Title

Slow NMDA-EPSCs are not required for song learning at two synapses critical for song development in zebra finches

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Slow NMDA-EPSCs at synapses critical for song development are not required for song learning in zebra finches

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Birdsong, like human speech, is learned via auditory experience during a developmentally restricted sensitive period. Within projection neurons of two avian forebrain nuclei, NMDA receptor-mediated EPSCs (NMDA-EPSCs) become fast during song development, a transition posited to limit learning. To discover whether slow NMDA-EPSCs at these synapses are required for learning, we delayed song learning beyond its normal endpoint, post-hatch day (PHD) 65, by raising zebra finches in isolation from song tutors. At PHD45, before learning, isolation delayed NMDA-EPSC maturation, but only transiently. By PHD65, NMDA-EPSCs in isolates were fast and adult-like, yet isolates presented with tutors readily learned song. Thus song learning did not require slow NMDA-EPSCs at synapses critical for song development.

The regulation of sensitive periods for activity-dependent neural plasticity, including learning, is an important issue. Over development, the decay times of NMDA receptor-mediated postsynaptic excitatory currents within diverse sensory and sensorimotor systems, including the avian song system, become fast[1–6]. These faster currents decrease synaptic integration times and duration of calcium influx, either of which might reduce synaptic plasticity, thereby ending sensitive periods. In support of this idea, overexpression of a slow form of the NMDA receptor within forebrain pyramidal neurons in mice enhances adult performance on learning and memory tasks[7]. Similarly, treatment of young zebra finches with androgens disrupts song learning[8] and causes NMDA-EPSCs in projection neurons of song-related forebrain regions to become prematurely fast[6]. Although both studies suggest that slow NMDA-EPSCs are critical for learning, neither resolves whether slow currents directly mediate learning or instead affect general developmental processes that subsequently facilitate learning. Resolving this issue requires that learning be temporally offset from neural development.

Here we tested whether slow NMDA-EPSCs were required for learning by manipulating whether and when juvenile zebra finches (*Taeniopygia guttata*) learned song and measuring NMDA-EPSC decay times at synapses critical for song development. Birdsong is ideal for this investigation because it is a naturally learned vocal behavior that proceeds through well characterized stages that can be experimentally manipulated[9] (**Fig. 1a**). In the first stage, called sensory acquisition, young male finches memorize the song of an adult male tutor. Normally, sensory acquisition ends by PHD65, but isolation of young birds from tutors extends this stage beyond PHD65 (refs. 10–13; **Fig. 1a**). If slow NMDA-EPSCs are required for learning, then isolated finches at PHD65 should have slow NMDA-EPSCs at synapses essential for song learning.

The neural circuitry implicated in song learning includes several interconnected forebrain nuclei known as the anterior forebrain pathway (**Fig. 1b**). NMDA receptors mediate synaptic transmission at several sites in this pathway. These include thalamic synapses on projection neurons of the lateral magnocellular nucleus of the anterior neostriatum (LMAN)[5] and synapses that LMAN neurons make onto projection neurons of the robust nucleus of the archistriatum (RA)[14,15]. LMAN is required for normal song development[16,17] as well as the extended learning shown by isolated finches[11], whereas RA is essential for singing and is the sole vocal premotor target of LMAN[18–20]. Within LMAN, NMDA receptors are implicated in song learning, as their blockade during tutoring decreases the number of song syllables learned[21]. Further, NMDA-EPSCs at DLM→LMAN synapses become faster during song development[5]. Neurotransmission between LMAN and RA projection neurons is mediated almost entirely by NMDA receptors[15], and these NMDA-EPSCs also become faster[6], suggesting that this synapse is another likely target for NMDA receptor modulation during song learning.

To determine if song learning requires slow NMDA-EPSCs, we either raised young finches in isolation from tutors or deafened them; we subsequently examined the effect of each treatment on NMDA-EPSC development in LMAN and RA. At PHD45, each form of auditory deprivation delayed NMDA-EPSC development within LMAN, but not in RA, and also depressed serum testosterone levels. These effects were transient, and by PHD65, NMDA-EPSCs in LMAN and circulating androgen levels were adult-like. Nonetheless, PHD65 isolates learned song when exposed to tutors. Therefore, although slow NMDA-EPSCs at these sites earlier in development may indirectly enhance learning, they were not needed when extended learning occurred.

