Early Sensory and Hormonal Experience Modulate Age-Related Changes in NR2B mRNA within a Forebrain Region Controlling Avian Vocal Learning

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ABSTRACT: **Male zebra finches are most apt to mimic songs heard between posthatch days (PHD) 35 and 65, and this vocal learning depends, in part, on the activation of *N*-methyl-D-aspartate receptors (NMDAR) within a discrete forebrain circuit that includes the lateral magnocellular nucleus of the anterior neostriatum (lMAN) and area X. Using *in situ* hybridization, we show that transcripts for both the constitutive NMDAR subunit NR1 and the modulatory subunit NR2B decrease abruptly in the lMAN between PHD20 and 40. This downregulation corresponds to the onset of song learning and a transition from slow to faster NMDAR currents in lMAN neurons. In area X, NR1 mRNA increases as NR2B mRNA decreases during song development. To understand how these changes in NMDAR mRNA might regulate song learning, we next investigated how manipulations that influence song development affect NMDAR mRNA expression. Early isolation from conspecific song (which delays closure of the sensitive period for song learning) selectively increases NR2B, but not NR1 mRNA, within lMAN at PHD60. In contrast, exposure to testosterone beginning at PHD20 (which impairs song development and hastens the developmental transition to faster NMDAR current kinetics within lMAN) accelerates the decline in NR2B mRNA in lMAN, again without affecting NR1 transcript levels. Neither manipulation significantly effects NR1 or NR2B mRNA levels in area X. Our data suggest that developmental changes in the expression of specific NMDAR subunits may regulate periods of neural and behavioral plasticity and that flexibility in the timing of these sensitive periods may be achieved through experience and/or hormone-dependent modulation of NMDAR gene expression.** © 2000 John Wiley & Sons, Inc. J Neurobiol 44: 82–94, 2000

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Avian song learning is one of several examples in which the timing of developmental sensitive periods may relate to alterations in the structure and function of *N*-methyl-D-aspartate receptors (NMDAR). During song development young male zebra finches (*Poephila guttata*) first memorize conspecific songs (*sensory acquisition*) and then translate those auditory memories to motor patterns of song production (*sensorimotor learning*). Sensory acquisition occurs between posthatch days (PHD) 30 and 65 (Eales, 1985, 1989; Jones et al., 1996) and is disrupted by infusions of the NMDAR antagonist APV into the lateral magnocellular nucleus of the anterior neostriatum (lMAN; Basham et al., 1996), a region critical for song learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Also, during song development there are changes in NMDAR mRNA expression (Basham et al., 1999), NMDAR receptor density (Aamodt et al., 1992, 1995), and NMDAR-mediated excitatory postsynaptic current (EPSC) kinetics (Livingston and Mooney, 1997) in lMAN that could affect thresholds for permanent synaptic change.

The NMDA subtype of glutamate receptor can contribute to fast synaptic transmission (Daw et al.,

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1993), but also has been implicated in various forms of synaptic plasticity both in development and in adulthood (Bliss and Collingridge, 1993; Fox and Daw, 1993; Collingridge and Bliss, 1995; Bear, 1996). The unique functional properties of NMDARs enable them to function as correlation detectors (Cotman et al., 1988; Seeburg et al., 1995) and their activation elevates postsynaptic Ca^{2+}, thereby initiating signaling pathways thought to modify synaptic strength (MacDermott et al., 1986; Davis et al., 1992; Bliss and Collingridge, 1993; Sakimura et al., 1995). Native NMDARs consist of the NR1 subunit, essential for channel activity, and one or more modulatory subunits (NR2A-D) that confer distinct functional properties (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Buller et al., 1994). In forebrain, NR2A and NR2B are the most prevalent modulatory subunits, and many regions express high levels of NR2B early in development and gradually replace or augment these with NR2A subunits (Monyer et al., 1994; Sheng et al., 1994). The resulting decrease in the NR2B:2A ratio reduces the decay time of NMDAR currents (Monyer et al., 1992; Flint et al., 1997), which could alter the probability or nature of subsequent Ca^{2+}-dependent biochemical cascades.

