# Developmental and Hormonal Regulation of NR2A mRNA in Forebrain Regions Controlling Avian Vocal Learning

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**ABSTRACT: Developmental changes in the composition of NMDA receptors can alter receptor physiology as well as intracellular signal transduction cascades, potentially shifting thresholds for neural and behavioral plasticity. During song learning in zebra finches, NMDAR currents become faster, and transcripts for the modulatory NR2B subunit of this receptor decrease in lMAN, a region in which NMDAR activation is critical for vocal learning. Using *in situ* hybridization, we found that NR2A transcripts change reciprocally, increasing significantly in both lMAN (59%) and in another song region, Area X (38%), between posthatch day (PHD) 20 and 40, but not changing further at PHD60 or 80. In adjacent areas not associated with song learning, NR2A mRNA did not change between PHD20–80. Although early song deprivation (which extends the sensitive period for song learning) delays changes in NR2B gene expression and NMDAR physiology within the lMAN, it did not alter NR2A mRNA levels measured at PHD40, 45, or 60. Early testosterone (T) treatment, which disrupts vocal development and accelerates the maturation of both NR2B levels and NMDAR physiology in lMAN, also significantly increased NR2A transcripts measured at PHD35 in lMAN. In Area X, a similar effect of T approached significance. Together with our previous studies, these results show that in a pathway critical for vocal plasticity, the ratio of NR2A:NR2B mRNA rises abruptly early during the sensitive period for song learning. Furthermore, androgen regulation of NMDAR gene expression may alter thresholds for experience-dependent synaptic change.**  J Neurobiol 51: 149–159, 2002;

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## INTRODUCTION

Sensitive periods are developmental windows during which the nervous system is especially susceptible to experience-dependent changes in functional organization. Avian song learning, ocular dominance column formation in visual cortex, and filial imprinting in the domestic chicken are examples of such developmentally regulated plasticity, and it is presumed that sensitive periods for these phenomena are determined by changes in the cellular events that regulate synaptic strength (Goodman and Shatz, 1993). One such event, activation of the *N*-methyl-D-aspartate receptor (NMDAR), has been implicated in various forms of learning and memory, and this receptor undergoes developmental changes in subunit structure that may

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impact synaptic plasticity during sensitive periods (Carmignoto and Vicini, 1992; Binns et al., 1999; Quinlan et al., 1999b; Roberts and Ramoa, 1999).

Many songbird species learn their songs during a discrete developmental period, and there is emerging evidence that NMDARs may modulate the capacity for song learning. Song behavior is learned in two distinct stages: during sensory acquisition, birds memorize songs produced by other members of their species, and during sensorimotor learning they use auditory feedback to match their own vocalizations to that memorized model. An anterior forebrain pathway (AFP) has been implicated in song learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991), and in one region of this pathway, the lateral magnocellular nucleus of the anterior neostriatum (lMAN), NMDAR activation during song exposure is critical for normal song learning (Basham et al., 1996). Furthermore, over the course of song development, NMDAR density, as well as mRNA levels for the constituent NR1 subunit of the NMDAR, decrease in lMAN (Aamodt et al., 1992; Singh et al., 2000). Coincident with this developmental decline in overall receptor density, expression of the NR2B modulatory subunit of the NMDAR within lMAN decreases (Basham et al., 1999; Singh et al., 2000) and NMDAR-mediated synaptic currents shorten (Livingston and Mooney, 1997).

There are several reasons for considering changes in the expression of NMDAR modulatory subunits as important correlates of the sensitive period for vocal learning. First, such changes influence receptor function in ways that could impact the threshold for initiating synaptic change. In a variety of brain regions, changes in the modulatory subunits of the NMDAR have been associated with a shortening of the NMDAR-mediated currents (Flint et al., 1997; Kuehl-Kovarik et al., 2000). Such a physiological change could affect the temporal stringency for coincidence detection, as well as the extent of NMDAR-mediated $Ca^{2+}$ influx (Brimecombe et al., 1997; Hoffmann et al., 2000; Philpot et al., 2001). Secondly, because NMDAR subunits differ in their associations with several key synaptic proteins (Kornau et al., 1995; Leonard et al., 1999), changes in NMDAR composition could affect the initiation of intracellular cascades that modulate changes in synaptic strength. Finally, changes in NMDAR composition are seen as promising candidates for regulating developmental plasticity because their timing is affected by manipulations that also impact sensitive periods. For instance, rearing zebra finches in isolation from conspecific song delays closure of the sensitive period for sensory acquisition (Price, 1979; Eales, 1987) and also retards both the down-regulation of NR2B mRNA expression in lMAN (Singh et al., 2000) and the shortening of NMDAR currents in this region (Livingston et al., 2000). In contrast, testosterone (T) exposure can promote early crystallization of song (Whaling et al., 1995) and impair song development (Korsia and Bottjer, 1991), and it also prematurely decreases NR2B mRNA (Singh et al., 2000) and NMDAR current durations within lMAN (White et al., 1999; White and Mooney, 2000).