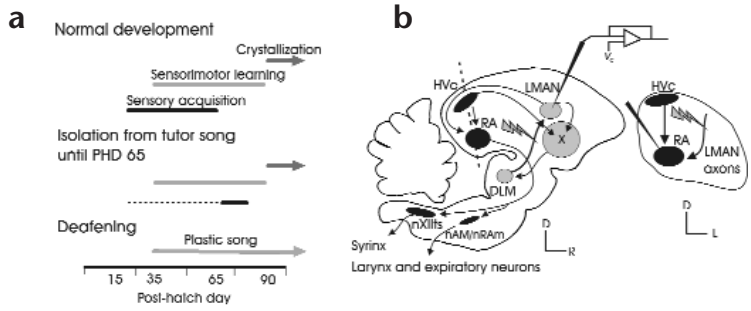

Fig. 1. Time line of song development and schematic of song system. (**a**) Time line showing the stages of song learning in the zebra finch. In normal song development, young male finches listen to and memorize the song of an adult male tutor during sensory acquisition, which begins at ~PHD20 and ends at ~PHD65 (black bar). Later, during sensorimotor learning (PHD35–90; light gray bar), the bird must hear his own vocalizations as he attempts to match them to the memorized model. Song learning ends with crystallization (dark gray arrow), when the previously variable song becomes highly stereotyped. Isolation of young finches from tutor song until PHD65 extends sensory acquisition (black bar, PHD65–86) beyond the normal period; dotted black line indicates normal period of sensory acquisition. Deafening prevents sensory acquisition and sensorimotor learning; consequently, only poorly formed and variable plastic song is produced (light gray arrow). (**b**) Schematics of the song system and the brain slice preparations used to record NMDA-EPSCs in LMAN and RA. Sagittal view (left) of the zebra finch brain. The vocal motor pathway for learned song production includes HVc, RA, the hypoglossal motor neurons (nXIIts) and respiratory areas (nAm, n. ambiguus; nRAm, n. retroambigualis). The anterior forebrain pathway includes area X, the dorsolateral part of the medial thalamus (DLM) and LMAN. NMDA-EPSCs were elicited in LMAN by electrically stimulating afferents from DLM at the site marked by the lightning bolt. To record NMDA-EPSCs from RA, coronal slices (right) were made at the plane of section shown by the dotted line (in the sagittal view); stimulation sites are shown by the lightning bolt. D, dorsal; R, rostral; L, lateral.

Results

Isolated zebra finches learned despite fast NMDA-EPSCs

Isolation of young zebra finches from tutors between PHD25 and PHD65 extended sensory acquisition beyond PHD65 without a simultaneous delay in NMDA-EPSC development within projection neurons at DLM→LMAN and LMAN→RA synapses (**Fig. 2**). After three weeks of tutoring (PHD65–86), previously isolated finches learned more song syllables from their tutors than did age- and tutor-matched controls (**Fig. 2a**), consistent with previous studies[10–13]. Analysis of songs from ~PHD86 birds revealed that on average, previously isolated finches learned 52% of their song syllables from tutors, whereas age-matched controls learned only 3% from the identical tutors ($p < 0.0025$; **Table 1**).

Extended learning did not require slow NMDA-EPSCs

Whole-cell recordings revealed that at DLM→LMAN synapses, NMDA-EPSCs of PHD65 isolates were as fast as those of control adults (PHD>90), as reflected by the time it took for the currents to decay to 1/e of their peak amplitude (**Fig. 2b**; e-fold-decay times for isolates versus adults, 47 ± 4 ms versus 48 ± 4 ms; $p = 0.98$), and were faster than those of PHD45 control juveniles (57 ± 3 ms, $p < 0.01$). These findings show that isolation did not alter NMDA-EPSCs in LMAN at PHD65 and suggest that these currents are normally mature by PHD65.

Maturation of NMDA-EPSCs in RA is more protracted than in LMAN, with greater developmental change in e-fold-decay times occurring between juvenile and adult life[6]. Consistent with this