Quantitative and qualitative changes in NMDARs are likely two factors that constrain sensitive periods. In the avian song system, the number of binding sites for the NMDA receptor antagonist MK-801 declines within lMAN between PHD30 and adulthood in zebra finches (Aamodt et al., 1995), and in mammalian visual cortex both NR1 expression and MK-801 binding decrease as NMDAR-mediated plasticity wanes (Bode-Greuel and Singer, 1989; Gordon et al., 1991; Reynolds and Bear, 1991). Yet, these changes are not sufficient for sensitive period closure, since early acoustic isolation extends song learning but does not affect overall MK-801 binding in lMAN (Aamodt et al., 1995), and dark-rearing extends visual cortical plasticity without affecting NMDAR density in visual cortex (Gordon et al., 1991; Reynolds and Bear, 1991). In contrast, qualitative changes in NMDAR subunit composition and physiology appear more tightly coupled to sensitive periods for neural plasticity. In the mammalian visual system, NR2B:2A ratios decline and NMDAR-EPSCs shorten during the sensitive period, and dark-rearing delays these changes (Carmignoto and Vicini, 1992; Quinlan et al., 1999). Within the lMAN of songbirds, NR2B mRNA and protein expression decrease during song learning (Basham et al., 1999) as does the duration of NMDAR-EPSCs (Livingston and Mooney, 1997). In the present study, we provide the first demonstration that experimental manipulations that alter the timing and/or pace of song learning also affect age-related changes in NR2B (but not NR1) mRNA expression within the lMAN.

Here, we report the results of several related experiments designed to test the relationship between changes in NMDAR gene expression in the lMAN and the sensitive period for avian song learning. We describe regionally specific changes in NR1 mRNA expression within the lMAN and area X (another forebrain song region that is necessary for normal song learning; Sohrabji et al., 1990; Scharff and Nottebohm, 1991), providing evidence that changes in gene expression underlie the decline in NMDAR density within the lMAN. We also clarify the timecourse of changes in NR2B mRNA expression in lMAN in relation to critical stages in song learning and show that rapid downregulation of NR2B mRNA expression accompanies the abrupt change in NMDA-EPSC duration that occurs within the lMAN between PHD25 and 40 (Livingston and Mooney, 1997). Finally, we show that the developmental decline in NR2B mRNA in lMAN is delayed by early isolation from conspecific song and is accelerated by early androgen treatment, which causes premature shortening of NMDA-EPSCs (White et al., 1999a), disrupts song learning (Korsia and Bottjer, 1991), and can accelerate the pace of song development (Whaling et al., 1995). These findings support the working hypothesis that alterations in NMDAR composition within the lMAN can affect the timing and propensity for vocal learning.

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 free-flight aviaries with both parents until they were sacrificed, except for birds surviving to PHD80, which were removed from the breeding aviaries at PHD60 and transferred to same-sex holding aviaries. Birds reared in this fashion preferentially acquire songs between PHD35 and 65 that are used as a template for their own song production (Eales, 1985; Jones et al., 1996). To delay the period of acquisition, several clutches were removed from the breeding aviaries by PHD8 and kept in single cages for 24 h with their parents. The father then was removed, and clutchmates were reared to independence by their mother in single cages visually and acoustically isolated from adult males. At PHD30, juvenile males from these clutches were visually isolated from one another. Such isolation enables male zebra finches to imitate songs first heard after PHD60 (Eales, 1985; Aamodt et al., 1995). Finally, to determine if

testosterone affects developmental changes in NMDAR gene expression, a separate group of PHD20 males was implanted subcutaneously with a 10-mm Silastic tube that was either empty or filled with 7 mm of crystalline testosterone (4-androsten-17β-ol-3-one; Steraloids, Newport, RI). Similar early androgen exposure disrupts normal song development in zebra finches (Korsia and Bottjer, 1991).

Tissue Preparation

At PHD20, 40, 60, or 80, normally reared male zebra finches ($n = 4$ or 5/group) were decapitated and their brains were removed, coated with OCT compound, frozen on dry ice, and stored at −70°C. These ages were chosen to represent several key stages in zebra finch song learning: before acquisition (PHD20), acquisition engaged and vocal practice beginning (PHD40), acquisition ending (PHD60), and vocal practice near completion (PHD80). Brains were taken from isolate males ($n = 5$) at PHD60, an age at which isolates (but not controls) can still learn new song material (Aamodt et al., 1995). Testosterone (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 μm) 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.

