Developmental Patterns of NMDAR Expression within the Song System Do Not Recur during Adult Vocal Plasticity in Zebra Finches

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ABSTRACT: All songbirds learn to sing during postnatal development but then display species differences in the capacity to learn song in adulthood. While the mechanisms that regulate avian vocal plasticity are not well characterized, one contributing factor may be the composition of N-methyl-D-aspartate receptors (NMDAR). Previous studies of an anterior forebrain pathway implicated in vocal plasticity revealed significant regulation of NMDAR subunit expression during the developmental sensitive period for song learning. Much less is known about the developmental regulation of NMDAR subunit expression in regions that participate more directly in motor aspects of song behavior. We show here that an increase in NR2A subunit mRNA and a decrease in NR2B subunit mRNA within the vocal motor pathway accompany song learning in zebra finches; however, manipulations that can alter the timing of song learning did not alter the course of these developmental changes. We also tested whether adult deafening, a treatment that provokes vocal change in songbirds that normally sing a stable song throughout adulthood, would render NMDAR subunit expression more similar to that observed developmentally. We report that NR2A and NR2B mRNA levels did not change within the anterior forebrain or vocal motor pathways after adult deafening, even after substantial changes in song structure. These results indicate that vocal plasticity does not require "juvenile patterns" of NMDAR gene expression in the avian song system. © 2003 Wiley Periodicals, Inc. J Neurobiol 58: 442–454, 2004

Keywords: **NMDA receptor; NR2A; NR2B; deafening; testosterone; birdsong**

INTRODUCTION

Neural and behavioral plasticity can be modulated by age, experience, and behavioral or hormonal state. Identifying how these factors alter cellular and synaptic processes is an important step in understanding how a system's aptitude for plasticity is regulated. In this context, the avian song system is an ideal model, because a variety of natural and experimental factors influence vocal plasticity. For instance, in many oscine songbirds, vocal learning is age-regulated; birds acquire and practice songs during an early developmental period characterized by substantial anatomical and molecular transformation within song-related neural circuitry (Nordeen and Nordeen, 1988; Alvarez-Buylla et al., 1990; Herrmann and Arnold, 1991; Kirn et al., 1991; Johnson and Bottjer, 1992; Nixdorf-Bergweiler et al., 1995; Clayton, 1997; Kittelberger et al., 1999). In addition, some oscine species exhibit seasonally regulated periods of neural and

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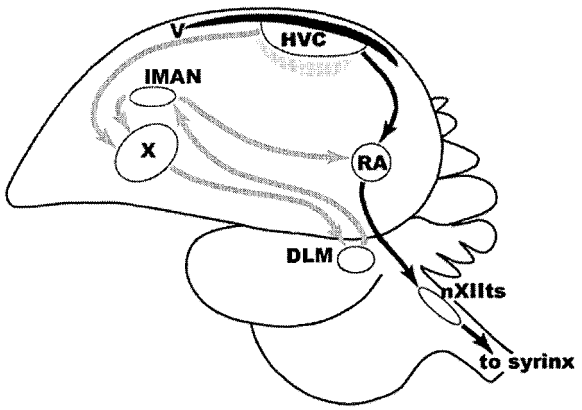


Figure 1 Simplified schematic of the avian song system. The vocal motor pathway, indicated in black, includes the HVC (used as proper name), the robust nucleus of the arcopallium (RA), and the nucleus of the tracheosyringeal nerve (nXIIts). The anterior forebrain pathway, indicated in gray, indirectly connects the HVC to the RA via area X (X), the dorsal-lateral nucleus of the medial thalamus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (lMAN). The ventricle (V) is also indicated. The gray region ventral to HVC, presumably the "shelf", also was analyzed.

vocal plasticity in adulthood that are coupled to fluctuating levels of gonadal androgens (Nottebohm et al., 1986, 1987; Kirn et al., 1991, 1994; Alvarez-Buylla and Kirn, 1997). And even in species where song learning normally is restricted to early postnatal development, altering sensory feedback in adulthood can reinitiate vocal change (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999).

Alterations in the composition and function of N-methyl-D-aspartate receptors (NMDAR) may be one variable that contributes to age and seasonal regulation of avian vocal learning. Activation of NMDARs in the lateral magnocellular nucleus of the anterior nidopallium (lMAN) is critical for normal song learning (Basham et al., 1996), consistent with the role these receptors play in other instances of behavioral learning (Bock et al., 1996; Fendt, 2001; Martinez et al., 2002) as well as in the formation and alteration of sensory representations (Cline and Constantine-Paton, 1989; Kano and Iino, 1991; Simon et al., 1992; Feldman et al., 1996; Jablonska et al., 1999; Myers et al., 2000; Rumpel et al., 2000; Ramoa et al., 2001). Furthermore, developmental studies of the anterior forebrain pathway (AFP), which includes lMAN (see Fig. 1), have revealed significant regulation of NMDAR modulatory subunits that likely influence thresholds for synaptic change. In the lMAN and area X, mRNA levels rise for NR2A and fall for NR2B during the sensitive period for song learning in male zebra finches (Basham et al., 1999; Singh et al., 2000; Heinrich et al., 2002). Correspondingly, NMDAR-mediated EPSCs in the lMAN shorten (Livingston and Mooney, 1997). Together, these data are consistent with a variety of studies demonstrating that increasing the NR2A:NR2B ratio shortens NMDAR-mediated currents and can reduce the likelihood of triggering Ca^{2+}-mediated synaptic strengthening (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Buller et al., 1994; Flint et al., 1997; Roberts and Ramoa, 1999).