In many developing systems the down-regulation of NR2B in forebrain regions is accompanied by increased expression of NR2A, another NMDAR modulatory subunit (Monyer et al., 1994; Laurie et al., 1997). In fact, the developmental regulation of visual cortical plasticity in mammals has been attributed largely to regulation of NR2A expression. Rearing animals in the dark prolongs the sensitive period for ocular dominance column plasticity, and specifically prevents the normal increase in NR2A expression (Quinlan et al., 1999a; Chen et al., 2000). In rat visual cortex, this effect is bidirectional; synaptically associated NR2A protein levels increase rapidly after a dark-reared animals' first exposure to light, and then decrease again after the animal is returned to the dark, yet levels of NR2B protein are unaffected by these manipulations (Quinlan et al., 1999a). The developmental increase in NR2A also appears to be the primary factor altering NMDAR current duration in mammalian neocortical neurons (Flint et al., 1997; Hoffmann et al., 2000). On the other hand, it is the decline in NR2B expression rather than the shortening of NMDAR currents that coincides most closely with the developmental decrease in the ability to induce long-term potentiation at thalamocortical synapses (Barth and Malenka, 2001), and the threshold for hippocampal LTP is reduced in adult transgenic mice overexpressing NR2B (Tang et al., 1999). These findings suggest that both the NR2B and NR2A subunits may affect NMDAR function in ways that could regulate plasticity. Consequently, to understand more fully how NMDARs within lMAN may affect the capacity for song learning, we determined whether NR2A expression within this region is developmentally regulated, and whether such expression is affected by manipulations that impact the timing of song development. We report that early in the sensitive period for song acquisition mRNA transcript levels for the NR2A subunit increase in both lMAN and Area X (another region of the AFP), and in lMAN this early increase is androgen sensitive. However, the developmental change in NR2A mRNA is not delayed

in either song region when the sensitive period is extended by early song deprivation.

## MATERIALS AND METHODS

### Subjects

Male zebra finches were raised in our laboratory and maintained on a 14:10 light/dark cycle. Normally reared birds were housed in breeding aviaries with both parents until they were sacrificed, with the exception of birds surviving to posthatch day (PHD) 80, which were segregated from their parents at PHD65 and transferred to same-sex holding aviaries. These conditions foster song acquisition between PHD35 and 65 (Eales, 1985; Jones et al., 1996). In other birds, the period of sensory acquisition was delayed by rearing them in isolation from conspecific song (Aamodt et al., 1995). These birds were placed by PHD8 into cages with their clutchmates and parents. When the oldest chick reached PHD9, the father was removed and the clutchmates remained together with their mother in a cage visually and acoustically isolated from adult males. At PHD30, juvenile males were moved into separate cages and visually isolated from one another. Finally, to determine if T regulates developmental changes in NR2A gene expression, a separate group of normally reared PHD20 males was implanted subcutaneously with a 10 mm Silastic tube that was either empty or filled with 7 mm of crystalline T (4-androsten-17$\beta$-ol-3-one; Steraloids, Newport, RI). Similar early androgen exposure disrupts normal song development in zebra finches (Korsia and Bottjer, 1991) and accelerates developmental changes in lMAN NR2B expression and NMDAR physiology (White et al., 1999; Singh et al., 2000).

### Tissue Preparation

Normally reared male zebra finches ($n$ = 5/group) were decapitated at PHD20, 40, 60, or 80, and their brains were removed, coated with OCT compound, frozen on dry ice, and stored at −70°C. These ages represent several key stages in zebra finch song learning: before acquisition (PHD20), early acquisition and vocal practice initiated (PHD40), acquisition ending (PHD60), and vocal practice near completion (PHD80). Brains were taken from isolate males ($n$ = 4–5/group) at PHD40 and 45 [when adult levels of NR2A mRNA normally are achieved (see Results)] and at PHD60 (when isolated, but not control birds can still acquire new song material). T-treated birds and their age-matched controls ($n$ = 6/group) were sacrificed at PHD35. On the day of sectioning, brains were warmed to −16°C for 1 h, and coronal sections (16 $\mu$m) were cut in a cryostat and serially mounted on Vectabond-coated slides. Sections were air-dried for 1 h at room temperature and stored in airtight boxes at −70°C.

### NR2A Oligonucleotide

We designed and used a 53 base synthetic oligonucleotide probe complementary to a partial NR2A sequence isolated from the zebra finch forebrain. RNA from zebra finch and control (rat forebrain) was subjected to RT-PCR analysis using forward and reverse primers targeted to a highly conserved portion of the rat NR2A gene (97% homology with mouse, 93% homology with human). The 301 bp zebra finch PCR product obtained using this strategy had 75% sequence similarity with the rat NR2A mRNA (Monyer et al., 1992; see Fig. 1).