NR1 Oligonucleotide

We used a synthetic 45-base oligonucleotide probe (GTGCTCAGCACCGCGCCGATGTTGACGATCTTGGGGTCGCAGCCG) to localize NR1 mRNA in zebra finch brain. This sequence is complementary to bases 522–566 of duck NR1 cDNA sequence (Kurosawa et al., 1994) and is 98% similar to rat NR1 cDNA (bases 325–369; Moriyoshi et al., 1991), 95% similar to mouse NR1 cDNA (bases 154–197; Yamazaki et al., 1992) and 93% similar to human NR1 cDNA (bases 231–275; Karp et al., 1993). In rats and humans, alternative splicing yields eight isoforms of the NR1 subunit, and our probe is based on a sequence present in all known splice variants (Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992; Zukin and Bennett, 1995). This sequence shows less than 60% similarity to other known sequences in the GenBank database.

NR2B Oligonucleotide

We designed a 45-base oligonucleotide probe (GGGCTTGCCAGAGCAGACACCCATGAAACAGTGGCGGAATTGCCA) based on a partial NR2B sequence isolated from zebra finch forebrain total RNA, as described previously (Basham et al., 1999). This sequence is 89% complimentary to bases 2587–2630 of rat NR2B mRNA sequence (Monyer et al., 1992) and less than 63% similar to any non-NR2B–related sequences in the GenBank. The amino acid sequence coded by this region is 100% similar to human, rat, and mouse NR2B sequences.

Probe Labeling

The HPLC purified NR1 and NR2B oligonucleotides were obtained from Genosys Biotechnologies and their 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 μg 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 to settle the probe. Probe 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 3 to 6 × 10^8 cpm/mL. Generally, radiolabeled probes were used within a week.

In Situ Hybridization

To control for variability in probe labeling, hybridization, and autoradiography, each hybridization experiment included sections from each age and treatment group. 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. Several sections through posterior levels of the brain were similarly processed to qualitatively compare regional patterns of NR1 hybridization to previous NMDA receptor binding studies in zebra finch. Sections were washed twice in 2× saline-sodium citrate (ssc) and then dehydrated in a graded series of ethanol and air-dried for 30 min. For both NR1 and NR2B hybridization, each slide was covered with 180–200 μL of hybridization buffer containing 1 × 10^7 cpm/mL purified ^{35}S-labeled probe, 50% deionized formamide, 4× SSC, 1× Denhardt's solution, 100 mg/mL denatured salmon sperm DNA, 250 μg/mL yeast tRNA, 100 μg/mL poly A, 5 μg/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/4× SSC, and incubated at 42°C for 16–18 h. After hybridization, coverslips were removed in 2× SSC at 42°C, and slides were washed twice in 2× SSC at 42°C (5 min each). Then, NR1 slides were washed in 1× SSC at 42°C (15 min), 0.5× SSC at 42°C (20 min), twice in 0.1× SSC at 60°C (20 min each), and finally in 0.1× SSC for 5 min at room temperature. NR2B slides were washed in 1× SSC at 42°C (15 min), twice in 0.5× SSC at 60°C (20 min each), and finally in

0.5× SSC for 5 min at room temperature. All slides were dehydrated for 2 min each in 50, 70, 95, and 100% ethanol and allowed to air dry for 30 min. These conditions optimized the signal/noise ratio and were established by a series of experiments in which we varied hybridization temperature, amount of probe, and stringency of posthybridization washes (salt and temperature).