Although it is not known how these changes in NMDAR composition and physiology within the AFP influence vocal plasticity (see Livingston et al., 2000; Heinrich et al., 2003), it is interesting that their time course is modified by environmental or hormonal manipulations that affect the timing and quality of song learning. That is, NMDAR maturation in the lMAN is delayed by early isolation from conspecific song (which delays closure of the sensitive period), and is accelerated by early testosterone treatment (which can accelerate and disrupt song development) (White et al., 1999; Basham et al., 1999; Livingston et al., 2000; Singh et al., 2000; Heinrich et al., 2002). Also, seasonal fluctuations in vocal plasticity in adult canaries are accompanied by changes in NR2B expression within the lMAN and in the robust nucleus of the arcopallium (RA), a region of the vocal motor pathway (Singh et al., 2003). These observations suggest that similar mechanisms may modulate the propensity for vocal change both during development and in adulthood.

In contrast to the significant body of information concerning regulation of NMDAR gene expression within the AFP of songbirds, much less is known about developmental regulation within song system nuclei such as RA and HVC (formal name) that participate more directly in motor aspects of song behavior (see Fig. 1). Physiological studies of RA have revealed developmental changes in NMDAR currents that are not altered by early isolation from conspecific song but can be accelerated by testosterone treatment (Stark and Perkel, 1999; Livingston et al., 2000). However, it is not known if these changes in function reflect regulation at the level of gene transcription. In HVC, NMDARs are expressed in both juveniles and adults (Aamodt et al., 1992), however the regulation of NMDAR phenotype within this region has not been explored previously. Thus, a primary goal of the present study was to describe the developmental regulation of NMDAR subunit expression within these nuclei of the descending vocal-motor pathway. In this context, we also examine how hormonal and rearing conditions that affect the timing of vocal plasticity

influence the time course of changes in NMDAR subunit expression within this circuitry.

A second goal of the present study was to further extend our examination of NMDAR expression in relation to adult vocal plasticity. In view of the relationship between changes in NMDAR expression and seasonal song plasticity in adult canaries, we were interested in whether manipulations that provoke vocal change in adult zebra finches would reinstate patterns of NMDAR subunit expression similar to those observed during song development. While song production in adult zebra finches normally is quite stable, prolonged perturbation of either auditory or proprioceptive feedback causes gradual decrystallization and deterioration of adult song patterns (Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Williams and Mehta, 1999; Hough and Volman, 2002). These gradual and persistent song modifications imply a reorganization of neural circuitry, and we examined whether such reorganization is accompanied by changes in NMDAR expression reminiscent of those seen during developmental and seasonal song plasticity.

METHODS

Animals

Male zebra finches (*Peophila guttata*) were hatched in free-flight aviaries, and maintained on a 14:10 light/dark cycle. NMDAR subunit expression was assessed at 20, 40, 60, 80, and >130 days (d) posthatch in males (n = 4–6/group) reared with free access to both parents and other conspecifics. These age groups correspond to several key stages of song learning: before learning (20 d), during sensory acquisition and early sensorimotor learning (40 d), the end of acquisition and during sensorimotor learning (60 d), during late sensorimotor learning (80 d), and after song crystallization (>130 d) (Eales, 1985, 1989; Jones and Westbrook, 1996). *Sensory acquisition* is the period during which a bird memorizes the song of a conspecific tutor; and, *sensorimotor learning* is when birds learn to integrate sensory-motor information and match their own vocal production to the memorized song. To extend the ability to imitate a song beyond 60 d (Eales, 1985; Jones et al., 1996), an additional group of birds was removed from the breeding aviary at 9 d, and placed in a cage with their clutchmates and mother in a room acoustically isolated from adult males. Beginning at 30 d, these males were visually isolated from one another. NMDAR subunit expression in isolated males was examined at 60 d (n = 5/group). In another group of young males, serum androgen levels were manipulated. Early androgen treatment disrupts song development in zebra finches (Korsia and Bottjer, 1991), can accelerate song crystallization in sparrows (Whaling et al., 1995), and both HVC and RA contain androgen-accumulating cells (Arnold et al., 1976; Brenowitz and Arnold, 1992). Therefore, to test the effect of exogenous androgen exposure on NMDAR expression in the vocal motor pathway, 20-day-old aviary-reared males were implanted with a 10 mm silastic tube (Dow Corning), which contained 7 mm of crystalline T (4-androsten-17B-ol-3-one; Steraloids, Newport, RI) and was sealed with silastic glue. Age-matched controls received an empty (blank) implant. Both T- and blank-implanted birds were subsequently housed with their parents and clutchmates in cages until they were sacrificed at 35 d (n = 5–6/group).

To examine the effects of adult deafening on NMDAR subunit expression, normally reared males (210–230 d) were anesthetized and either deafened or sham operated. To deafen animals, an incision was made in the tympanic membrane, the middle ear bones removed, and a fine wire hook was used to extract the cochlea bilaterally. All cochlea were extracted with the distal end (lagena) intact. Sham operates were anesthetized but otherwise unmanipulated so as to preserve normal hearing. Animals were sacrificed 48 h or 3 months after surgery (n = 4–6/group). Another group of deafened animals was sacrificed 7 days after surgery to assess the time course of any induced changes in NMDAR gene expression. In both the developing and young adult visual systems, modified sensory input alters NMDAR subunit composition within hours to days (Quinlan et al., 1999a,b; Philpot et al., 2001). Thus, the 48-h and 7-day time points were chosen to probe for rapid and perhaps transient changes in NMDAR expression. To confirm the presence of song degradation after deafening, the songs of the long-survival group (3 months) were recorded prior to deafening and then every 4 weeks after deafening until several days before sacrifice.