### Probe Labeling

The HPLC purified NR2A oligonucleotide was obtained from Genosys Biotechnologies and the 3′ end was labeled with [$^{35}$S]dATP (New England Nuclear, Boston, MA) using a terminal deoxy-nucleotidyl transferase (tdt) kit (Boehringer Mannheim, Indianapolis, IN). Five picomoles of oligonucleotide was incubated with 50 pmol of [$^{35}$S]dATP (≅1250 Ci/mmol), 5 m*M* cobalt chloride, and 50 units of tdt in tdt buffer at 37°C for 60 min. Following incubation, 200 $\mu$g of glycogen (Boehringer Mannheim) was added as a carrier and the labeled oligo-probe was precipitated in 0.4 *M* lithium chloride (Sigma, St. Louis, MO) and ethanol at −20°C (overnight). The reaction mixture was centrifuged at 14,000 rpm for 30 min at 4°C. The pellet was washed once in 80% ethanol and then dissolved in 10 m*M* Tris-HCL (pH 8.0), 1 m*M* EDTA, and 5 m*M* dithiothretiol (DTT; Sigma) solution, and stored at −20°C. Radiolabeling using this procedure generated probes ranging from 4 to 8 × $10^8$ cpm/mL. Radiolabeled probes were used within a week of preparation.

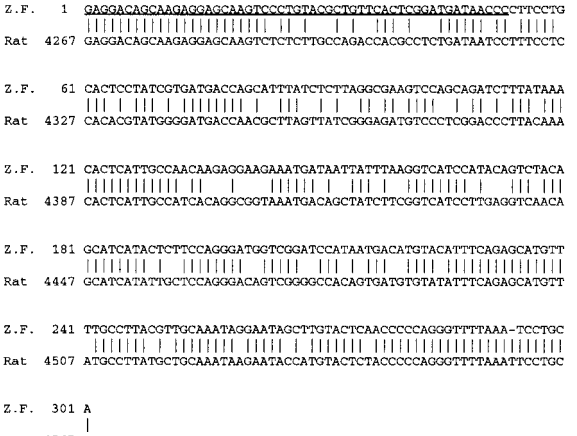


**Figure 1** Comparison of partial nucleotide sequences (301 bp) for zebra finch and rat NR2A mRNA (Monyer et al., 1992). The underlined portion of the zebra finch sequence was used as the template for design of the NR2A oligoprobe used in these studies.

## *In Situ* Hybridization

To control for variability in probe labeling, hybridization, and autoradiography, all analyses and comparisons involved tissue run within a single hybridization experiment. PHD20, 40, 60, and 80 animals were run together in an individual hybridization, as were PHD35 T-treated and control animals together with PHD60 isolates and controls. The final experiment contained PHD40 and 45 isolates and controls. The following conditions were established to optimize the signal/noise ratio by experiments in which we varied the hybridization temperature, amount of probe, and stringency of posthybridization washes (salt concentration and temperature). On the day of hybridization, slides containing sections through the anterior forebrain (including all of lMAN and Area X) were warmed to room temperature, fixed in cold 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.6) for 15 min, washed twice in PBS (5 min each), equilibrated in 0.1 *M* tri-ethanol amine saline (pH 8.0) for 5 min, and acetylated in freshly prepared 0.25 acetic anhydride in 0.1 *M* tri-ethanol amine saline (pH 8.0) for 10 min with shaking. Sections were washed twice in 2X saline-sodium citrate (SSC), dehydrated in a graded series of ethanol, and air-dried for 30 min. Each slide was covered with 180–200 $\mu$L of hybridization buffer containing 0.5 $\times$ $10^7$ cpm/mL purified $^{35}$S-labeled probe, 50% deionized formamide, 4X SSC, 1X Denhardt's solution, 100 mg/mL denatured salmon sperm DNA, 250 $\mu$g/mL yeast tRNA, 100 $\mu$g/mL poly A, 5 $\mu$g/mL poly dA, 50 m*M* DTT, 10% dextran sulfate, and 10 m*M* Tris-HCL/1 m*M* EDTA buffer (pH 7.6). Slides were coverslipped, arranged in a chamber humidified with 50% formamide/4X SSC, and incubated at 42°C for 16–18 h. After hybridization, coverslips were removed in 2X SSC at 42°C, and slides were washed twice in 2X SSC at 42°C (5 min each). Then the slides were washed in 1X SSC at 42°C (15 min), twice in 0.5X SSC at 55°C (15 min each), and finally in 0.5 SSC for 5 min at room temperature. Slides were dehydrated for 2 min each in 50, 70, 95, and 100% ethanol and allowed to air dry for 30 min.

The NR2A oligoprobe used in these studies hybridized specifically with its target mRNA. No specific hybridization was detected after incubating the sections with $^{35}$S-labeled sense probe. Hybridization signals were not altered by the addition of 100-fold excess cold NR2B probe to the hybridization buffer prior to hybridization. However, the addition of 100-fold excess unlabeled NR2A probe to the hybridization buffer before hybridization abolished all hybridization signals. NR2A hybridization was greater over cell somata than neuropil (see Results).