Both the NR1 and NR2B oligoprobes used in this study hybridized specifically with their respective target mRNAs. No specific hybridization was detected after incubating the sections with ^{35}S-labeled sense oligoprobes. Treatment of sections with RNAse A (50 μg/mL) before hybridization abolished all hybridization signals from the tissue sections as did the addition of 100-fold excess unlabeled probe to the hybridization buffer. Specificity of the NR2B probe was further confirmed by the similarity in hybridization pattern to that obtained using a different NR2B oligoprobe (Basham et al., 1999). For both the NR1 and NR2B probes, 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 × 2) and left overnight in xylene before being washed in 100% ethanol (10 min × 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 for 5 h or overnight. They then were placed in airtight boxes with desiccant, sealed with black electrical tape, and stored at 4°C for 2 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 4 min. Slides were washed in running tap water for at least 30 min and then fixed in 10% formalin solution, washed in water, stained in thionin solution, dehydrated, and 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 analyzed that represented randomly selected 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 40× magnification. Then the area occupied by silver grains was measured within this defined somal area. For this analysis a blue filter (transmission wavelengths ≤ 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. For each subject, the eight measurements for each region (4 each from the left and right hemispheres) were averaged because no systematic hemispheric or rostrocaudal variations were evident. For some groups (see Figs. 2 and 4) 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 neostriatum," an area of neostriatum ventrolateral to lMAN and lateral to the center of 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., 1997), background grain density for each animal was calculated as 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. The 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 post hoc Bonferroni tests on selected groups. Isolates and controls at PHD60, and 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 average cell density or clumping rather than differences in the proportion of cells expressing a particular message or the average expression per cell. To tease apart these possibilities, we also measured hybridization levels in single, nonoverlapping cells. This was done for NR2B mRNA expression in lMAN of normally reared PHD20, 40, and 60 birds, and PHD60 isolates, thus allowing a fuller description of those developmental and isolation-induced changes in hybridization level that were particularly striking (see Results). A similar cellular analysis was not possible in tissue hybridized with the NR1 oligoprobe because dense labeling precluded the identification of isolated neurons and also was not conducted for NR2B transcript expression in area X because of extensive cellular overlap within this region. At 100×, fields within the central portion of lMAN were surveyed for neurons, identified by their darkly staining 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. For each animal, 24 lMAN neurons drawn equally from each hemisphere were averaged, and the effects of age were evaluated by a one-way ANOVA (2-tailed) followed by post hoc Bonferroni tests on selected groups. Isolates and controls at PHD60 were compared by an independent *t* test (2-tailed). Finally, to examine age- and

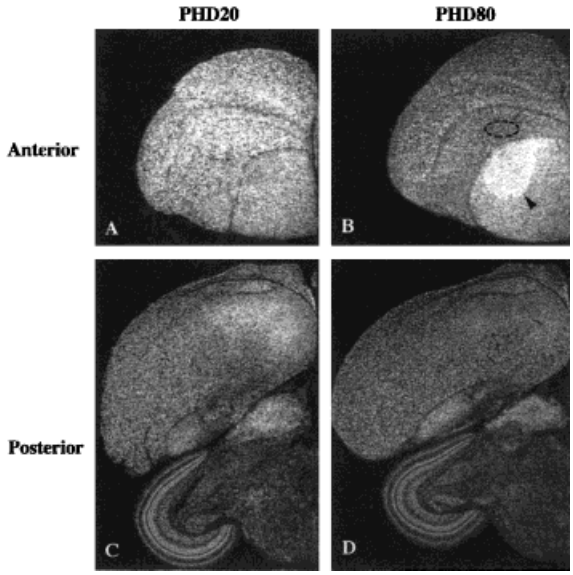

Figure 1 Dark field images of *in situ* hybridization showing age-related changes in NR1 mRNA expression in male zebra finch brain. In most regions of the anterior forebrain, including lMAN (dotted outline in B), NR1 message levels decreased between PHD20 (A) and PHD80 (B). However, in area X (arrow in B), NR1 mRNA increased during adolescence. Within posterior forebrain, NR1 message levels were regionally heterogeneous, but generally declined between PHD20 (C) and 80 (D).

experience-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/treatment group and created frequency histograms by sorting neurons according to labeling intensity (bin width = 10 grains) or cell size (bin width = 50 μm^2).