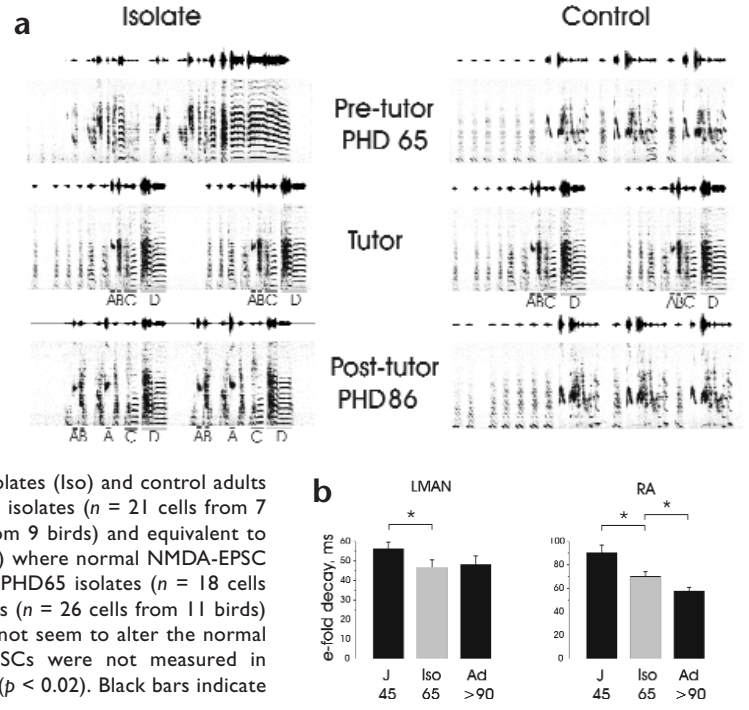

Fig. 2. Isolation from tutor song extends the period of sensory acquisition into the time when NMDA-EPSCs are fast and adult-like. (**a**) Spectrograms for one isolate, one control and one tutor. Top, pre-tutor spectrograms of PHD65 pupils; left, male raised in isolation from PHD25–PHD65; right, control male raised in the breeding colony. Middle, spectrogram of an adult male used to tutor both the isolate and the control for three weeks. Bottom, post-tutor spectrograms of the above two pupils at ~PHD86; left, isolate; right, control juvenile. The previously isolated pupil (left) has learned four syllables from the tutor, whereas the control juvenile retained his pre-tutor song. Learned notes are identified by letters and bars shown underneath the spectrograms. Audio files for these spectrograms can be found at http:/www.nature.com/neuro/web_specials/ (**b**) NMDA-EPSC development of isolates is not delayed at PHD65. Bar graphs show the average e-fold-decay times of control juveniles at PHD45 (J), PHD65 isolates (Iso) and control adults (PHD>90; Ad). In LMAN (left), NMDA-EPSCs of PHD65 isolates (n = 21 cells from 7 birds) are faster than those of juveniles (n = 20 cells from 9 birds) and equivalent to those of adults (n = 23 cells from 10 birds). In RA (right) where normal NMDA-EPSC development is more protracted[6], e-fold-decay times in PHD65 isolates (n = 18 cells from 5 birds) are intermediate between those of juveniles (n = 26 cells from 11 birds) and adults (n = 20 cells from 12 birds). As isolation did not seem to alter the normal developmental trajectory of NMDA-EPSCs, NMDA-EPSCs were not measured in PHD65 controls. Asterisks denote significant differences ($p < 0.02$). Black bars indicate previously published data[6].

Table 1. Syllable learning.

		Total syllables		Learned syllables		Percent learned	
	Birds	Range	Average	Range	Average	Range	Average
Isolates	$n = 8$	2–5	4.0 ± 1.2	0.5–3.5	2.1 ± 1.2	15–70%	52 ± 8%
Controls	$n = 6$	3–4	3.3 ± 0.5	0–0.5	0.1 ± 0.2	0–17%	3 ± 2%

These values are the averages of the authors' values and those of an observer blind to the experimental manipulation.

protracted development, the average e-fold-decay time of NMDA-EPSCs in RA of PHD65 isolates lies along a single-exponential decay curve fitted from developmental values[6] (data not shown). Within RA, NMDA-EPSCs of PHD65 isolates were faster than those of PHD45 control juveniles (91 ± 6 ms versus 70 ± 5 ms; $p < 0.01$) and slower than those of control adults (**Fig. 2b**; 70 ± 5 ms versus 58 ± 3 ms; $p < 0.02$). Thus, isolation extended song learning without concurrently extending NMDA-EPSC development.

Isolation delayed early synaptic development in LMAN

Slow NMDA-EPSCs are neither present nor required at DLM→LMAN and LMAN→RA synapses for learning at PHD65. Rather than being directly needed during learning, slow NMDA-EPSCs early in development could indirectly permit extended learning by enhancing developmental processes that subsequently facilitate learning. To determine whether isolation delayed synaptic maturation earlier in development, we measured NMDA-EPSCs in isolates before PHD65. NMDA-EPSCs within LMAN of PHD45 isolates were slower than those of control juveniles (**Fig. 3**; 70 ± 6 ms versus 57 ± 3 ms, $p < 0.025$). This effect was specific to LMAN, as NMDA-EPSCs within RA did not differ between PHD45 isolates and control juveniles (98 ± 8 ms versus 91 ± 6 ms; $p = 0.68$).

Although isolation between PHD25 and 45 resulted in NMDA-EPSCs in LMAN that were slower than those of control juveniles, it did not completely arrest synaptic development: NMDA-EPSCs of these PHD45 isolates were still faster than those of PHD25 control fledglings (**Fig. 3**; 70 ± 6 ms versus 94 ± 7 ms, $p < 0.01$). To determine whether more prolonged isolation could more severely retard synaptic maturation in LMAN, finches were isolated from tutors beginning at PHD10. As with the short isolation protocol, longer isolation produced slower NMDA-EPSCs at PHD45 in LMAN relative to those of age-matched controls (**Fig. 3**; 83 ± 9 ms versus 57 ± 3 ms, $p < 0.025$). However, NMDA-EPSCs of finches isolated from PHD10 until 45 did not differ from those isolated from PHD25 until 45 (83 ± 9 ms versus 70 ± 6 ms, $p = 0.35$). In RA at PHD45, the longer isolation protocol did not alter NMDA-EPSC e-fold-decay times relative to age-matched control values (100 ± 9 ms versus 91 ± 6 ms, $p = 0.39$, **Fig. 3**). Therefore, neither isolation protocol affected LMAN→RA synapses, whereas both affected DLM→LMAN synapses, indicating that isolation affected NMDA-EPSCs selectively within the song system.