## Autoradiography

Hybridized slides were washed in 95% ethanol for 5 min and then twice (10 min each) in 100% ethanol. Slides were defatted in xylene (10 min $\times$ 2) and left overnight in xylene before being washed in 100% ethanol (10 min $\times$ 2) and air dried for 30 min. Slides were dipped in Kodak NTB2 emulsion at 42°C for 2–3 s, placed vertically on wet tissue papers for 1 h, and then air dried in the dark overnight. They then were placed in airtight boxes with desiccant, sealed with black electrical tape, and stored at 4°C for 3 weeks. Emulsion-coated slides then were warmed to room temperature and developed in Kodak D-19 developer (15°C) for 2.5 min, washed in water (15°C) for 30 s, and fixed in Kodak fixer (15°C) for 5 min. Slides were run under tap water for at least 30 min, fixed in 10% formalin solution, washed in water, lightly stained in thionin solution, dehydrated, and then coverslipped.

## Analysis

lMAN and Area X were identified in the Nissl stained slides. The analysis of hybridization levels initially involved a calculation of silver grain area/total somal area using a computer-assisted image analysis system (Image; NIH). For each subject, two different sections were selected for analysis representing random anterior/posterior locations in each nucleus. In these sections, two adjacent fields were sampled from the approximate center of the nucleus in each hemisphere. In each field sampled, the total area occupied by intact soma was measured at 40X magnification. Then the area occupied by silver grains was measured within this defined somal area. For this analysis a blue filter (transmission wavelengths $\leq$490 nm) was placed over the light source to render the Nissl staining invisible, thus making it possible to measure silver grain area based on gray-level thresholding. Somal grain density was calculated as the area occupied by silver grains divided by the total somal area measured. For each subject, the eight measures for each region (four each from the right and the left hemispheres) were averaged because no systematic hemispheric or rostrocaudal variations were evident. For some groups (see Figs. 2 and 3), two other nonsong regions were measured similarly. These were "medial LPO" (a portion of the lobus parolfactorius medial to the center of Area X) and lateral

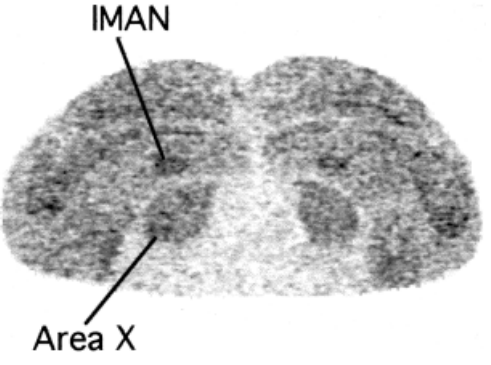


**Figure 2** Photomicrograph of X-ray film NR2A *in situ* hybridization demonstrating typical regional patterns in an anterior section of male zebra finch brain (PHD40).

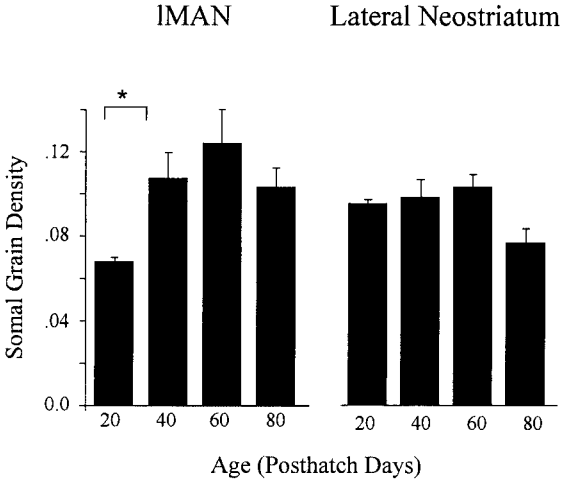


**Figure 3** Developmental regulation of NR2A mRNA in lMAN (left) and adjacent neostriatum (right) of male zebra finches. In lMAN, NR2A mRNA expression increased between PHD20–40 and did not change significantly thereafter. In contrast, within the lateral neostriatum, there was no effect of age on levels of NR2A transcripts. Data shown are means ± S.E.M. $*p < .01$.

neostriatum (part of the neostriatum ventrolateral to lMAN and lateral to Area X). Because control experiments showed that neuropil labeling included specific hybridization [perhaps reflecting mRNA expression in dendrites, as has been shown in other systems (Benson, 1997; Gazzaley et al., 1996)], background grain density for each animal was determined by calculating the average of eight measurements (area occupied by silver grains/total area) taken from nontissue portions of the slides adjacent to the sections chosen for analysis. This estimated background grain density was subtracted from the somal grain densities for each animal. For each region, the effects of age were evaluated by separate one-way ANOVAs (2-tailed) followed by posthoc Bonferroni tests on selected groups. Isolates and controls at PHD40, 45, and 60, as well as T-treated and controls at PHD35 were compared by independent *t* tests (2-tailed).