RESULTS

Developmental Profile of NR1 mRNA Expression

With the notable exception of area X in adult birds (see below), the expression of NR1 mRNA in male zebra finches was qualitatively similar to the pattern of NMDA receptor expression revealed by MK-801 binding autoradiography (Aamodt et al., 1992). At all ages, NR1 mRNA expression was relatively high in the telencephalon, dorsal thalamus, optic tectum, and granular cell layer of the cerebellum. In contrast, hybridization levels were relatively modest within the brainstem. As shown in Figure 1, between PHD20 and 80 a noticeable decline in NR1 mRNA was evident throughout much of the telencephalon. In contrast to this general ontogenetic pattern, area X within the LPO exhibited a marked increase in NR1 mRNA expression between PHD20 and 80.

Quantitative analyses of average somal grain density confirmed that NR1 mRNA expression declined throughout both phases of song development in lMAN but not area X (Fig. 2). NR1 mRNA expression in lMAN decreased more than 50% between PHD20 and 80, and a one-way ANOVA revealed a significant main effect of age [$F(3,16) = 54$; $p < .001$]. Much of this downregulation occurred very early in song development: 56% of the total decline occurred between PHD20 and 40, 12% occurred between PHD40 and 60, and 32% occurred between PHD60 and 80 (Fig. 2, top left). Importantly, in the lateral neostriatum, NR1 mRNA decreased by only 28% between PHD20 and 80 [$F(2,12) = 11.4$; $p < .002$], and the decline was much more gradual than that observed in

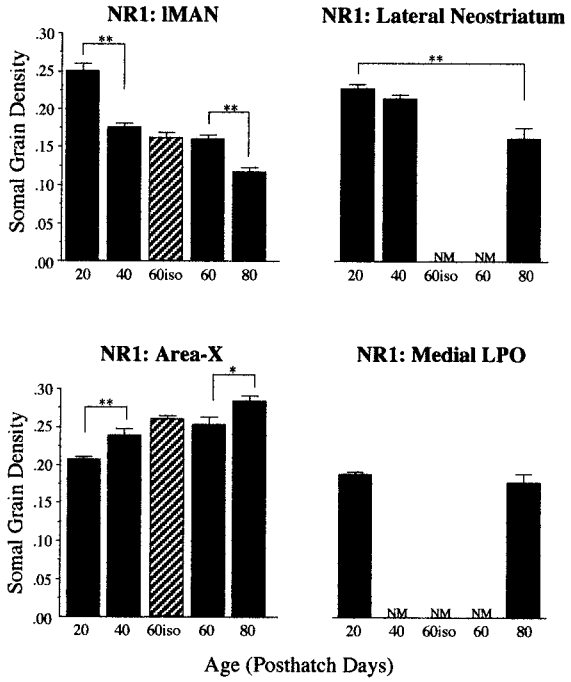

Figure 2 Developmental regulation of NR1 mRNA expression in anterior forebrain of male zebra finches. Data shown (mean ± S.E.M.) are from PHD20, 40, 60, and 80 control birds, and PHD60 birds reared in isolation from conspecific song (60iso). In lMAN (top left), NR1 mRNA expression decreased markedly between PHD20 and 80, with the majority of the decline occurring between PHD20 and 40. A more moderate age-related decline in NR1 mRNA was evident in neostriatum ventrolateral to lMAN (top right). In contrast, NR1 mRNA expression increased within area X (bottom left) but not LPO medial to area X (bottom right) between PHD20 and 80. Early isolation from conspecific song (hatched bars) did not affect NR1 mRNA expression at PHD60 in either lMAN or area X. NM, not measured. *$p < .05$ **$p < .01$.

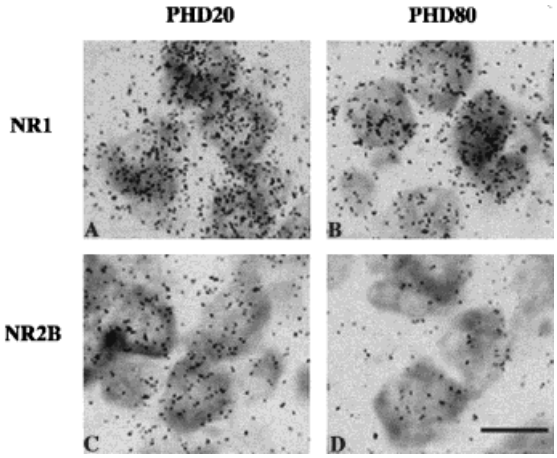

Figure 3 Photomicrographs illustrating expression of NR1 and NR2B mRNA within individual lMAN cells. For both mRNA species, the hybridization signal was greater over cell somata than neuropil. Scale bar = 25 μm.

lMAN (Fig. 2, top right). In fact within this region, the downregulation of NR1 mRNA between PHD20 and 40 (11%) was not significant.