In Situ Hybridization

Radio-isotopic *in situ* hybridization was completed as described previously (Singh et al., 2000; Heinrich et al., 2002) to examine the expression of NR1 (constitutive subunit), and NR2A and NR2B (modulatory subunits most prevalent in the telencephalon) mRNA (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Ishii et al., 1993; Buller et al., 1994). In brief, animals were rapidly decapitated and the brains were harvested, immediately frozen, and stored at −70°C. Serial coronal sections (16 μm) were cut through HVC and RA and mounted such that each slide contained both song nuclei. In addition, for adult deafened animals and their sham controls, anterior sections including lMAN and area X were mounted on separate slides. We used synthetic oligonucleotides (53 or 45 mer) complimentary to zebra finch NR2A or NR2B sequences (Singh et al., 2000; Heinrich et al., 2002) or complementary to the duck NR1 sequence (Kurosawa et al., 1994) to localize and measure NMDAR mRNA expression. Sections were fixed in 4% paraformaldehyde, incubated in .25% acetic anhydride, dehydrated in graded ethanols, and air-dried. Then, they were hybridized overnight at 42°C with purified [35]S-labeled probe (1

$\times 10^7$ CPM/mL for NR2B and NR1 probe; 0.5×10^7 CPM/mL for NR2A). Replicate sets of slides were incubated with NR1, NR2A, or NR2B probes that hybridize specifically to their respective mRNAs in zebra finch tissue (Basham et al., 1999; Singh et al., 2000; Heinrich et al., 2002). After hybridization, sections were washed with saline-sodium citrate under conditions that optimize the signal/noise ratio (see Singh et al., 2000; Heinrich et al., 2002) and then were prepared for autoradiography. Slides were dipped in NTB2 emulsion, stored for 10–21 days depending upon probe type, developed (Kodak D-19), fixed, and lightly stained with thionin. Nissl-defined song regions were identified in each hemisphere, and for each animal eight randomly selected fields per region (16 for HVC) were analyzed. A region ventral to HVC (see Fig. 1) also was analyzed; this region likely was within the HVC "shelf" (Vates et al., 1996; Mello et al., 1998). Within each field (40X), a computer-assisted analysis system (NIH Image) was used to measure the total area occupied by soma, and then the Nissl stain was rendered invisible with a blue filter so that gray level thresholding could be used to measure the total somal area occupied by silver grains. Background grain density obtained from a nontissue portion of the slide then was subtracted to obtain the final measure of somal grain density. Although in previous developmental studies of the AFP we conducted an individual cell analysis of NR2B mRNA in lMAN (Singh et al., 2000), it was not possible to investigate developmental changes in RA or HVC in this way due to extensive cell overlap that was exaggerated in young animals.

For the developmental study, single-factor ANOVAs compared transcript levels across age. Post-hoc comparisons (two-tailed) were Bonferroni-corrected. The effects of song experience and T treatment on mRNA levels were assessed with independent t tests (two-tailed). The effect of adult deafening was assessed by two-way ANOVA (survival time and hearing condition). Only the 48-h and 3-month survival groups were entered in this analysis because the 7-day survival group did not have a separate sham-control group. A one-way ANOVA comparing mRNA levels by survival time among deaf animals further explored whether deafening promoted a time-dependent change in mRNA expression.

Behavioral Analysis

To confirm song degradation after adult deafening, female-directed songs produced by birds in the long-survival groups (deaf and control) were recorded, amplified, and digitized by Avisoft Recorder software. Sonograms were produced using Avisoft SAS lab Pro (fast-Fourier transform length = 256 points, frame size = 100%, and FlatTop window with 50% overlap). Song syllables were defined as acoustic units surrounded by intervals of baseline energy lasting at least 10 ms. Two preoperative recording timepoints were used to confirm that songs were stable prior to deafening. Behavioral analysis focused on changes in the acoustic structure (phonology) of song syllables. Song recorded just prior to deafening was compared with a 10 s sample of postoperative song recorded 3 months after surgery. The phonological similarity between each syllable in the postoperative sample and the preoperative syllable it most resembled in contour, frequency, and duration was given a score of 0 (no similarity), 1 (slight similarity), 2 (high similarity), or 3 (apparent identity). An observer blind to experimental group scored and analyzed the percent of postoperative syllables *matched* (scored 2–3) to a preoperative syllable. To further assess the retention of preoperative syllables, we measured the proportion of unique preoperative syllables that could be identified in postoperative song. For example, if a bird had a five-syllable preoperative song, and four of these syllables matched (score = 2 or 3) a syllable in postoperative song, 80% of the preoperative song was *maintained*. The percent of postoperative song syllables not found in preoperative song was also assessed. If this same bird had a six-syllable song 3 months after deafening and two of these syllables were unmatched (score = 0) to a preoperative syllable, one-third of the postoperative song was *unmatched*. Group differences in the production of matched postoperative syllables were assessed using a one-tailed Mann-Whitney U test. One-tailed Wilcoxon signed-rank tests assessed group differences in maintained preoperative syllables and unmatched postoperative syllables because the control group exhibited no variation in these dependent measures. Group data are presented as average $\pm$SEM.

RESULTS

Developmental changes in NR1 gene expression were evident in RA [$F(4,19) = 7.08$; $p < 0.005$] but not HVC (Fig. 2). In RA, NR1 transcript levels increased transiently by 34% between 20–40 d ($t = 2.893$; $p < 0.05$), then fell by almost 40% between 40–60 d ($t = 3.73$; $p < 0.01$) and remained constant thereafter. In contrast, NR1 transcript levels did not change significantly across age in either HVC or the region just ventral to HVC.

A developmental increase in the expression of NR2A mRNA was evident in both RA [$F(4,18) = 36.8$; $p < 0.0001$] and HVC [$F(4,18) = 4.10$; $p < 0.05$], although the magnitude and profile of these changes differed across regions. In RA (Fig. 3, top), NR2A transcripts rose 258% between 20 and 40 d ($t = 10.9$; $p < 0.001$), an increase that overlaps with early aspects of both song acquisition and sensorimotor learning. Subsequently, NR2A mRNA levels decreased moderately and gradually between 40 d and adulthood ($t = 4.57$; $p < 0.01$). Nonetheless, NR2A levels in adult RA remained significantly higher than levels at 20 d ($t = 7.82$; $p < 0.001$). In HVC (Fig. 3, middle), a more protracted and modest age-related increase in NR2A gene expression was evident. Here,

transcript levels doubled between 20–60 d ($t = 3.83$; $p < 0.05$) and then remained stable into adulthood. There was no effect of age on NR2A message expression in the region just ventral to HVC (Fig. 3, bottom).

