tutored as compared to control birds ($p < 0.0001$, F (6,6) = 83.06). Although the live tutoring paradigm employed here produced substantial variation in the number of song bouts heard during the 2-h tutoring session (mean ± S.E.M. = 13 ± 4; range = 3–35), the amplitude of the pCaMKII signal did not relate systematically to the total number of tutor bouts heard ($p > 0.50$), or to the number of tutor bouts heard during the last 30 (range = 1–13) or 60 min (range = 1–17) prior to sacrifice. Whereas pCaMKII levels were higher in father-tutored vs. untutored birds, total CaMKII protein levels were similar in the two groups. Thus, when expressed as a proportion of total CaMKII, pCaMKII levels were eightfold higher in father-tutored as compared to control birds ($p < 0.01$, U = 4.0).

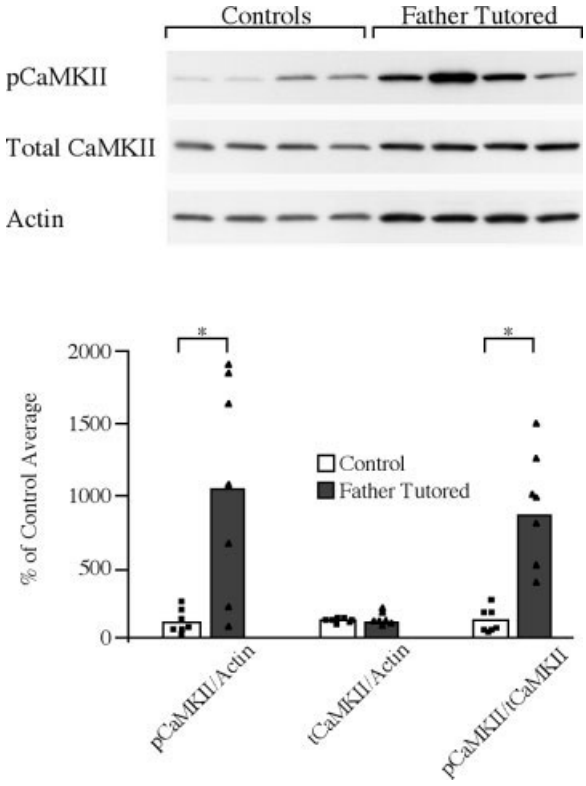

Figure 3 Tutoring by a familiar male (father) elevates pCaMKII levels in Area X of aviary-reared males. Top panel shows representative immunoblots from a single experiment comparing 35d-old untutored and father-tutored males that had been previously isolated at 30d. Each lane consists of Area X homogenate from an individual bird. The membrane was probed sequentially with antibodies specific to pCaMKII, total CaMKII (tCaMKII), and actin. Bar graph shows group means (individual data superimposed) expressed relative to average intensity in untutored controls run on the same gel. Two hours of tutoring by father markedly increased pCaMKII (left), but not total CaMKII (middle) protein levels within Area X. The proportion of tCaMKII that was phosphorylated (pCaMKII/tCaMKII) also was significantly greater in father tutored as compared to untutored birds. *$p < 0.02$.

Tutored males experienced a variety of stimuli that untutored controls did not encounter, and so we next tested whether song behavior was a necessary aspect of the stimulus set that elevates pCaMKII in Area X. As shown in Table 1, measures of pCaMKII, total CaMKII, and the pCaMKII/total CaMKII ratio in juvenile males housed with their father in the absence of song (Father: No song) were not significantly different from those in untutored controls. Thus, 2 h of social interaction with a non-singing father is not sufficient to increase pCaMKII in Area X.

Next, we explored whether hearing song alone is a sufficient stimulus for elevating pCaMKII in Area X. Zebra finches do not readily imitate a tutor's song if that tutor is visually separated from the pupil (Eales, 1989; Morrison and Nottebohm, 1993), and so we tested whether 2 h of song exposure would alter pCaMKII levels in Area X of juvenile males that could hear, but not see their father. As shown in Table 1, hearing father's song was not sufficient to elevate pCaMKII levels in Area X. Pupils heard a similar number of song bouts (18 ± 4 , range = 7–35) as did birds caged with their father during tutoring, however pCaMKII levels in the ''hearing only'' group of males were actually 45% lower, while total CaMKII levels were about 40% higher than their untutored controls. Although neither of these group differences was statistically significant ($p = 0.10$ ($U = 16$) and $p = 0.06$ ($U = 14$), respectively), the ratio of pCaMKII/total CaMKII was significantly *depressed* in the ''hearing only'' treatment group compared to the untutored controls ($p < 0.02$, $U = 10$).