Group differences in the somal grain density measurements described above could reflect differences in cell clustering and overlap rather than differences in the proportion of cells expressing the message, or the average expression per cell. To tease apart these possibilities, we also measured hybridization levels in single nonoverlapping cells in lMAN of PHD20 and 40 birds. This allowed a fuller description of the particularly striking developmental changes in hybridization found between those ages (see Results). A similar cellular analysis could not be conducted in Area X because nonoverlapping cells were rare in this region. At 100X, fields within the central portion of lMAN were surveyed for neurons, identified by their darkly stained cytoplasm, clear nucleus, and prominent nucleolus. For each isolated neuron encountered, the total somal area and the somal area occupied by silver grains were calculated. Background grain density for each animal was calculated as the average of eight measurements taken from nontissue portions of the slides. For each neuron analyzed, background grain density was multiplied by the neuron's somal area, and this value then was subtracted from the cell's total silver grain area. The average size of a silver grain was then calculated and used to transform the data to number of grains/neuron. Finally, somal area was used to convert that measurement to grains/100 $\mu m^2$. For each animal, 24 lMAN neurons drawn equally from each hemisphere were averaged, and the effects of age were compared by independent *t* tests (2-tailed). To examine age-related changes in the distribution of labeling intensity and to explore whether effects were evident among all cell size classes, we pooled all neurons within each age group and created frequency histograms by sorting neurons according to labeling intensity (bin width = 2 grains/100 $\mu m^2$) or cell size (bin width = 50 $\mu m^2$).

## RESULTS

Qualitatively, the developmental and regional profile of NR2A mRNA in adult male zebra finch brain was consistent with NR2A expression patterns described in adult mammalian brain (Riva et al., 1994; Shi et al., 1997; Yamakura and Shimoji, 1999). NR2A hybridization was relatively high throughout the telencephalon, cerebellum, and optic tectum, and lower in the dorsal thalamus and brainstem. In the song system, NR2A transcript levels were higher in lMAN than in surrounding neostriatum, and also higher in Area X than in the surrounding LPO (Fig. 2). Similar to developmental profiles of NR2A mRNA in mammalian brain, there was a generalized increase in NR2A hybridization over the course of development in the zebra finch.

### Developmental Profile of NR2A mRNA Expression

Within lMAN, NR2A mRNA expression increased early during song learning and remained relatively constant after PHD40 (Fig. 3). A one-way ANOVA revealed a main effect of age on NR2A somal grain density in this region [$F(3, 16) = 5.30$; $p < .01$]. Posthoc analysis confirmed that a significant increase (59%) in NR2A transcripts occurred between PHD20 and 40 [$t = -3.52$; $p < .05$ (Bonferroni *t* tests)] and that expression did not differ significantly among the remaining age groups. In contrast, in the lateral neostriatum adjacent to lMAN there was no effect of age on NR2A mRNA expression.

Within Area X, there also was a developmental up regulation of NR2A mRNA [main effect of age: $F(3,$

16) = 6.351; $p = .005$], although this increase was less dramatic than that which occurred within lMAN. In Area X, NR2A transcripts increased by 38% between PHD20 and 40 and expression levels did not change significantly thereafter (Fig. 4). In the LPO medial to Area X, there was no effect of age on NR2A mRNA.

To evaluate whether the increase in NR2A mRNA expression between PHD20–40 might merely reflect an increase in cellular overlap we measured the NR2A hybridization signal over individual lMAN neurons (see Materials and Methods). As shown in Figure 5, this single cell analysis also revealed a significant increase in NR2A transcripts in lMAN neurons between PHD20 and 40 ($t = -4.1$; $p < .01$). The developmental pattern of NR2A mRNA expression was evident among all cell size classes in lMAN, however, it was somewhat magnified in the largest cells (see Fig. 6).

## Effects of Early T Exposure and Song Experience on NR2A mRNA Expression

In zebra finches, chronic exposure to T beginning prior to PHD40 disrupts normal song development (Korsia and Bottjer, 1991) and also accelerates the developmental decrease in NR2B expression within lMAN (Singh et al., 2000). As shown in Figure 7, early T exposure (PHD20–35) also accelerated the developmental increase in NR2A mRNA expression

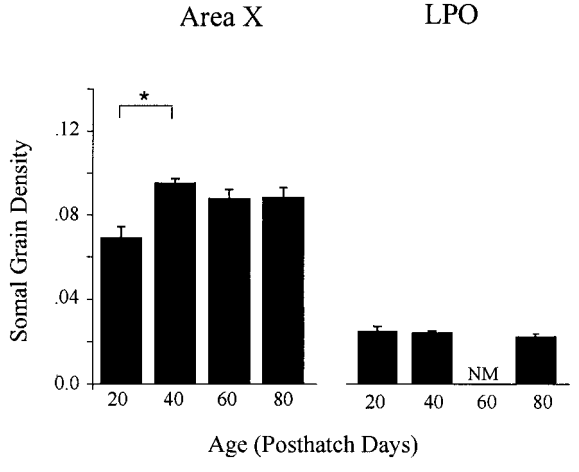


**Figure 4** Developmental regulation of NR2A mRNA in Area X (left) and adjacent LPO of male zebra finches. In Area X, NR2A transcripts increased between PHD20–40 and then remained stable through PHD80. In the LPO medial to Area X, there was no effect of age on NR2A mRNA expression. Data shown are means ± S.E.M. $p < .01$.