In contrast to NR2A, NR2B mRNA levels decreased in both RA (Fig. 4, top) and HVC (Fig. 4, middle) during development. In RA, the age-related decline in NR2B expression [$F(3,14) = 4.66$; $p < 0.05$] occurred abruptly between 40–60 d ($t = 3.09$; $p < 0.05$), just after the primary increase in NR2A transcripts. In HVC, the more gradual decline in NR2B expression was not statistically significant when analyzed by a one-way ANOVA [$F(3,14) = 2.51$; $p = 0.10$], but post-hoc analysis did reveal a significant decline between 20–80 d ($t = 2.53$; $p < 0.05$). In the sample region ventral to HVC, NR2B mRNA levels were not developmentally regulated (Fig. 4, bottom).

Manipulations that alter the course of song learning did not significantly affect the developmental regulation of NR2A or NR2B mRNA expression in either RA or HVC at the ages evaluated (Table 1). These data are in sharp contrast to previous investigations within the AFP, where both early isolation and T treatment modified NMDAR physiology and transcript expression of NMDAR modulatory subunits (White et al., 1999; Livingston et al., 2000; Singh et al., 2000; Heinrich et al., 2002). T treatment beginning at 20 d, which disrupts song learning in zebra finches (Korsia and Bottjer, 1991) and can accelerate song crystallization in at least some species (Whaling et al., 1995), did not significantly alter NR2A or NR2B subunit expression in RA or HVC at 35 d. Also, while early isolation extends the ability to learn song beyond 60 d (Eales, 1985; Jones et al., 1996), this manipulation did not alter the levels of NR2A or NR2B mRNA in RA or HVC at 60 d. NR1 mRNA expression in RA also was not affected by T treatment or isolation. However, in HVC these manipulations were associated with small group differences in NR1 mRNA expression that approached significance. Compared to control animals, NR1 transcripts in HVC were slightly elevated in T-treated birds at 35 d ($t = 2.04$; $p = 0.068$), and were slightly depressed in isolates at 60 d ($t = 2.33$; $p = 0.053$).

To explore whether adult deafening reinstates patterns of NMDAR gene expression characteristic of younger birds, NR2A and 2B message levels were assessed at 48 h and 3 months after adult deafening or sham-operation. For both subunits, there was no effect of hearing condition, survival time, or interaction in any of the four song nuclei measured (Figs. 5 and 6). Similarly, a one-way ANOVA including deafened

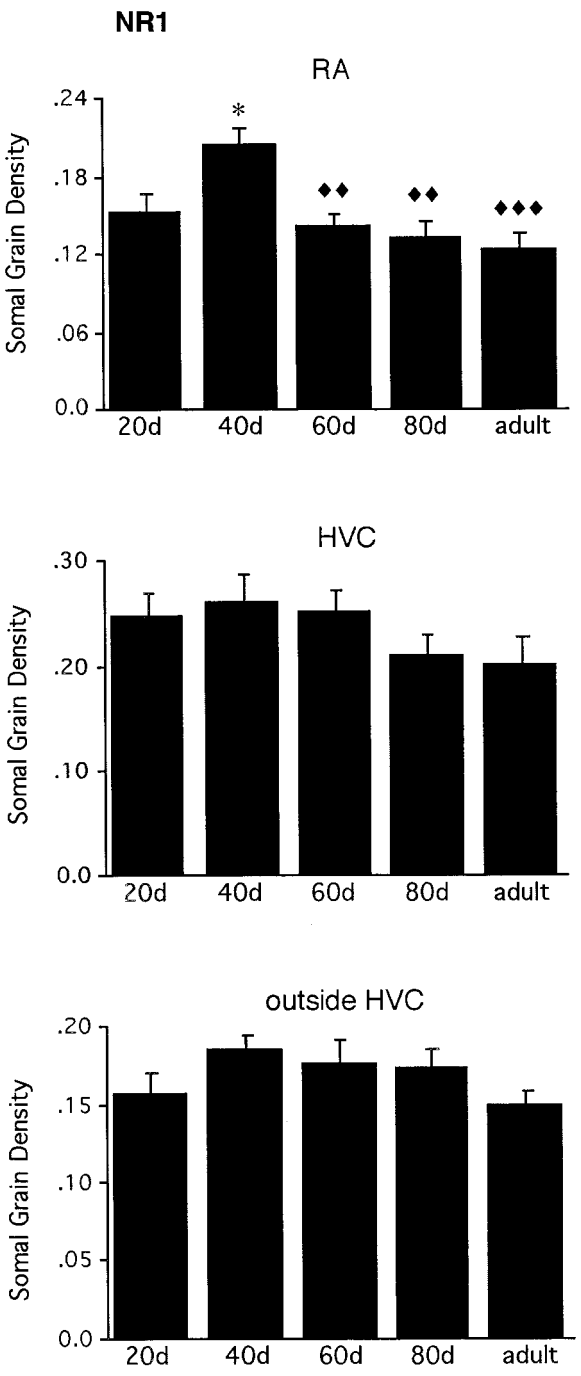


Figure 2 Developmental regulation of NR1 mRNA expression in the vocal-motor pathway of normal male zebra finches. In RA (top), NR1 mRNA expression rises between 20 and 40 d and then declines from 40 d to adulthood. NR1 mRNA expression is not altered during song development in HVC (middle) or the region just ventral to HVC (bottom). Asterisks indicate a significant increase over 20 d levels. Diamonds indicate a significant decline from 40 d levels. *$p < 0.01$; ◆◆$p < 0.01$; ◆◆◆$p < 0.001$. Data shown are mean ±SEM.