In order to gain insight into the functional significance, and circuitry underlying, the tutoring-induced pCaMKII signal, we next tested whether tutoring promotes in young females a pCaMKII signal in the dorsolateral portion of the medial striatum that corresponds to Area X in males. Female zebra finches do not produce learned song, and the neural circuitry that supports song behavior is markedly reduced in this sex (Wade and Arnold, 2004). In fact, Area X is not histologically distinct in female zebra finches, and this part of the striatum does not receive the large afferent projection that connects the vocal motor pathway (via HVC) to Area X in males (Simpson and Vicario, 1991; Burek et al., 1993). Yet, females develop a sexual preference that is based in part on father's song, and this preference is established during an early developmental period that overlaps with the sensitive period for sensory acquisition in males (Miller, 1979; Clayton, 1988). As shown in Table 1, aviary-reared females tutored by their father (# song bouts = 15 ± 3; range = 9–30) for 2 h on d35 did not exhibit significantly altered pCaMKII levels in the dorsolateral portion of medial striatum. Levels of total CaMKII and the ratio of pCaMKII/total CaMKII also were not significantly different between tutored and untutored females.

One final manipulation with aviary-reared males revealed that the pCaMKII signal in Area X discriminates between a familiar and unfamiliar tutor. While tutoring by father elevated pCaMKII levels in Area X, tutoring by an unfamiliar tutor did not. As shown in Figure 4, pCaMKII levels in males tutored by an unfamiliar male were not significantly different from untutored controls (the same controls used in experi-

Table 1 Summary of Treatment Effects in Aviary-Reared and Early Isolated Male and Female 35-Day-Old Zebra Finches[1]

Variable		pCaMKII	tCaMKII[2]	pCaMKII/tCaMKII
Aviary-reared				
Males				
Father-tutored	Untutored (N = 7)	100 ± 31	100 ± 7	100 ± 32
	Tutored (N = 7)	1024 ± 286*	117 ± 17	841 ± 203*
Father: no song	Untutored (N = 8)	100 ± 42	100 ± 10	100 ± 41
	Tutored (N = 8)	83 ± 39	131 ± 16	65 ± 27
Father: hearing only	Untutored (N = 8)	100 ± 21	100 ± 16	100 ± 18
	Tutored (N = 8)	55 ± 16	138 ± 11	38 ± 14*
Unfamiliar-tutored	Untutored (N = 7)	100 ± 28	100 ± 12	100 ± 33
	Tutored (N = 8)	133 ± 38	211 ± 27*	50 ± 12
Females				
Father-tutored	Untutored (N = 7)	100 ± 23	100 ± 11	100 ± 23
	Tutored (N = 7)	115 ± 34	108 ± 13	112 ± 36
Early isolate				
Males				
Unfamiliar-tutored	Untutored (N = 9)	100 ± 19	100 ± 7	100 ± 18
	Tutored (N = 12)	279 ± 115†	125 ± 14	250 ± 99
Unfamiliar: no song	Untutored (N = 8)	100 ± 26	100 ± 21	100 ± 32
	Tutored (N = 7)	38 ± 9	82 ± 6	35 ± 8
Females				
Unfamiliar-tutored	Untutored (N = 7)	100 ± 12	100 ± 10	100 ± 10
	Tutored (N = 5)	109 ± 27	101 ± 7	106 ± 29

[1] Data shown are mean ± S.E.M. (expressed as % of average signal intensity in untutored controls run on the same gel).
[2] tCaMKII is total CaMKII.
* $p \leq 0.02$; † $p \leq 0.05$.

ments with father-tutored males). Interestingly, the amplitude of the pCaMKII signal in males tutored by an unfamiliar male was positively correlated ($r^2 = 0.65$, $p < 0.02$) with the number of tutor bouts heard (mean song bouts = 25 ± 4; range = 13–43). Given this relationship, the lack of an overall effect of tutoring on pCaMKII levels is particularly notable since the mean # song bouts heard was nearly 2× greater in this data set than in birds tutored by their father. In contrast to pCaMKII, total CaMKII levels in tutored birds were twofold higher than in the untutored controls ($p < 0.01$, U = 5). This elevation resulted in a nonsignificant ($p = 0.33$) decrease in the pCaMKII/total CaMKII ratio in birds exposed to an unfamiliar tutor. Thus, whereas tutoring by a familiar male (i.e., father) increased levels of phosphorylated CaMKII nearly 10-fold, tutoring by an unfamiliar male did not promote any significant change in levels of this phosphoprotein.

pCaMKII Signaling in "Early Isolates"