Deafening delayed synaptic development sequentially

A live tutor provides a young zebra finch with more than auditory stimulation. To test whether visual and tactile cues from the tutor are sufficient for normal synaptic development in the absence of auditory stimulation, young zebra finches were deafened at PHD15 and reared in the colony. Consistent with previous reports[22,23], deafened finches developed abnormal songs that remained plastic throughout life (**Fig. 4a**). NMDA-EPSCs in deafened finches were measured at PHD45 and after PHD90 and compared with those of age-matched controls. Within LMAN, NMDA-EPSCs of deafened juveniles were slower than

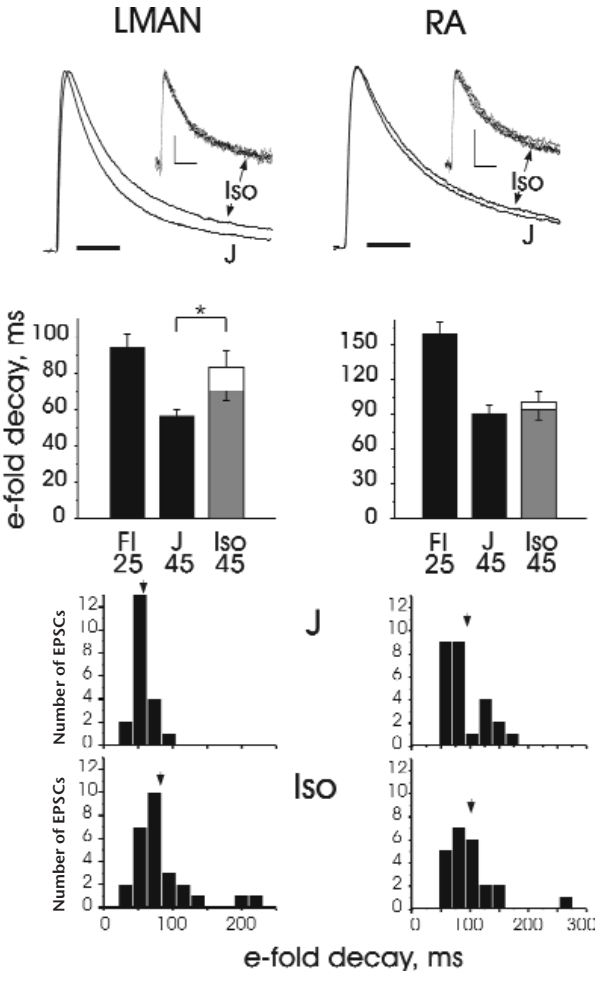

Fig. 3. NMDA-EPSC development in LMAN is delayed at PHD45 in isolates. Top, average of normalized NMDA-EPSCs from LMAN and RA in PHD45 isolates (Iso; short isolation protocol) and age-matched control juveniles (J). Insets show five consecutive raw traces from a single cell in each nucleus from the isolate group. Horizontal scale bars, 50 ms; vertical scale bars, 50 pA. Middle, bar graphs show the average e-fold-decay times of the above groups, including both isolate protocols (gray, isolation from tutors from PHD25–45, $n = 22$ cells from 7 birds for LMAN and 20 cells from 3 birds for RA; white, isolation from tutors from PHD10–45, $n = 27$ cells from 7 birds for LMAN and 23 cells from 7 birds for RA), as well as fledglings (Fl, PHD25, $n = 18$ cells from 8 birds for LMAN and 32 cells from 14 birds for RA). Black bars indicate previously published data[6]. Bottom, histograms show the distribution of e-fold-decay times for juveniles (J) and isolates (Iso; short isolation protocol). Arrows indicate the mean. *$p < 0.025$.