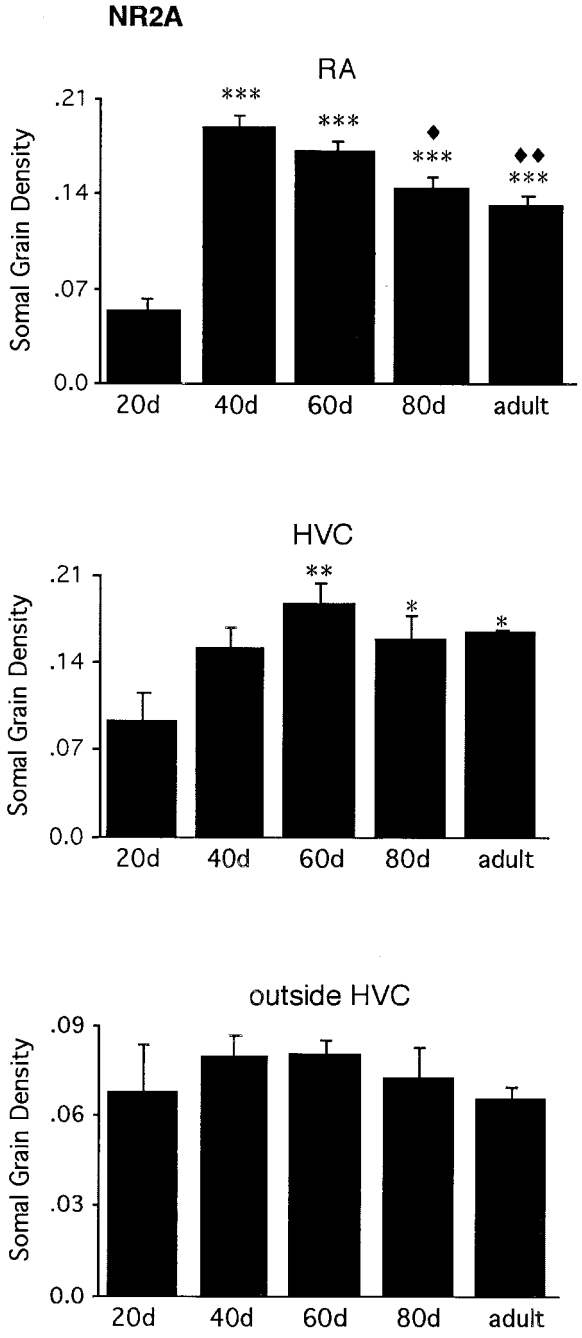


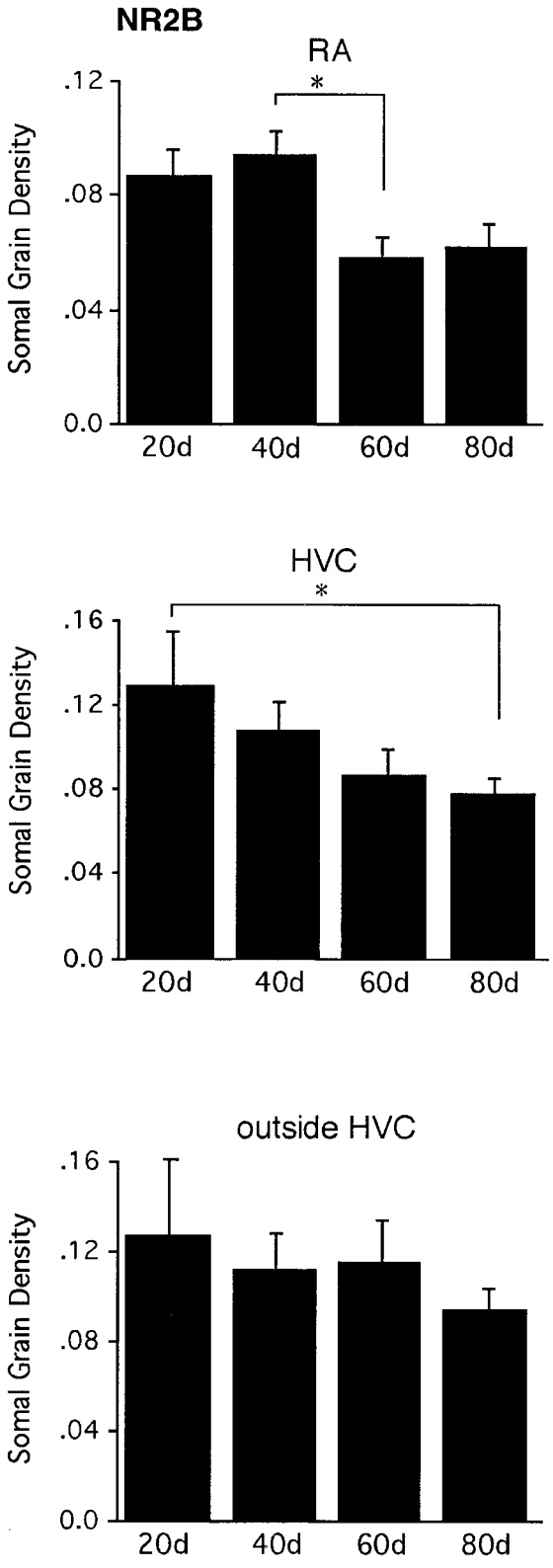
Figure 3 Developmental regulation of NR2A mRNA expression in the vocal-motor pathway of normal male zebra finches. In RA (top), NR2A message expression rises between 20 and 40 d and then declines moderately into adulthood. In HVC (middle), NR2A mRNA expression rises between 20 and 60 d. NR2A mRNA expression is not regulated developmentally in the region just ventral to HVc (bottom). Asterisks indicate a significant increase over 20 d levels. Diamonds indicate a significant decrease from 40 d levels. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ◆$p < 0.05$; ◆◆$p < 0.01$. Data shown are mean ± SEM.