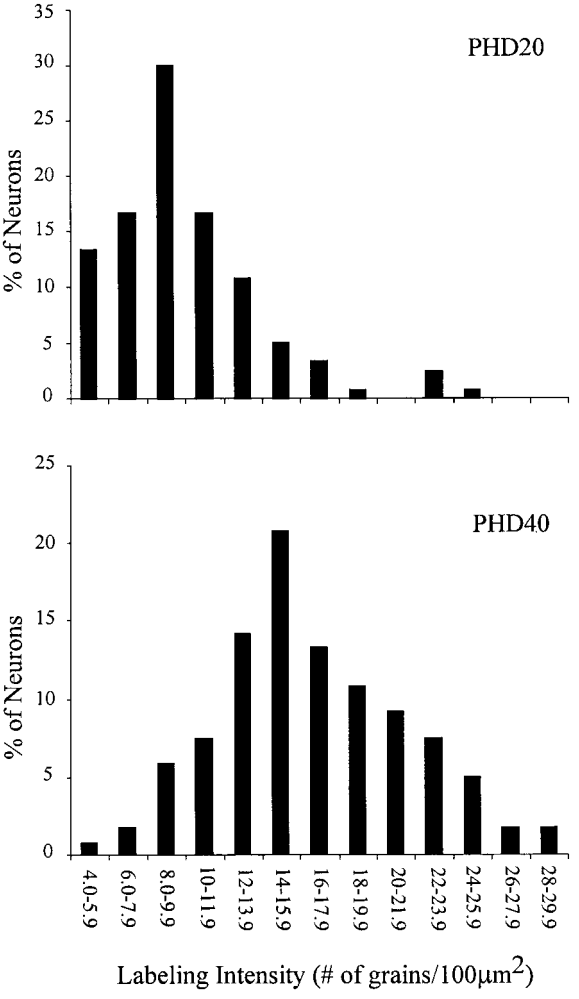


**Figure 5** Frequency distributions of NR2A hybridization levels over individual lMAN neurons at PHD20 ($n = 5$) and 40 ($n = 5$). The number of silver grains over individual nonoverlapping lMAN neurons (24/animal) was analyzed at 100X. All of the neurons within each group were pooled and sorted according to labeling density (bin width = 2 grains/100 $\mu m^2$). Between PHD20 and 40 there was a uniform shift in the distribution to higher labeling intensity per 100 $\mu m^2$ of neuronal area.

within lMAN. At PHD35, the level of NR2A transcripts in this region was 17% greater in T-treated birds than in age-matched controls ($t = 3.1$; $p = .011$). Within Area X, NR2A hybridization levels at this age were about 11% higher in T-treated birds than in controls, a difference that approached significance ($t = 2.53$; $p = .055$). Within the neighboring medial LPO and neostriatum lateral to lMAN, early T treatment had no effect on NR2A mRNA expression at PHD35 (data not shown).

Early isolation from conspecific song extends the

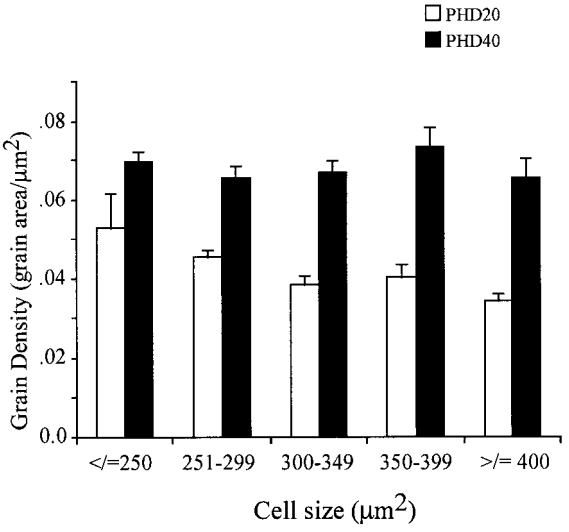


**Figure 6** NR2A transcript levels in lMAN neurons at PHD20 and 40 within each cell size class. The NR2A hybridization densities recorded from individual lMAN neurons for each age group were pooled and sorted according to cell size (bin width = 50 $\mu m^2$). For all cell sizes, NR2A mRNA expression was higher in cells from PHD40 animals, with larger neurons exhibiting a particularly prominent increase in hybridization density between PHD20 and 40.

sensitive period for song learning (Immelmann, 1969; Kroodsma and Pickert, 1980; Eales, 1985, 1987; Aamodt et al., 1995) and delays the developmental down-regulation of NR2B mRNA expression in lMAN (Singh et al., 2000). However, this manipulation did not affect levels of NR2A transcripts in anterior forebrain song regions (Fig. 8). Levels of NR2A mRNA expression measured in lMAN and Area X at PHD40, 45 (data not shown), and 60 did not differ between isolation-reared and age-matched control birds.

## DISCUSSION

The present report extends our characterization of NMDAR gene expression during the sensitive period for vocal learning in zebra finches. We find that in lMAN and Area X, two forebrain regions implicated specifically in song learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991), NR2A transcripts increase markedly between PHD20 and 40, just as song learning begins. Importantly, these increases in NR2A mRNA levels coincide with reciprocal changes in transcript levels for NR2B (Singh et al., 2000). We also find that the developmental increase in NR2A expression within lMAN and Area X can be accelerated by early exposure to T, but is not affected by exposure to conspecific song. Together with our previous studies describing developmental down-regulation of NR2B mRNA within these same regions, our results show that in a pathway critical for vocal plasticity, the ratio of NR2A:NR2B mRNA rises markedly during the sensitive period for song learning. Furthermore, androgen and/or auditory regulation of these subunits may alter thresholds for experience-dependent synaptic change (Singh et al., 2000).