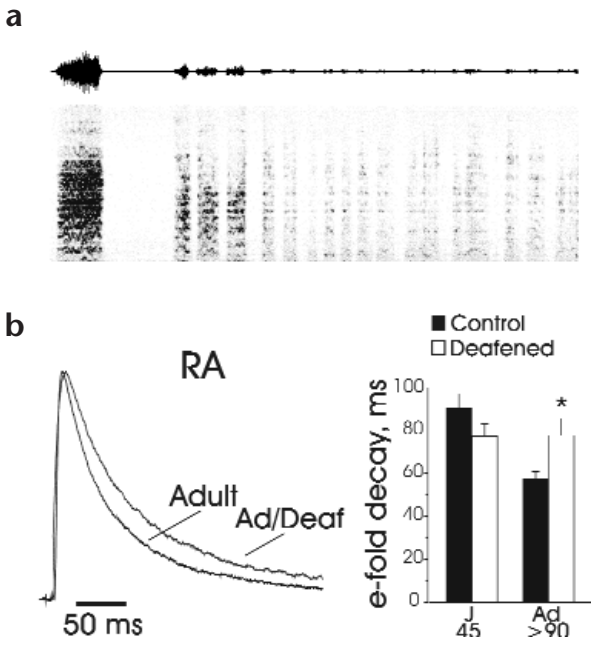

Fig. 4. Early deafening delays late NMDA-EPSC maturation in RA. (**a**) An example of song from a bird deafened ~PHD15 and recorded as an adult (PHD132). Note the lack of defined song structure as compared with control adults (**Fig. 2a**, tutor song). (**b**) Left, average NMDA-EPSCs recorded in RA in adult deafened birds (Ad/Deaf, $n = 25$ cells from 10 birds) and age-matched controls (Adult, $n = 20$ cells from 12 birds). Right, bar graphs show the e-fold-decay times of deafened ($n = 20$ cells from 5 birds) and control ($n = 26$ cells from 11 birds) juveniles(J) at PHD45 and deafened and control adults at PHD>90 (Ad). *$p < 0.025$.

those of control juveniles (65 ± 3 ms versus 57 ± 3 ms, $p < 0.05$), but by PHD>90, they were as fast as those of control adults (48 ± 4 ms versus 48 ± 3 ms, $p = 0.54$). Thus, visual and tactile stimuli without audition were insufficient for normal NMDA-EPSC development, suggesting that an auditory deficit contributed to the delay in NMDA-EPSC development at PHD45 in isolates and deafened birds. In this regard, the specific auditory deficit could be tutor song, because either deafening or isolation produced similar effects (deafening versus PHD15–45 isolation, $p = 0.14$; versus PHD25–45 isolation, $p = 0.37$). However, these results cannot distinguish whether the effects of deafening were due to removal of auditory stimuli or to abnormal visual and tactile interactions that could result from deafening; we note that deafened juveniles seemed well fed, suggesting that they had normal feeding interactions with adult birds.

In RA at PHD45, NMDA-EPSCs of deafened juveniles did not differ from those of control juveniles (76 ± 6 ms versus 91 ± 6 ms, **Fig. 4b**). By PHD > 90, however, deafened finches had slower NMDA-EPSCs relative to control adults (78 ± 8 ms versus 58 ± 3 ms, $p < 0.025$). Thus in RA, removal of auditory stimuli disrupted later stages of synaptic development, arresting NMDA-EPSC e-fold-decay times of deafened adults in an immature state. This late effect of deafening on RA NMDA-EPSCs was not observed following either isolation protocol (isolate data PHD>90 not shown). Together with data from LMAN, these results show that different forms of auditory deprivation had distinct effects on the two song nuclei: isolation specifically affected NMDA-EPSCs within LMAN, whereas deafening affected NMDA-EPSCs in both LMAN and RA, albeit at different developmental stages.

Auditory deprivation depresses testosterone at PHD45

In addition to auditory experience, the hormonal milieu regulates both song and synaptic development in zebra finches: early castration[24,25] and exogenous testosterone[8] each disrupt song learning, and exogenous testosterone accelerates the maturation of NMDA-EPSCs in LMAN and RA[6]. Given this androgen sensitivity, one possibility is that slower NMDA-EPSCs in isolates and deafened birds at PHD45 stem from depressed testosterone levels. Therefore, serum testosterone titers were measured at PHD45, 65 and >90 in controls and isolates (short isolation protocol) and at PHD45 and >90 in deafened finches. Although testosterone levels at PHD65 and PHD>90 were equivalent across all groups (**Fig. 5**), both isolates and deafened birds had lower testosterone levels relative to control values at PHD45 (**Fig. 5a**; Wilcoxon rank sum, $p < 0.05$, one-tailed). The depression in testosterone levels at PHD45 coincided with the transient delay in NMDA-EPSC development in LMAN produced by auditory deprivation (**Fig. 5b**).

The slower NMDA-EPSCs in isolates and deafened birds at PHD45 could be due to their reduced androgen levels; alternatively, slow NMDA-EPSCs could arise independently of altered steroid levels. To discover whether NMDA-EPSCs in isolates were sensitive to androgens, we implanted finches isolated at PHD10 at with the nonaromatizable androgen dihydrotestosterone (DHT) ~PHD35, ten days before recording NMDA-EPSCS from LMAN. Similar steroid treatment causes NMDA-EPSCs in normally reared birds to become prematurely fast in LMAN and RA[6]. Here, PHD45 isolates treated with DHT had NMDA-EPSCs in LMAN that were as fast as those of control juveniles (59 ± 3 ms versus 57 ± 3 ms, $p = 0.55$). Thus NMDA-EPSCs in isolates remained androgen sensitive, suggesting that impoverished auditory experience exerted its effects on synaptic development by depressing levels of sex steroid.