birds surviving 48 h, 7 d, and 3 months showed that NR2A and NR2B mRNA expression did not vary at these time points after deafening. Despite this stability in NMDAR gene expression, significant behavioral change was evident in the song patterns produced by birds that survived 3 months after deafening, while control birds exhibited few if any vocal changes during the same time period. During a 10 s sample of postoperative song bouts, deafened animals sang fewer syllables matching a preoperative syllable than did sham-operated birds (69.3 ± 9.5% vs. 99.4 ± 0.4%; one-tailed $p < 0.005$). Also, deafened birds produced significantly more unmatched syllables than did sham-operates (15.7 ± 9.5% vs. 0%; one-tailed $p < 0.03$). Overall, deaf birds maintained only 66.4 ± 8.8% of their preoperative song while sham-operates maintained all of their preoperative song (one-tailed $p < 0.03$).

DISCUSSION

We show here that vocal learning in zebra finches is accompanied by marked changes in NMDAR gene expression within the vocal motor pathway. Specifically, within both HVC and RA, NR2A mRNA increases and NR2B mRNA decreases during song acquisition and sensorimotor learning, changes similar to those that occur within the developing AFP (Basham et al., 1999; Singh et al., 2000; Heinrich et al., 2002). NR1 mRNA expression does not change significantly within the HVC, and exhibits only a transient elevation within RA, patterns that support earlier observations that MK801 binding in these regions is similar in 30 d as compared to adult zebra finches (Aamodt et al., 1992).

Changes in mRNA expression are functionally meaningful only when they are accompanied by changes in synaptic protein, and NMDAR protein expression appears to be tightly regulated in subunit-specific ways. Regarding NR1, large intracellular pools of the NR1 protein are not incorporated into the cell surface (Huh and Wenthold, 1999; Hall and Soderling, 1997), indicating that synthesis rates may not be primary regulators of NR1 synaptic expression. In contrast, a high percentage of NR2 protein is incorporated into the surface membrane (Hall and Soderling, 1997), suggesting that transcription and translation can be important regulators of the synaptic expression of these subunits. Although synaptic NMDAR composition and number also can be regulated by post-transcriptional mechanisms (Carroll and Zukin, 2002; Barria and Malinow, 2002), within song-related brain regions there is thus far excellent



agreement between developmental changes in NR2A subunit gene expression and predicted changes in NMDAR physiology. Studies relating NMDAR subunit composition to receptor physiology predict that the developmental increase in NR2A:NR2B within HVC and RA would shorten NMDAR current durations, perhaps affecting thresholds for synaptic change (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Buller et al., 1994; Flint et al., 1997; Roberts and Ramoa, 1999). Individual subunit contributions to receptor physiology further predict that NR2A expression would relate most closely to changes in current duration (Flint et al., 1997; Hoffmann et al., 2000). Indeed, in RA, a steep decrease in NMDAR EPSC decay times (Stark and Perkel, 1999; Livingston et al., 2000) occurs in tandem with the steep increase in NR2A gene expression between 20 and 40 d posthatch, while the decrease in NR2B expression is more protracted. A similar relationship between maturation of NMDAR gene expression and physiology is evident in the lMAN (White et al., 1999; Livingston et al., 2000; Singh et al., 2000; Heinrich et al., 2002). Although NMDAR-mediated currents in HVC have not been studied, the changes in NR2A:NR2B gene expression reported here predict that currents also would shorten in this region during the course of song learning.

The developmental regulation of NMDAR subunit expression within the vocal motor pathway is accompanied by robust changes in the anatomy of these same regions. In RA, the early rise in NR1 and NR2A mRNA expression overlaps with the arrival of HVC axons within RA (Mooney, 1992) and significant growth of RA projection neurons as evidenced by heightened spine density, local collateral branching, and distal dendritic branching (Kittelberger and Mooney, 1999). Although a causal relationship has not been tested, a significant portion of the increase in NR2A mRNA and the transient rise in RA's NR1 expression may be associated with increased synaptic input associated with the arrival with the HVC-RA axons (Kittelberger and Mooney, 1999). Moreover,

Figure 4 NR2B mRNA expression during song learning in the vocal-motor pathway of normal male zebra finches. In RA (top), NR2B mRNA expression declines markedly between 40 and 60 d during early sensorimotor learning. In HVC (middle), NR2B mRNA expression falls gradually between 20 and 80 d. Unlike expression in HVC and RA, NR2B message levels ventral to HVC (bottom) do not change during song development. $*p < 0.05$. Data shown are mean ±SEM.

Table 1 Effect of Hormonal and Rearing Conditions on NMDAR Subunit Expression in the RA and HVC

			NR2A	NR2B	NR1
RA	(35 d)	Blank	.054 ± .004	.172 ± .011	.174 ± .020
		T-implant	.056 ± .007	.151 ± .010	.195 ± .012
	(60 d)	Control	.170 ± .008	.063 ± .009	.141 ± .011
		Isolate	.160 ± .011	.069 ± .008	.128 ± .007
HVC	(35 d)	Blank	.044 ± .003	.117 ± .009	.219 ± .021
		T-implant	.050 ± .005	.135 ± .016	.275 ± .024
	(60 d)	Control	.184 ± .019	.087 ± .011	.250 ± .022
		Isolate	.154 ± .013	.078 ± .009	.196 ± .014

Data shown are average (± SEM) somal grain density for each oligoprobe.