The data described above suggests that the early song tutoring in aviary-reared birds modifies neural circuitry such that subsequently, tutoring by a familiar rather than unfamiliar tutor more effectively triggers pCaMKII signaling within Area X. To determine whether initial song tutoring provokes pCaMKII signaling, we tested whether naive song circuitry mounts a detectable pCaMKII signal in response to tutoring by an unfamiliar male. In males that were isolated prior to the onset of vocal learning (by d9), exposure to a song tutor on d35 produced a threefold increase in average pCaMKII levels within Area X (Fig. 5, $p < 0.05$, U = 25). Variability in pCaMKII levels of tutored early isolates was significantly greater than in untutored controls ($p < 0.0001$, F(11,8) = 48.79), but this difference was due to one tutored bird (20 song bouts heard) with pCaMKII levels that were 5.5 × higher than the group average. Interestingly, in contrast to the pattern observed in aviary reared birds tutored by an unfamiliar male, pCaMKII levels in early isolates tutored by an unfamiliar male did not vary systematically with the number of song bouts heard (mean = 32 ± 5; range = 10–70). In fact, among these early isolates, pCaMKII levels actually tended to be lower in those that heard more than the mean number of song bouts (range = 36–70) as compared to those that heard fewer song bouts (range = 10–30). In contrast to the tutoring-induced increase in pCaMKII levels, there was no effect of tutoring on

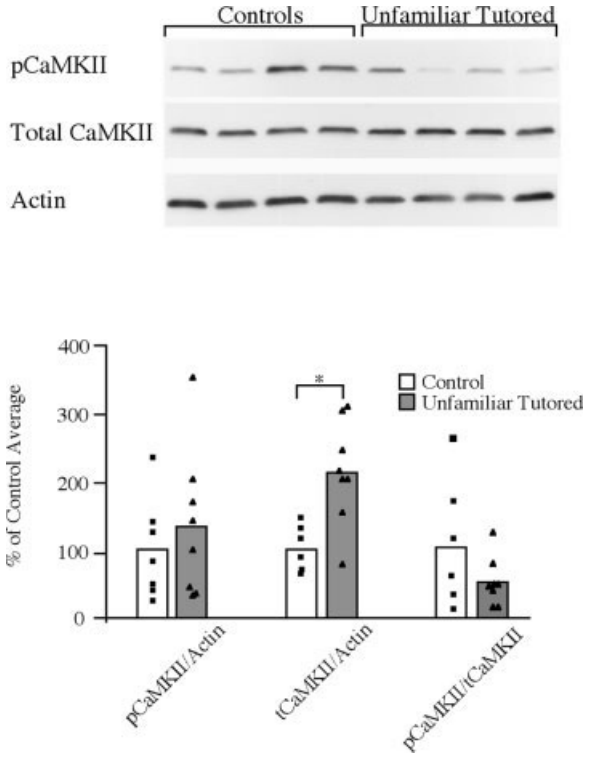

Figure 4 Tutoring by an unfamiliar male does not elevate pCaMKII in aviary-reared males. Top panel shows a representative immunoblot from a single experiment comparing 35d-old untutored and unfamiliar-tutored males that had been previously isolated at 30d. Each lane consists of Area X homogenate from an individual bird. Bar graph shows group means (individual data superimposed) expressed relative to average intensity in untutored controls run on the same gel. Two hours of tutoring by an unfamiliar male did not significantly alter pCaMKII (left) levels in Area X. Total CaMKII (middle) in the tutored group was elevated above control levels, but there was not a significant group difference in the proportion of pCaMKII/tCaMKII. $*p < 0.01$.

total CaMKII levels, and although the ratio of pCaMKII/total CaMKII in tutored males was nearly threefold higher than in untutored males, this group difference in the mean was not statistically significant ($p = 0.19$, $U = 35$).

While exposure to a singing tutor produced a threefold elevation in pCaMKII levels in Area X of early isolates, exposure to a non-singing adult male did not elevate pCaMKII levels (see Table 1). Early isolate males housed with a non-singing adult male also did not differ from their controls in either total CaMKII levels, or the pCaMKII/total CaMKII ratio.

Finally, tutoring in early isolate females did not alter pCaMKII levels in the portion of the striatum that corresponds to Area X in males (see Table 1). Despite hearing similar amounts of song as did early isolate males (mean # song bouts = 21 ± 6; range = 5–35), females first exposed to a male tutor on d35 did not differ from untutored controls in levels of pCaMKII in the dorsolateral portion of medial striatum. There also was no effect of tutoring on levels of total CaMKII, or on the ratio of pCaMKII/total CaMKII in these females.