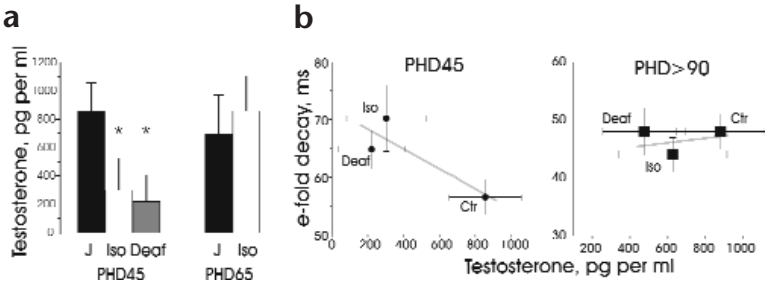

Fig. 5. Serum testosterone levels are depressed in isolates and deafened birds at PHD45. (**a**) Testosterone levels (pg per ml) from PHD45 and PHD65 birds. Control juveniles (J), birds isolated at PHD25 (Iso), birds deafened at PHD15 (deaf). *$p < 0.05$ (difference from juvenile; one-tailed). (**b**) Left, relationship (gray line) between testosterone levels and the corresponding average e-fold-decay times in LMAN from PHD45 experimental groups shown in (**a**). Right, a similar plot from PHD>90 isolated, deafened and control groups.

Discussion

These results demonstrate that extended sensitive periods for song learning did not require slow NMDA-EPSCs at two synaptic sites critical to song development. Nonetheless, auditory experience is essential for normal synaptic development at these sites as well as for maintenance of normal androgen levels. Sex steroids could synchronize synaptic development within the song system to sexual maturation. The transient sensitivity of androgen levels to auditory experience shown here may additionally allow synaptic development to be regulated by tutor song availability.

The correlations among auditory deprivation, low testosterone and slow NMDA-EPSCs in LMAN at PHD45 indicate that, during normal development, auditory experience affected testosterone, which may have promoted the transition from slow to fast NMDA-EPSCs in LMAN. Testosterone increases with maturation in zebra finches[6,26] and, additionally, fluctuates in response to environmental stimuli, particularly those relevant to reproduction. For example, among songbirds that sing seasonally, photoperiod is a major regulator of testosterone, but steroid levels also surge in response to threats, including singing, made by conspecifics[27]. Here we show that auditory cues, in addition to maturational state, affected serum testosterone levels in young zebra finches. The common stimulus eliminated both by deafening and by isolation is tutor song; deafened finches may additionally have abnormal visual or tactile interactions because they cannot respond to salient auditory cues. Although the social environments of isolated versus deafened finches clearly differ, their auditory environments each lack tutor song, suggesting it as the stimulus regulating juvenile testosterone levels.

The correlation in LMAN between delayed synaptic development and depressed testosterone at PHD45 (**Fig. 5b**) suggests that androgens mediated the effects of auditory deprivation on NMDA-EPSCs. This interpretation is strengthened by the known sensitivity of these currents to exogenous testosterone[6]. Among alternate possibilities, auditory-driven activity of LMAN neurons could directly regulate changes in NMDA-EPSCs. Actually, LMAN neurons in young finches (PHD30–45) are activated by a wide variety of auditory stimuli[28]; therefore deafening might be expected to more profoundly delay NMDA-EPSC development. However, isolates had NMDA-EPSCs that were as slow at PHD45 as those from deafened birds, indicating that NMDA-EPSC maturation in LMAN did not simply mirror the degree of general auditory stimulation. Further, finches isolated at PHD10 and treated with DHT at PHD35 had NMDA-EPSCs at PHD45 that were faster than those of untreated age-matched isolates, despite a similarly impoverished auditory experience. Androgenic regulation of synaptic development in LMAN is suggested by the parallel recovery of NMDA-EPSCs and testosterone to control values at PHD65, which occurred even in deafened birds, despite their lack of auditory stimulation. The recovery in testosterone levels indicates that, regardless of auditory activity, maturational increases in androgens ultimately occur and could drive the transition to fast NMDA-EPSCs. These observations support a model whereby gross maturational changes in androgens[6,26] influenced by experience of the tutor song serve to finely tune androgen-sensitive synaptic development in LMAN during a limited developmental window.