the formation of new RA synapses during this early period of song learning could be promoted by NR2B-containing synaptic NMDARs, because synaptic NR2B expression facilitates long-term potentiation (Barth and Malenka, 2001), which in turn is associated with spine formation (Engert and Bonhoeffer, 1999). Consistent with this idea, NR2B mRNA in RA does not decline until after 40 d, when the density and number of dendritic spines in RA recess moderately (Kittelberger and Mooney, 1999). In HVC, the developmental increase in NR2A mRNA and decline in NR2B mRNA are more protracted. Many new neurons are incorporated into the HVC during song learning (Nordeen and Nordeen, 1988; Kirn and DeVoogd, 1989), and to facilitate synapse formation these new neurons may initially express high levels of NR2B. If so, the ongoing insertion of new neurons into HVC could partially mask the maturational change from high NR2B to high NR2A expression. Because neuron addition in HVC continues throughout adulthood,

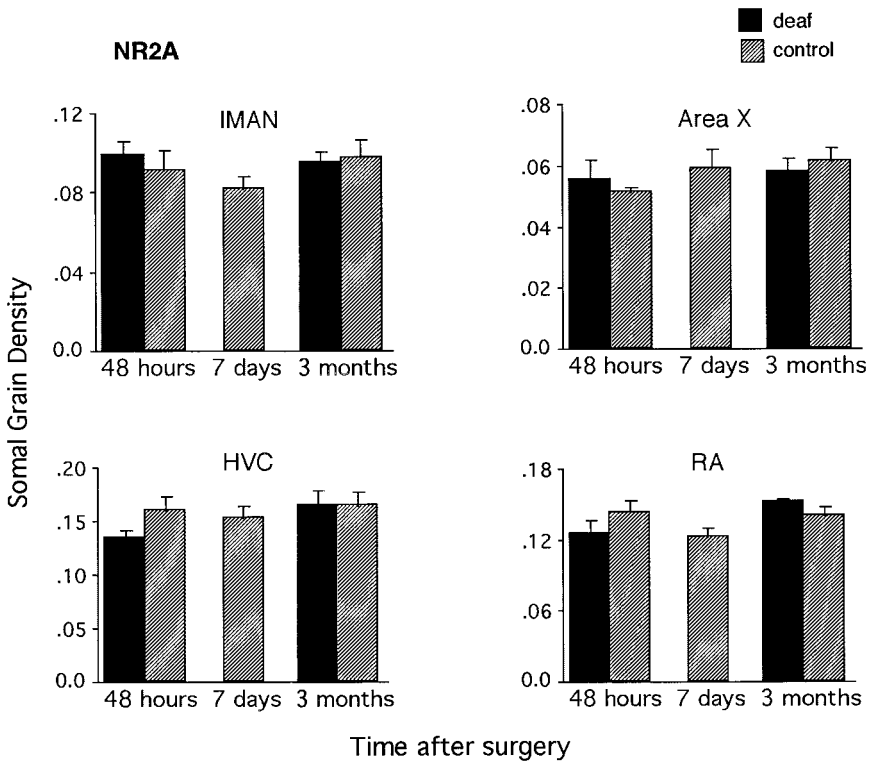


Figure 5 NR2A mRNA expression in several key song nuclei of sham-operated (black bars) or adult deafened (open bars) zebra finches sacrificed 48 h, 7 days, or 3 months after surgery. NR2A mRNA expression in lMAN, area X, HVC, and RA is not affected by the removal of auditory feedback in adulthood. Data shown are mean ±SEM.

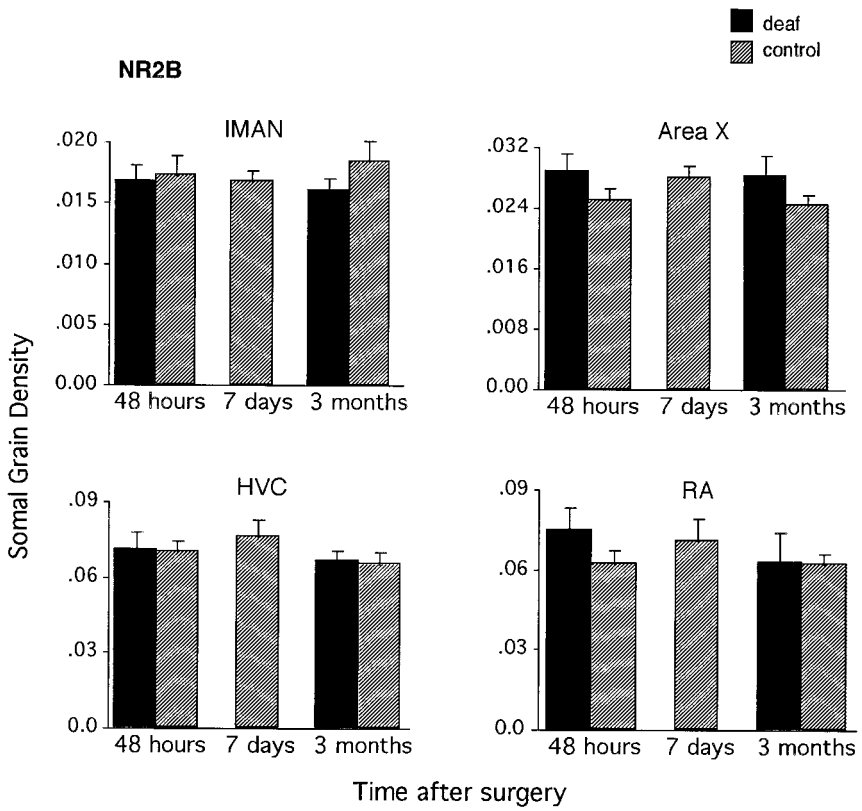


Figure 6 NR2B mRNA expression in several key song nuclei of sham-operated (black bars) or adult deafened (open bars) zebra finches sacrificed 48 h, 7 days, or 3 months after surgery. NR2B mRNA expression in lMAN, area X, HVC, and RA is not affected by the removal of auditory feedback in adulthood. Data shown are mean ±SEM.

it would be interesting to determine if newborn neurons arriving in HVC exhibit a transient elevation in NR2B expression regardless of when they are incorporated.