In summary, the overall pattern of results obtained from aviary-reared and early isolated birds was remarkably consistent. Relative to untutored controls, pCaMKII expression in Area X of males was dramatically elevated in aviary-reared birds tutored by their father, and was elevated more modestly in early isolates first exposed to a song tutor. Interestingly, in

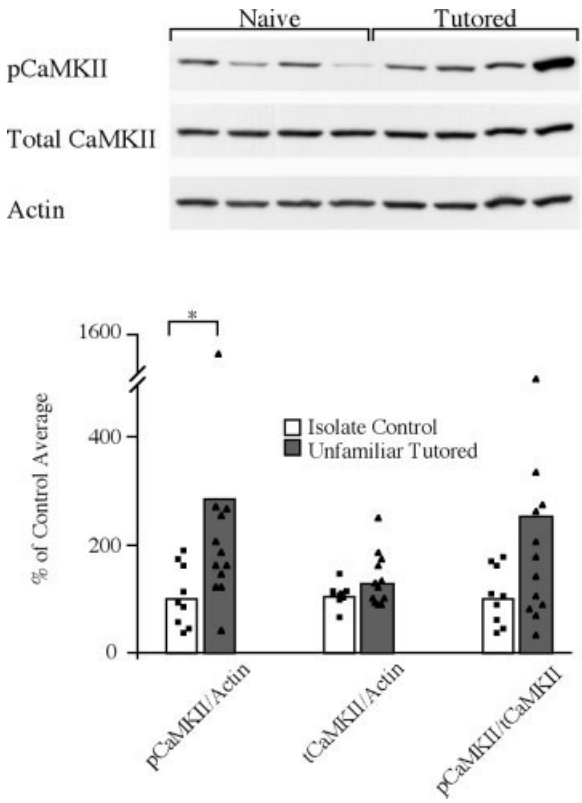

Figure 5 First exposure to a song tutor elevates pCaMKII levels in Area X of early isolated males. Top panel shows a representative immunoblot from a single experiment comparing untutored and tutored males that had been previously isolated from posthatch d9. Each lane consists of Area X homogenate from an individual bird. Bar graph shows group means (individual data superimposed) expressed relative to average intensity in untutored controls run on the same gel. Average pCaMKII levels (left) within Area X were significantly higher in tutored birds as compared to untutored controls. Song exposure did not significantly alter total CaMKII (middle) or the proportion of pCaMKII/total CaMKII within Area X. $*p < 0.05$.

aviary-reared birds (which are likely to have already begun sensory acquisition), the pCaMKII response was mounted to familiar but not unfamiliar song. Regardless of rearing condition, females did not exhibit any effect of tutoring on pCaMKII levels in that portion of the striatum that corresponds to Area X in males. Similarly, pCaMKII levels were not elevated by social interaction with a non-singing male, or by hearing song while visually isolated from the tutor. Thus, hearing song in a context that affords visual/social interaction with the tutor appeared necessary to elevate pCaMKII expression in Area X.

DISCUSSION

Autophosphorylation of CaMKII at Thr286 is critical for the lasting changes in synaptic efficacy that underlie certain forms of learning and developmental plasticity (Lisman et al., 2002; Colbran and Brown, 2004). The studies described here indicate that in young male zebra finches, exposure to a live tutor during song learning promotes such phosphorylation of CaMKII within Area X, a specialized region of the avian basal ganglia. The findings add to an increasing number of studies implicating Area X, and the AFP generally, in avian vocal learning. More specifically, our results suggest that elevations in pCaMKII within Area X may reflect synaptic changes associated with the sensory acquisition phase of learning (i.e., encoding a representation of the song model). Even in the absence of vocal practice, exposure to a tutor's song behavior triggered CaMKII phosphorylation. While this does not preclude a role for CaMKII signaling in sensorimotor learning, the biochemical changes reported here were not initiated by this latter phase of vocal development. Furthermore, the stimulus conditions that provoked CaMKII phosphorylation were precisely those that optimize song acquisition in this species; i.e., social interaction with a live, singing tutor (Eales, 1989; Morrison and Nottebohm, 1993; Jones and Slater, 1996). And perhaps most convincingly, prior experience with a song tutor (aviary-reared birds) in effect ''tuned'' this pCaMKII response, making the familiar tutor a highly effective stimulus for driving CaMKII phosphorylation while diminishing the response to a novel song tutor.