The developmental changes in NR2A and NR2B message expression within lMAN provide a plausible explanation for important changes in NMDAR physiology that occur within this region during song learning. The *e*-fold decay time of NMDAR-mediated EPSCs within the lMAN in response to stimulation of DLM afferents decreases markedly between PHD20 and 45, and more modestly between PHD45 and

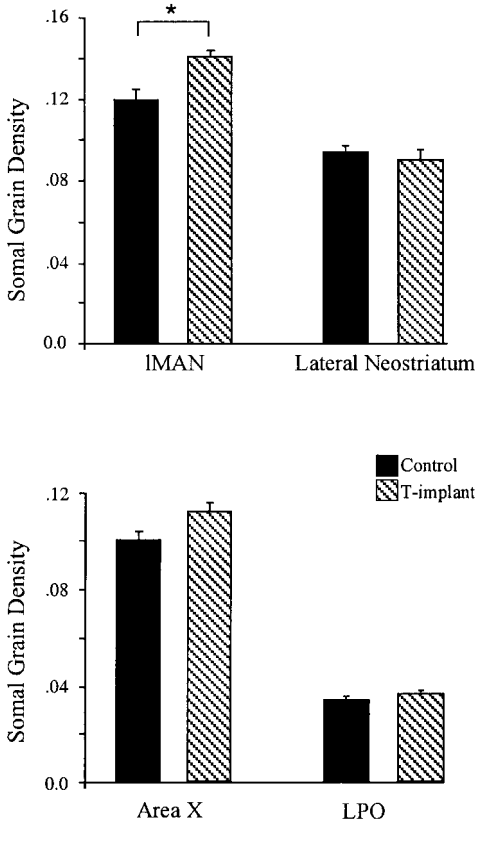


**Figure 7** Effects of early testosterone treatment on NR2A mRNA expression within lMAN and Area X at PHD35. Within lMAN (top), T implants given on PHD20 significantly increased the expression of NR2A compared to controls ($p < .02$). In Area X (bottom), a similar effect of T on NR2A expression only approached significance ($p < .055$).

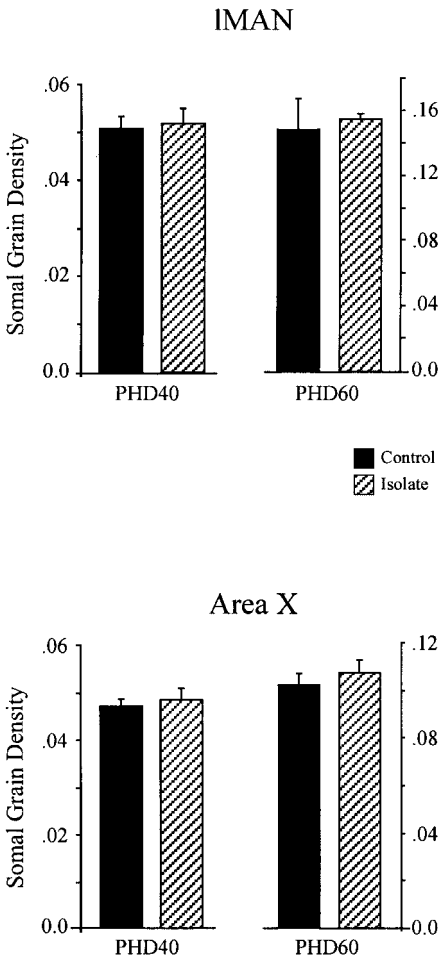


**Figure 8** Early isolation from song did not affect NR2A mRNA expression in anterior forebrain regions at PHD40 or 60. At PHD40, NR2A transcript levels in isolates did not differ from age-matched controls in either lMAN (top left) or Area X (bottom left). Likewise, in a separate hybridization experiment using tissue from PHD60 animals, neither lMAN (top right) nor Area X (bottom right) exhibited effects of rearing with respect to NR2A mRNA.

adulthood (White et al., 1999). Likewise, EPSC decay time among the NMDAR-rich recurrent collateral synapses of lMAN neurons decreases sometime between PHD20 and 60 (Boettiger and Doupe, 2001). Developmental alterations in NMDAR subunit composition are known to alter receptor physiology in mammalian neocortex (Flint et al., 1997), and oocyte expression studies have linked decreases in NMDAR current duration specifically to increases in the NR2A:NR2B ratio (Monyer et al., 1994). Our data suggest that transcriptional regulation of both NR2A and NR2B contributes to the shortening of NMDAR-mediated currents observed in lMAN neurons during song learning.