Within the LPO, there were marked regional differences in the developmental regulation of NR1 mRNA expression. Unlike the neostriatal regions, analyses of average somal grain density confirmed a significant *increase* in NR1 hybridization in area X during song development [main effect of age: $F(3,17) = 17.2$, $p < .001$]. The overall increase in NR1 mRNA expression between PHD20 and 80 was 37% with most of the change concentrated between PHD20 and 40, and PHD60 and 80 (Fig. 2, bottom left). However, while NR1 transcript levels increased within area X, they did not change significantly within the surrounding medial LPO (Fig. 2, bottom right).

Developmental Profile of NR2B mRNA Expression

As described previously (Basham et al., 1999), NR2B mRNA is expressed predominantly in the telencephalon and dorsal thalamus of zebra finch brain. In most if not all forebrain regions, NR2B hybridization was visibly less than NR1 hybridization, as is evident in Figure 3, which shows expression of both transcripts over individual cells within lMAN. Widespread reduction in NR2B transcript levels was apparent between PHD20 and 80 within the anterior forebrain, and this downregulation was confirmed for all four regions in which quantitative measurements were obtained.

In lMAN, analyses of average somal grain density revealed a 70% decline in NR2B mRNA expression between PHD20 and 80 [main effect of age: $F(3,14) = 112.6$, $p < .0001$]. Much of this decrease occurred very early in song development: 52% of the total decline occurred between PHD20 and 40, with the remaining decrease occurring equally between PHD40 and 60, and PHD60 and 80 (Fig. 4, top left). NR2B mRNA also declined in the lateral neostriatum adjacent to lMAN, but the magnitude of this reduction was less than that which occurred within lMAN. In the lateral neostriatum, NR2B mRNA decreased by only 35% between PHD20 and 80 [main effect of age: $F(3,14) = 3.8$; $p < .05$], although post hoc analysis revealed no significant differences between neighboring age groups (Fig. 4, top right).

Within regions of the LPO, the expression of

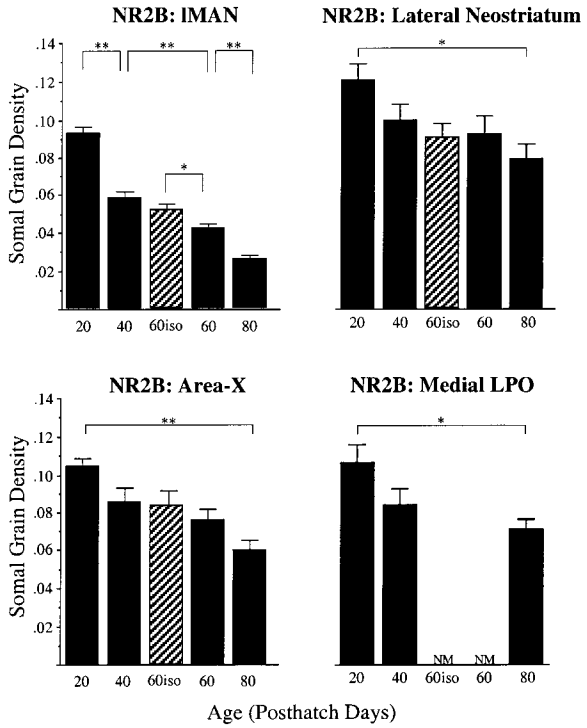

Figure 4 Effects of age and isolation on NR2B mRNA expression in anterior forebrain. Data shown (mean ± S.E.M.) are from PHD20, 40, 60, and 80 control birds, and PHD60 birds reared in isolation from conspecific song (60iso). In lMAN (top left) but not neostriatum ventrolateral to lMAN (top right), NR2B mRNA expression decreased markedly in normally reared birds between PHD20 and 40, and early isolation from conspecific song (hatched bars) increased significantly the expression of NR2B transcripts at PHD60. In area X (bottom left) and LPO medial to area X (bottom right), NR2B mRNA decreased more gradually with age and no effect of isolation was evident at PHD60 in area X. NM, not measured. *$p < .05$, **$p < .01$.