A direct test of whether the tutoring-induced CaMKII phosphorylation relates specifically to template encoding and/or storage, as opposed to other aspects of the tutoring paradigm, will require behavioral pharmacology. However, the overall pattern of results makes many alternative interpretations less likely. For instance, the possibility that the pCaMKII response is a reflection of generalized changes in behavioral state is hard to reconcile with the complete absence of a pCaMKII response in females and in aviary-reared birds exposed to a novel tutor. Another possibility is that the pCaMKII response reflects the activation of neurons tuned to the bird's own song (BOS), rather than the encoding of tutor's song. However, several observations make this interpretation unlikely. First, male zebra finches are only producing highly variable and rudimentary songs at 35d. Secondly, even if these vocalizations in the aviary-reared males already bear some feature similarity to father's song (thus causing neurons tuned to BOS to also respond preferentially to father's song), this interpretation cannot account for the increase in pCaMKII levels seen in naive (early isolate) birds first exposed to a tutor. Finally, the stimulus specificity of the pCaMKII response cannot be explained by simple auditory activation of BOS neurons, since this response is not evident in birds that merely hear familiar tutor song from a visually separated adjacent cage.

The fact that the pCaMKII response discriminates between familiar and unfamiliar song in aviary reared birds suggests that early song tutoring changes the intrinsic circuitry of Area X (and/or its afferents) in ways that strengthen the ability of this same stimulus to promote intracellular signaling cascades that could further modify synapses within Area X. Relevant to this idea is the observation that song exposure prior to d30 is sufficient to tune the auditory responsiveness of lMAN neurons in male zebra finches (Yazaki-Sugiyama and Mooney, 2004). Importantly, subsequent tutor exposure can retune these neurons (Yazaki-Sugiyama and Mooney, 2004) and promote imitation of that more recent tutor's song (see also Heinrich et al., 2003), and male zebra finches exposed to a series of tutors often imitate at least some of the more recently heard tutors (Slater et al., 1991). While these later observations seem at odds with the lack of a pCaMKII response to unfamiliar song in our aviary reared birds, the brief 2-h tutoring regime used to study pCaMKII signaling in the present study may be insufficient to overwrite prior tuning of AFP circuitry. It will be interesting to determine if more prolonged exposure to an unfamiliar tutor would eliminate or reverse the discriminative nature of the pCaMKII response in Area X.

Lesions of Area X in juvenile males cause song behavior to remain plastic into adulthood (Sohrabji et al., 1990; Scharff and Nottebohm, 1991), an observation that may reflect a role for Area X in establishing or refining a song template that is then referenced

during sensorimotor learning. Area X contains neurons that respond selectively to both the tutor's song and BOS (Solis and Doupe, 1999, 2000), and the initial synaptic changes that establish this representation of tutor song could entail modest song-induced elevations in pCaMKII such as those observed in our naive birds. Then, as this neural template takes shape (within Area X or its afferents), re-exposure to that same tutoring experience would be an even more powerful stimulus for provoking molecular and synaptic changes that further refine the template. At first glance, this interpretation seems inconsistent with a report that auditory selectivity in Area X neurons develops only after 45d (Doupe, 1997), but it may be important that selectivity was measured in this earlier study by presenting tape-recorded songs to anesthetized birds. In the present study, the constellation of acoustic and visual/social stimuli associated with live tutoring proved critical for provoking the pCaMKII response within Area X. Furthermore, recent work indicates that anesthesia may mask neuronal selectivity for tutor's song in young birds (Nick and Konishi, 2004), at least within HVC, the most likely source of auditory input to Area X (Mooney, 2000; Mooney et al., 2002).

Establishing how regions afferent to Area X contribute to tutoring-induced CaMKII phosphorylation may aid our understanding of how tutor song information is processed and/or encoded during acquisition. Even if a representation of tutor's song is encoded within Area X, it probably is not the only, or initial, encoding of that stimulus. In particular, the discriminative pCaMKII response observed in the aviary-reared males may require readout or transfer of template information initially encoded and stored elsewhere. While it is unfortunate that we were not able to adapt our regionally specific pCaMKII assay to other song regions, especially HVC and lMAN, it is important to reiterate the relative lack of CaMKII immunoreactivity within these regions. If synaptic plasticity associated with template encoding occurs first within these regions, it may involve different signal transduction cascades (e.g., cAMP-dependent protein kinase, extracellular signal regulated kinase). Interestingly, HVC neurons in awake juvenile zebra finches are strongly selective for tutor song during the sensory and early sensorimotor phase of song learning (Nick and Konishi, 2004). HVC–>X neurons could, in turn, convey this auditory information into the AFP (Mooney, 2000), thus contributing to the discriminative pCaMKII response that emerges there. Such a role for HVC would be consistent with the absence of a tutoring-induced increase in pCaMKII within the female LPO, as the projection from HVC to Area X is greatly reduced in females, even early in sensory learning (Burek et al., 1993).