Changes in NMDAR subunit composition and physiology are believed to be one factor contributing to the developmental regulation of neural and behavioral plasticity. While it is not yet certain how such changes impact the propensity for song learning, several recent findings restrict the possibilities. For instance, it seems almost certain that the developmental changes in NMDAR subunit expression and physiology in lMAN are not sufficient for closing the sensitive period. The rise in NR2A mRNA occurs entirely between PHD20–40, and over 50% of the total down-regulation of NR2B transcripts takes place during that same period. Yet, song acquisition normally extends until PHD60 (Slater et al., 1991). Also, while early isolation from conspecific song can extend the sensitive period for acquisition, this manipulation does not affect the timing of the developmental up-regulation of NR2A mRNA, and acquisition can persist well after maturational changes in NMDAR current duration have occurred both within lMAN and within the vocal motor pathway (Stark and Perkel, 1999; Livingston et al., 2000; White and Mooney, 2000).

An alternative hypothesis is that changes in NMDAR structure and function facilitate an experience-dependent sculpting of neural circuitry. In ferret visual cortex, NR2A levels increase and NMDAR-mediated currents shorten at the opening of the critical period for ocular dominance plasticity (Roberts and Ramoa, 1999), and dark rearing delays both the maturation of NR2A levels (Quinlan et al., 1999b) and the opening of the sensitive period (Mower, 1991). Likewise, in lMAN the NR2A:NR2B ratio rises and, at least among DLM-derived synapses, NMDAR current durations shorten most dramatically at the onset of the critical period for song learning. Thus, we hypothesize that an initially low NR2A:NR2B ratio and long NMDAR current duration in lMAN may increase the probability of activity-dependent synaptic strengthening and favor the formation and maintenance of dense synaptic connections (see also Goldin et al., 2001). Then, as the NR2A:2B ratio increases, the resulting shorter synaptic currents would raise the threshold for Hebbian synaptic strengthening and help trigger the sensitive period for learning by optimizing the ability of song exposure to competitively eliminate synaptic connections within the anterior forebrain loop. That is, synaptic pruning would be facilitated as shorter NMDAR-mediated currents decrease the probability that inputs are detected as coincident, resulting in the strengthening of only those synapses with the most highly correlated activity. In fact, recent work from Boettiger and Doupe (2001) indicates such a shift in lMAN synaptic plasticity. At PHD 20, pairing recur-

rent collateral EPSPs with postsynaptic action potentials produces long-term potentiation (LTP) of intrinsic lMAN synapses, while at PHD60 the same protocol elicits long-term depression (LTD). It is not known precisely when this change in the threshold for LTP versus LTD occurs between PHD20 and 60, but if it is driven by the shift to a higher NR2A:NR2B ratio within lMAN neurons, it would occur by PHD40 or earlier (i.e., towards the beginning of the sensitive period). If so, it could drive the experience-driven elimination of dendritic spines on lMAN neurons that occurs after PHD35 (Nixdorf-Bergweiler et al., 1995).

It was somewhat surprising that the developmental increase in NR2A mRNA within lMAN was not influenced by rearing birds in isolation from conspecific song. In mammalian visual cortex, early visual experience regulates the expression of the NR2A (but not the NR2B) subunit (Quinlan et al., 1999b; Chen et al., 2000), and this is presumed to underlie the effects of experience on NMDAR current durations within this region. Within the lMAN of the avian song system, NMDAR expression is also regulated by early experience, because isolation from conspecific song delays the developmental decline in NR2B mRNA (Singh et al., 2000) and initially retards the normal developmental hastening of NMDAR currents (White and Mooney, 2000). While we cannot rule out a very early effect (prior to PHD40) of isolation on NR2A transcription, even if present, such an effect could not account directly for the differences in current kinetics of isolated and control animals at PHD45. Thus, our data suggest that the impact of early song exposure on developmental changes in NMDAR current duration in lMAN cannot be accounted for by transcriptional regulation of the NR2A subunit. However, it remains possible that isolation impacts NMDAR function by affecting NR2A protein levels post-transcriptionally.

In contrast, the developmental pattern of NR2A transcription can be impacted by the timing and/or degree of early androgen exposure. T treatment at PHD20 accelerates the increase in NR2A levels within lMAN, and to some extent, within Area X. Similar early androgen exposure also hastens both the down-regulation of NR2B message levels (Singh et al., 2000) and the maturation of the NMDAR mediated currents in lMAN (White et al., 1999), and disrupts normal song development (Korsia and Bottjer, 1991). Given that isolation rearing is reported to depress androgen levels measured at PHD45 (Livingston et al., 2000), it seems puzzling that we observed no effect of isolation on NR2A mRNA levels. Perhaps even the depressed androgen levels of isolates are sufficient to trigger a normal pattern of NR2A expression in lMAN and Area X. Alternatively, isolation rearing may not alter androgen levels until after NR2A mRNA expression has reached adult levels (i.e., PHD40). In any case, androgen's ability to alter NMDAR subunit expression and current kinetics raises the possibility that individual differences in the early hormonal environment impact the time course of changes in synaptic function that support learning. Furthermore, it points to androgens as a potentially powerful tool to manipulate the timing of these changes in receptor structure and function to elucidate their functional role in song learning.

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