NR2B mRNA also declined with age; however, the magnitude of the developmental downregulation was markedly less than that which occurred within lMAN. In area X, NR2B transcripts decreased by 42% between PHD20 and 80 [main effect of age: $F(3,14) = 9.4$; $p < .01$; Fig. 4, bottom left]. In the LPO medial to area X, NR2B mRNA expression decreased by 33% between PHD20 and 80 [main effect of age: $F(2,10) = 5.3$; $p < 0.05$; Fig. 4, bottom right]. In neither of these regions were the differences between neighboring age groups statistically significant.

Effects of Experience on NR1 and NR2B mRNA Expression

Early isolation from conspecific song extends the sensitive period for song learning (Immelmann, 1969; Kroodsma and Pickert, 1980; Eales, 1985, 1987), and we found that this manipulation also affects the developmental decline in NMDAR mRNA expression. Importantly, the effects observed at PHD60 were specific to both region and mRNA species. That is, analyses of average somal grain density failed to reveal any effects of isolation on NR1 mRNA expression at PHD60 in either lMAN or area X (Fig. 2, left panels). However, in these same birds, levels of NR2B mRNA expression in lMAN (Fig. 4, top left) were 25% greater in isolate than in control birds ($t = 2.64$; df $= 7.4$; $p < .05$). Within area X, NR2B hybridization levels were about 10% higher in isolates than in controls (Fig. 4, bottom left), but this difference was not statistically significant ($p > .2$). Also, in the lateral neostriatum, early isolation from conspecific song had no effect on NR2B mRNA expression at PHD60 (Fig. 4, top right).

Individual Cell Analysis

Theoretically, changes in our measure of somal grain density could be the result of changes in intercellular organization (e.g., cell density and/or overlap) rather than intracellular expression. Although the former are unlikely to account for the effects of development and sensory experience reported above because lMAN and area X neuron density is constant or increases somewhat during development (Bottjer et al., 1985; Nordeen and Nordeen, 1988; Bottjer and Sengelaub, 1989), we explored further the developmental and experiential regulation of NR2B mRNA expression in lMAN by measuring the hybridization signal over individual neurons (see Materials and Methods). This neuronal analysis confirmed that the expression of NR2B transcripts within lMAN neurons declined significantly with age between PHD20 and 60 [main effect of age: $F(2,10) = 16.2$; $p < .001$], with most of the decline (64%) occurring between PHD20 and 40. Moreover, this neuronal analysis also revealed that the expression of NR2B transcripts at PHD60 was significantly greater in isolate than in control birds ($t = 3.2$; df $= 6.2$; $p < .02$). Distribution of labeling intensity demonstrated an age-related decline in the range of values observed. At PHD20, labeling intensity varied widely but the majority of neurons were moderately to heavily labeled (Fig. 5). A downward shift and narrowing of the distribution was evident by PHD40, and this trend continued to PHD60. As shown in Figure 5, the distribution of labeling intensity in PHD60 isolates was more similar to that of PHD40 than PHD60 controls.

Qualitatively, the expression of NR1 and NR2B transcripts was not obviously restricted to particular cell size classes in either lMAN or area X. Moreover, our individual cell analysis of NR2B mRNA expression within the lMAN showed that the effects of development and experience were evident among all cell size classes. As shown in Figure 6, all size classes exhibited a decrease in NR2B mRNA labeling intensity between PHD20 and 60. The magnitude of this decrease ranged from 56 to 70%, and in all size classes the decline was most prominent between PHD20 and 40. Also, early isolation from a conspecific song model affected all neuronal size classes except the very largest (>400 μm^2): the intensity of NR2B hybridization over individual neurons at PHD60 was greater in isolates than in control (Fig. 6). This effect of isolation at PHD60 was most prominent among neurons with soma measuring 300–400 μm^2, where the hybridization signal in isolates was 60–80% greater than in age-matched controls and was at least as great as in PHD40 controls.