While changes in NMDAR physiology and subunit expression within the vocal motor pathway coincide with vocal learning and song system development, their functional relationship to behavior remains elusive. It has been speculated that longer NMDAR synaptic currents associated with a lower NR2A:NR2B ratio might be permissive for experience-dependent synaptic strengthening (Carmignoto and Vicini, 1992; Quinlan et al., 1999a,b; Tang et al., 1999), and in some systems changes in receptor composition have been put forward as a possible mechanism for constraining critical periods for developmental plasticity (Carmignoto and Vicini, 1992; Roberts and Ramoa, 1999; Singh et al., 2000). However, we show here that isolation from conspecific song, which extends the sensitive period for song acquisition beyond 60 d (Aamodt et al., 1992; Jones et al., 1996; Eales, 1985), does not prevent the normal developmental increase in NR2A:NR2B ratio in either HVC or RA. Similarly, isolation rearing does not alter NMDAR decay times within the RA (Livingston et al., 2000). Together, these results indicate that song acquisition does not require "juvenile patterns" of NMDAR gene expression or physiology within the vocal motor pathway. Thus, while we cannot rule out the possibility that neural mechanisms subserving song learning differ fundamentally in isolate and normal birds, it presently seems likely that these maturational changes in NMDAR receptor structure and function are not sufficient for terminating the sensitive period for song acquisition. Similar conclusions recently have been reached regarding developmental changes in NMDAR expression and physiology within the lMAN (Livingston et al., 2000; Heinrich et al., 2003).

Although early testosterone treatments that disrupt song learning in zebra finches can accelerate the maturation of NMDAR currents in RA (White et al., 1999), we failed to detect a significant effect of T treatment on NMDAR modulatory subunit gene expression in either HVC or RA. It is likely that physiological recordings in RA are biased towards the large neurons that are androgen target cells, whereas

our somal grain analysis included all cell types (neurons as well as glia). Only a fraction of RA neurons are androgen-accumulating (Brenowitz and Arnold, 1992), and it is possible that our overall analysis would not be able to detect an effect of T that is specific to only the subset of RA neurons that are androgen targets. Unfortunately, we were unable to conduct a cell-by-cell analysis of NMDAR subunit expression in this region (see Methods). However, while the data from RA hinted at an effect of T on NR2B transcript levels, there was no evidence of an effect on NR2A mRNA levels. Because NMDAR current durations are influenced particularly by changes in NR2A expression (Flint et al., 1997; Hoffmann et al., 2000), a more plausible explanation for the discrepancy between the physiological and molecular data is that early T treatment alters NMDAR function in RA through post-transcriptional mechanisms (Carroll and Zukin, 2002). For example, if T promotes an increase in neural activity within RA, this could accelerate the insertion of NR2A subunits into synaptically localized NMDARs (Barria and Malinow, 2002).

In the present investigation, we also were interested in whether induced vocal plasticity in adulthood is accompanied by changes in the expression of genes encoding the NR2A or NR2B subunits. It was recently shown that adult canaries respond to changes in photoperiod (a manipulation that promotes change in T levels and vocal plasticity) with altered NR2B expression in both the lMAN and RA (Singh et al., 2003). This observation in particular led us to question whether deafening-induced vocal change in an age-regulated learner also would entail changes in NMDAR modulatory subunits that could encourage growth and refinement of connections within the AFP or motor pathway. Vocal plasticity in adult deafened zebra finches requires an intact lMAN (Brainard and Doupe, 2000), suggesting that the AFP may convey an instructive or permissive signal to RA that can promote change in vocal behavior (Mooney, 2000; Solis et al., 2000; Troyer and Doupe, 2000). Also, it has been suggested that adult deafening may result in reorganization of an auditory-motor map in HVC (Troyer and Doupe, 2000; Troyer and Bottjer, 2001). Thus, "permissive" changes in the expression of NMDAR modulatory subunits might be anticipated in HVC, RA, and/or the AFP after deafening. However, we detected no changes in transcript levels for either NR2A or NR2B, within HVC, RA, lMAN, or area X, even 3 months following adult deafening, by which time song structure had changed substantially. Interestingly, deafening early in life can alter NMDAR physiology in both the AFP and vocal motor pathway. This manipulation transiently prolongs NMDAR currents in lMAN, and slows NMDAR currents in RA once birds reach adulthood (Livingston et al., 2000). The present study suggests that the effects of deafening on NMDAR subunit expression may be developmentally restricted. Alternatively, deafening at any time may alter NMDAR current durations, but through mechanisms that do not involve changes in gene transcription. Further molecular studies in young animals and physiological studies in adult deafened birds will be needed to evaluate these possibilities.

Developmental and seasonal vocal plasticity correlate with a number of other behavioral, neural, and endocrine changes, and their absence in the adult deafening paradigm could account for the lack of regulatory changes in NMDAR gene transcription. For instance, in young zebra finches and adult canaries, the transition from a low to high NR2A:NR2B ratio within both the AFP and vocal motor pathway is associated with substantial neural growth (Bottjer et al., 1985; Nottebohm et al., 1986) that most likely is not associated with adult deafening (Wang et al., 1999). And, both developmental and seasonal changes in NMDAR composition are accompanied by substantial changes in androgen secretion (Hutchison et al., 1984; Nottebohm et al., 1987). It may be that structural and physiological changes in the NMDAR relate to these changes, rather than to vocal plasticity per se. Perhaps by studying NMDAR expression in species that exhibit seasonal changes in androgen levels and the size of song nuclei without vocal remodeling (e.g., white crowned sparrows), we can learn more about the functional significance of changes in receptor composition and function.

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