Although exogenous testosterone accelerates NMDA-EPSC development in LMAN and RA[6], such regulation must be indirect at the LMAN→RA synapse, as our results here reveal that NMDA-EPSC development in RA was not delayed while testosterone was depressed. Further, NMDA-EPSCs in RA were slower in deafened adults compared with age-matched controls, despite similar levels of testosterone. One indirect action of exogenous testosterone could be to promote singing[29]; resultant increases in self-induced auditory stimulation might accelerate NMDA-EPSC development in RA. Regardless, the late effects of deafening and the production of songs with low stereotypy by deafened adults[22,23] (**Fig. 4**) suggest that the final maturation of NMDA-EPSCs in RA is related to sensorimotor learning and/or crystallization.

Although LMAN is necessary for isolate learning[11], slow NMDA-EPSCs in LMAN at PHD65 were not. This contradicts the hypothesis that learning requires the longer windows of coincidence detection or calcium influx afforded by slow NMDA-EPSCs. Future work could address whether the discordant timing between occurrence of slow NMDA-EPSCs and occurrence of extended learning also pertains to NMDA receptors at other synapses within the song system. The idea that slow NMDA-EPSCs facilitate activity-driven synaptic remodeling, including learning, is derived from studies in the visual system, where dark-rearing prolongs the period of plasticity of ocular dominance columns[30] and delays the transition from slow to fast NMDA-EPSCs[1]. This correlation extends to the molecular level: exposure of dark-reared rats to light rapidly induces expression of NR2A[31], the subunit that confers fast kinetics on the NMDA receptor[32]. In isolated zebra finches, the normal developmental decline in NR2B levels is delayed (T.D. Singh, E.J. Nordeen & K.W. Nordeen, *Soc. Neurosci. Abstr.* **25**, 551.18, 1999); if paralleled by low NR2A levels, this delay could underlie the slower NMDA-EPSCs observed here in PHD45 isolates. Transgenic mice provide a more direct test of the link between NMDA receptor subunits, slow currents and learning. Overexpression of NR2B subunits within hippocampal pyramidal neurons enhances performance on learning and memory tasks, suggesting that increased NR2B expression directly facilitates learning[7]. Because transgenic expression was not temporally regulated in this case, an alternate but not mutually exclusive possibility is that denser NR2B expression is not required at the time of learning but acts indirectly, perhaps by promoting changes during neural development that then enable the enhanced learning.

Our results here support such an indirect role of slow NMDA-EPSCs in learning, as do measurements of NMDA receptor development in the ferret visual cortex revealing that the transition from slow to fast NMDA-EPSCs occurs before the peak of plasticity in formation of ocular dominance columns[33]. If slow NMDA-EPSCs in early development promote song learning, then they must do so via an intermediary process (such as synaptic growth and connectivity) that persists after NMDA-EPSCs become fast. In the songbird, one such intermediary process could be an abundance of dendritic synapses, which might provide a substrate for auditory feedback-dependent synaptic refinement. Indeed, Golgi staining demonstrates that the decline in dendritic spine densities on LMAN projection neurons that normally occurs by PHD60 is delayed by isolation[34]. In this light, it would be interesting to see if these elevated spines are pruned during subsequent song learning, as this would strengthen the correlations among NMDA-EPSCs, anatomical plasticity and learning.

Song is an androgen-regulated reproductive behavior that also depends on auditory experience. By rendering endocrine state sensitive to auditory stimuli, the songbird may ensure that sensitive periods for learning, as well as sexual maturation, are not entirely dictated by experience-independent mechanisms. The experience-dependent changes in e-fold-

decay times of NMDA-EPSCs observed here were small compared with developmental changes that happen before[35] and during normal song learning and with those that occur in primary sensory areas of other species[1–3]. These smaller changes, however, seem more relevant to learning because they respond to the learning stimulus and are affected by the same hormonal manipulations that alter song development in zebra finches[8]. Thus, in addition to maturational and/or seasonal changes in androgens, the changes produced by auditory deprivation could provide a limited window of flexibility in development of androgen-sensitive NMDA-EPSCs. An influence of slow NMDA-EPSCs on learning must be indirect, however, as the developmental delay produced here by auditory deprivation was transient, and slow NMDA-EPSCs were not required at the time of learning.

Methods

Subjects. Brain slices were made from 46 male zebra finches, in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. Finches were raised in our breeding colony on a 14 h:10 h day:night light cycle. Three age groups were used, juveniles at PHD38–49 (referred to as PHD45), adults at PHD90–200 (PHD>90) and juveniles at an intermediate time, PHD63–67 (PHD65). Control data from 'PHD25' fledglings (PHD21–32), juveniles and adults were previously reported[6].

Deafening. The cochleae were surgically removed under anesthesia, as described[36].

Isolation protocols. In the short protocol, ~PHD25 fledglings were removed from breeding cages and housed alone in small stainless steel cages (22 × 22 × 25 cm; Prevue DB; Hornbeck's, Rosemont, Illinois) until PHD45 or PHD65. Groups of six cages were placed in sound isolation chambers (Industrial Acoustics, Bronx, New York); siblings could hear but not see one another. In the long protocol, fathers were removed from breeding cages when nestlings were ~PHD10. Siblings were raised together with their mother and another adult female in a sound isolation chamber. At PHD35, young finches were transferred to the individual cages used in the first protocol.