Auditory regions upstream of HVC also may participate in the initial encoding of song-related auditory memories. Song presentations markedly increase expression of the immediate early gene ZENK (aka zif-268 or egr-1) within high level auditory regions (NCM and CMM) that are afferent to HVC (Mello et al., 1992; Mello and Clayton, 1994). In mammals, this gene has been implicated in the molecular cascade leading to the late phase of LTP and memory (Jones et al., 2001; Bozon et al., 2002), and in birds, song-induced ZENK induction emerges in correlation with the onset of sensory acquisition (Jin and Clayton, 1997; Mello et al., 2004). However, in young birds, the ZENK response habituates to repeated presentation of the same song, does not discriminate between conspecific and heterospecific song (although electrophysiological measures do), and occurs in females as well as in males (Jin and Clayton, 1997; Stripling et al., 2001; Mello et al., 2004). This suggests that ZENK expression in NCM/CMM may not reflect the formation of templates used specifically for vocal mimicry, but information encoded in these regions could be used to establish such templates elsewhere.

The present results also have implications for interpreting an earlier finding implicating lMAN in sensory acquisition. Specifically, infusions of an NMDAR antagonist into the vicinity of lMAN just prior to bi-daily tutoring sessions impair vocal learning while identical infusions delivered on non-tutoring days do not (Basham et al., 1996). We originally interpreted this result as suggesting that NMDAR-mediated synaptic plasticity within lMAN proper is important for encoding and/or storing a song template. The present results suggest the intriguing possibility that NMDAR-mediated processes in lMAN are essential for normal CaMKII signaling cascades in Area X, and disruption of these cascades may underlie the behavioral disruptions observed after antagonism of NMDARs in lMAN. Alternatively, it is possible that NMDAR-mediated plasticity within Area X may have been disrupted directly, through diffusion of the NMDAR antagonist into this region. However, this interpretation seems unlikely, because the pallial-subpallial lamina that separates lMAN and Area X creates a significant barrier to diffusion between these regions (personal observations).

In addition to the information received via projections from HVC and lMAN, Area X derives input from dopaminergic (DA) neurons of the ventral tegmental area (Lewis et al., 1981; Bottjer, 1993; Nixdorf-Bergweiler et al., 1995; Soha et al., 1996). The

Hebbian nature of striatal LTP could allow for synaptic changes based on the coincident activation of any or all of these inputs. In fact, LTP within Area X involves glutamatergic synapses from HVC and/or lMAN onto medium spiny neurons, and depends on coactivation of NMDARs and D1 dopamine receptors (Ding and Perkel, 2004). As discussed below, DA input could be critical for associating reward value with specific auditory inputs during song acquisition. In this regard, it is interesting that preliminary immunocytochemical studies in our laboratory indicate virtually complete overlap between Area X neurons expressing CaMKII, and those expressing DARPP-32, a DA signaling protein expressed in medium spiny neurons of the striatum. In contrast, CaMKII immunoreactivity is absent among large LANT6 immunopositive cells that are presumed to be pallidal neurons (Perkel et al., 2002; Carrillo and Doupe, 2004; Reiner et al., 2004). It will be important to determine if CaMKII autophosphorylation in Area X, like LTP, requires activity in both the nidopallial (i.e., HVC and lMAN) and DA inputs. We must note, however, that Ding and Perkel (2004) did not observe LTP in Area X of birds younger than 37d, and stimulus parameters that successfully induced LTP in birds >37d instead elicited synaptic depression in younger birds. While this leaves open the possibility that the pCaMKII response to tutoring reflects events unrelated to LTP, perhaps the LTP/LTD threshold in Area X is developmentally regulated, and the initiation of molecular cascades provoking striatal LTP requires specific patterns of activity that are achieved by live tutoring.

A possible role for avian basal ganglia in song acquisition is intriguing. In addition to its role in motor behavior, the mammalian basal ganglia has been implicated clearly in learning and memory (Setlow, 1997; Middleton and Strick, 2000; Packard and Knowlton, 2002). Moreover, both imaging and clinical studies implicate these structures in human language acquisition and processing (Callan et al., 2003; Ullman, 2004). Striatal dopamine modulates synaptic changes that could reflect attachment of reward value to primary stimuli, as well as signal prediction errors of reward during learning (Schultz, 2002). Reinforcement models of song learning necessarily would include such processes (Doya and Sejnowski, 1994). Thus, if the tutoring-induced phosphorylation of CaMKII described here proves to reflect such DA-modulated plasticity (e.g., LTP), the present results suggest that Area X may be critically involved in encoding and/or attaching reward value to a representation of tutor song that is accessed later to guide motor learning.

We are indebted to Dr. William Tank and Dr. Kerry O'Banion for their advice on Western blot methodology and analysis, and to Heather Bradstreet and Adam Neidert for providing invaluable technical support. We also thank two anonymous reviewers for their thoughtful comments on a previous version of this manuscript.

REFERENCES

Basham ME, Nordeen EJ, Nordeen KW. 1996. Blockade of NMDA receptors in the anterior forebrain impairs sensory acquisition in the zebra finch (Poephila guttata). Neurobiol Learn Mem 66:295–304.

Boettiger CA, Doupe AJ. 2001. Developmentally restricted synaptic plasticity in a songbird nucleus required for song learning. Neuron 31:809–818.

Bohner J. 1990. Early acquisition of song in the zebra finch, Taeniopygia guttata. Anim Behav 39:369–374.

Bottjer SW. 1993. The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. J Neurobiol 24:51–69.

Bottjer SW, Miesner EA, Arnold AP. 1984. Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science 224:901–903.

Bozon B, Davis S, Laroche S. 2002. Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. Hippocampus 12:570–577.

Burek MJ, Nordeen KW, Nordeen EJ. 1993. Ontogeny of sex differences among HVC projection neurons. Soc Neurosci Abst 19:1312.

Callan DE, Tajima K, Callan AM, Kubo R, Masaki S, Akahane-Yamada R. 2003. Learning-induced neural plasticity associated with improved identification performance after training of a difficult second-language phonetic contrast. Neuroimage 19:113–124.

Carrillo GD, Doupe AJ. 2004. Is the songbird Area X striatal, pallidal, or both? An anatomical study. J Comp Neurol 473:415–437.

Clayton DF. 1997. Role of gene regulation in song circuit development and song learning. J Neurobiol 33:549–571.

Clayton NS. 1988. Song discrimination learning in zebra finches. Anim Behav 36:1016–1024.

Colbran RJ, Brown AM. 2004. Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. Curr Opin Neurobiol 14:318–327.

Ding L, Perkel DJ. 2004. Long-term potentiation in an avian basal ganglia nucleus essential for vocal learning. J Neurosci 24:488–494.

Doupe AJ. 1997. Song- and order-selective neurons in the songbird anterior forebrain and their emergence during vocal development. J Neurosci 17:1147–1167.

Doupe AJ, Konishi M. 1991. Song-selective auditory circuits in the vocal control system of the zebra finch. Proc Natl Acad Sci USA 88:11339–11343.

Doupe AJ, Kuhl PK. 1999. Birdsong and human speech: common themes and mechanisms. Annu Rev Neurosci 22:567–631.

Doya K, Sejnowski TJ. 1994. A novel reinforcement model of birdsong vocalization learning. In: Tesauro G, Touretzky D, Leen T, editors. Advances in neural information processing systems. Cambridge, MA: MIT Press. p 101–108.

Eales LA. 1985. Song learning in zebra finches: some effects of song model availability on what is learnt and when. Anim Behav 33:1293–1300.

Eales LA. 1987. Song learning in female-raised zebra finches: another look at the sensitive phase. Anim Behav 35:1356–1365.

Eales LA. 1989. The influences of visual and vocal interaction on song learning in zebra finches. Anim Behav 37: 507–520.

Funabiki Y, Konishi M. 2003. Long memory in song learning by zebra finches. J Neurosci 23:6928–6935.

Heinrich JE, Singh TD, Nordeen KW, Nordeen EJ. 2003. NR2B downregulation in a forebrain region required for avian vocal learning is not sufficient to close the sensitive period for song learning. Neurobiol Learn Mem 79:99–108.

Hessler NA, Doupe AJ. 1999. Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. J Neurosci 19:10461–10481.

Immelmann K. 1969. Song development in the zebra finch and other estrildid finches. In: Hinde RA, editor. Bird vocalizations. Cambridge, UK: Cambridge University Press. p 61–77.

Jin H, Clayton DF. 1997. Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. Neuron 19:1049–1059.

Jones AE, Slater PJB. 1996. The role of aggression in song tutor choice in the zebra finch: cause or effect? Behavior 133:103–115.

Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S, Davis S. 2001. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. Nat Neurosci 4:289–296.

Lewis JW, Ryan SM, Arnold AP, Butcher LL. 1981. Evidence for a catecholaminergic projection to area X in the zebra finch. J Comp Neurol 196:347–354.

Lisman J, Schulman H, Cline H. 2002. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Marler P, Peters S. 1981. Sparrows learn adult song and more from memory. Science 213:780–782.

Marler P, Peters S. 1982. Long-term storage of learned birdsongs prior to production. Anim Behav 30:479–482.