Androgen manipulations and measurements. Androgens were augmented using implants (~50 μg) of dihydrotestosterone (DHT) and endogenous androgen levels were measured as previously described[6]; except that testosterone antibody and extracted serum were allowed to incubate at room temperature for one hour before addition of trace, which improved the linearity of the standard curve at the lower end.

Brain slices and electrophysiological recordings. Brain slices and electrophysiological techniques are only briefly described here; detailed accounts can be found in prior studies[5,6,15]. Recording electrodes were pulled from borosilicate tubing (VWR, West Chester, Pennsylvania) and filled with an internal solution consisting of 3.19% (v/v) 50% D-gluconic acid (Sigma), 10 mM EGTA (Sigma), 5 mM $MgCl_2$ (Sigma), 40 mM HEPES (Fluka, Ronkonkoma, New York), 2 mM Na^+-ATP (Sigma), 0.3 mM Na^+-GTP (Boehringer-Mannheim, Indianapolis, Indiana), 1 mM QX-314 (RBI/Sigma, Natick, Massachusetts). The pH was adjusted to 7.25 with CsOH (50% g per ml H_2O; Aldrich Chemical, Milwaukee, Wisconsin). The final electrode impedances ranged from 2–5 MΩ. NMDA-EPSCs were recorded in 50 μM picrotoxin (Sigma) and 5 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide disodium (NBQX, RBI/Sigma), applied by bath perfusion. NMDA-EPSCs were electrically evoked with bipolar stimulating electrodes while holding the membrane potential 20 mV more positive than the empirically determined EPSC reversal potential to remove the voltage-dependent blockade of NMDA receptors by extracellular magnesium[37,38].

Data acquisition and analysis. Data acquisition and analysis for intracellular recordings were performed with a National Instruments (Austin, Texas) data acquisition board (AT-MIO-16E2), controlled by custom Labview software developed by F. L., Rob Neummann and S. W. Five to ten individual events were collected from a single neuron, digitally filtered, and then averaged to obtain a representative cellular EPSC. The time from the peak amplitude to 1/e of the amplitude was calculated for each representative cellular EPSC using automated Labview software and is reported in the text. Peak amplitudes of evoked EPSCs are not reported because they vary with the slice preparation, stimulating electrodes and stimulus intensity. Cellular EPSCs were normalized by their peak amplitudes, and then averaged by treatment group to create the EPSCs shown in the figures. Statistical analyses were conducted using JMP IN software (SAS Institute, Cary, North Carolina). Nonparametric statistical tests were used because the data were not assumed to be normally distributed. Mann-Whitney *U*-tests and the Wilcoxon Rank Sum were used to assess the significance. In all cases, the minimum significance level was set at $p < 0.05$ using two-tailed comparisons except when previous findings allowed the test to be protected in one direction, as noted. Averages are reported with the standard error of the mean (± s.e.).

Song analysis. Song was collected by placing a male zebra finch in a soundproof recording chamber with a female finch and using digital audio recording techniques (sampled at 20 kHz). Examples were transferred via .wav files to Avisoft Software (SASLab Pro 3.4, Raimond Specht, Berlin, Germany) for analysis and generation of spectrograms (frequency intensity versus time). A song syllable was defined as a continuous marking on the spectrogram. Spectrograms were generated before tutoring, at PHD65, for all pupils, then again three weeks after tutoring. Tutor spectrograms were from >120 PHD. The pre-tutor songs of pupils were analyzed to ensure that they did not already contain elements from the selected tutor. Twenty to forty spectrograms for each bird at each age were scanned to obtain a representative exemplar (2.5 s) that contained all the syllables produced by the bird during the recording period. Exemplars were selected in a blind fashion such that the tutor-pupil pairing was not revealed, and were labeled by the authors to indicate the different song syllables. Two of the authors and an observer blind to the experimental condition of the pupil examined the labeled hard-copy of the 2.5-s spectrogram from an individual pupil and from its tutor. Judges determined which, if any, syllables were learned from the tutor. Any ambiguous notes and syllables were additionally judged by listening to audio playback of the pupil's and tutor's songs. For each pupil, the percentage of syllables learned from the tutor was calculated by dividing the number of learned syllables by the total number of syllables in the bird's repertoire. Averages for control and experimental groups were generated. These averages are reported in the text and table. There was no difference between the values obtained by blind and non-blind ratings (paired *t*-test, $n = 14$ pairs, $p = 0.21$).

Note: audio files of example songs from Fig. 2a can be found on the Nature Neuroscience web site (http://www.nature.com/neuro/web_specials/).

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