Mello CV, Clayton DF. 1994. Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J Neurosci 14:6652–6666.

Mello CV, Velho TA, Pinaud R. 2004. Song-induced gene expression: a window on song auditory processing and perception. Ann NY Acad Sci 1016:263–281.

Mello CV, Vicario DS, Clayton DF. 1992. Song presentation induces gene expression in the songbird forebrain. Proc Natl Acad Sci USA 89:6818–6822.

Middleton FA, Strick PL. 2000. Basal ganglia output and cognition: evidence from anatomical, behavioral, and clinical studies. Brain Cogn 42:183–200.

Miller DB. 1979. Long-term recognition of father's song by female zebra finches. Nature 280:389–391.

Mooney R. 2000. Different subthreshold mechanisms underlie song selectivity in identified HVc neurons of the zebra finch [published erratum appears in J Neurosci 2000 Aug 1; 20(15) following table of contents]. J Neurosci 20:5420–5436.

Mooney R, Rosen MJ, Sturdy CB. 2002. A bird's eye view: top down intracellular analyses of auditory selectivity for learned vocalizations. J Comp Physiol [A] 188:879–895.

Morrison RG, Nottebohm F. 1993. Role of a telencephalic nucleus in the delayed song learning of socially isolated zebra finches. J Neurobiol 24:1045–1064.

Nick TA, Konishi M. 2004. Neural song preference during vocal learning in the zebra finch depends on age and state. J Neurobiol 30:30.

Nixdorf-Bergweiler BE, Lips MB, Heinemann U. 1995. Electrophysiological and morphological evidence for a new projection of LMAN-neurones towards area X. Neuroreport 6:1729–1732.

Nottebohm F, Stokes TM, Leonard CM. 1976. Central control of song in the canary, Serinus canarius. J Comp Neurol 165:457–486.

Packard MG, Knowlton BJ. 2002. Learning and memory functions of the basal ganglia. Annu Rev Neurosci 25: 563–593.

Perkel DJ. 2004. Origin of the anterior forebrain pathway. Ann NY Acad Sci 1016:736–748.

Perkel DJ, Farries MA, Luo M, Ding L. 2002. Electrophysiological analysis of a songbird basal ganglia circuit essential for vocal plasticity. Brain Res Bull 57:529–532.

Price PH. 1979. Developmental determinants of structure in zebra finch song. J Comp Physiol Psych 93:268–277.

Reiner A, Perkel DJ, Mello CV, Jarvis ED. 2004. Songbirds and the revised avian brain nomenclature. Ann NY Acad Sci 1016:77–108.

Scharff C, Nottebohm F. 1991. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. J Neurosci 11:2896–2913.

Schultz W. 2002. Getting formal with dopamine and reward. Neuron 36:241–263.

Setlow B. 1997. The nucleus accumbens and learning and memory. J Neurosci Res 49:515–521.

Simpson HB, Vicario DS. 1990. Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci 10:1541–1556.

Simpson HB, Vicario DS. 1991. Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations. J Neurobiol 22:777–793.

Slater PJB, Richards C, Mann NI. 1991. Song learning in zebra finches exposed to a series of tutors during the sensitive phase. Ethology 88:163–171.

Soha JA, Shimizu T, Doupe AJ. 1996. Development of the catecholaminergic innervation of the song system of the male zebra finch. J Neurobiol 29:473–489.

Sohrabji F, Nordeen EJ, Nordeen KW. 1990. Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behav Neural Biol 53:51–63.

Solis MM, Doupe AJ. 1999. Contributions of tutor and bird's own song experience to neural selectivity in the songbird anterior forebrain. J Neurosci 19:4559–4584.

Solis MM, Doupe AJ. 2000. Compromised neural selectivity for song in birds with impaired sensorimotor learning. Neuron 25:109–121.

Stripling R, Kruse AA, Clayton DF. 2001. Development of song responses in the zebra finch caudomedial neostriatum: role of genomic and electrophysiological activities. J Neurobiol 48:163–180.

Ullman MT. 2004. Contributions of memory circuits to language: the declarative/procedural model. Cognition 92: 231–270.

Wade J, Arnold AP. 2004. Sexual differentiation of the zebra finch song system. Ann NY Acad Sci 1016:540–559.

Wild JM. 2004. Functional neuroanatomy of the sensorimotor control of singing. Ann NY Acad Sci 1016:438–462.

Yazaki-Sugiyama Y, Mooney RD. 2004. Sequential learning from multiple tutors and serial retuning of auditory neurons in a brain area important to birdsong learning. J Neurophysiol 30:30.