Effects of Early Testosterone Exposure on NR1 and NR2B mRNA Expression

Chronic testosterone (T) exposure beginning before PHD40 severely disrupts normal song development (Korsia and Bottjer, 1991), and we found that this treatment alters the developmental decline in NMDAR mRNA expression. As was the case for early isolation, the effects of early hormone treatment were specific to both mRNA species and region. That is, analyses of average somal grain density failed to reveal any effects of T treatment on NR1 mRNA expression in either lMAN or area X at PHD35 [Fig. 7(A)]. In contrast, in these same birds, levels of NR2B mRNA expression in lMAN were about 25% lower in the T-treated group than in their age-matched controls [$t = 3.1$; df $= 11.6$; $p < .02$; Fig. 7(B)]. Thus,

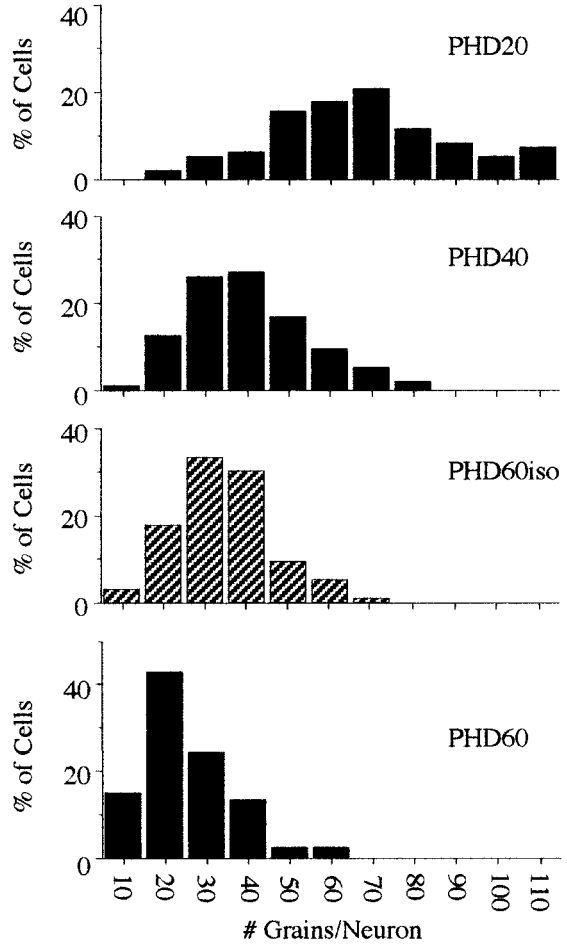

Figure 5 Frequency distributions of the intensity of NR2B hybridization over individual lMAN neurons during development. In tissue hybridized to the NR2B probe, the number of silver grains over individual nonoverlapping lMAN neurons (24/animal) were analyzed at 100×. Four to five animals were analyzed for each group of control (PHD20, 40, and 60) and isolate (PHD60iso) birds, and then all of the neurons within each group were pooled and sorted according to their labeling intensity (bin width = 10 grains). At PHD20, many neurons exhibited relatively high levels of NR2B expression, but variability in labeling intensity was large. By PHD40, the distribution had become narrower and was shifted toward lower levels of expression, a trend that continued to PHD60. Early isolation from conspecific song retarded the developmental change in labeling intensity such that PHD60 isolates (PHD60iso) were more similar to PHD40 than to PHD60 controls.

although NR2B transcript levels in lMAN of PHD35 control birds were intermediate between levels measured in PHD20 and PHD40 controls, expression levels in PHD35 T-treated birds were intermediate between those for PHD40 and PHD60 controls. Within area X, NR2B hybridization levels were about 13% lower in T-treated than in controls [Fig. 7(B)], but this difference was not statistically significant ($t = 2.0$; $p = .07$). Also, within the neighboring medial LPO and neostriatum lateral to lMAN, early T treatment had no effect on NR2B mRNA expression at PHD35.

DISCUSSION

We present several new findings concerning the developmental regulation of NMDAR gene expression within the avian song system: (1) NR1 mRNA decreases within lMAN but increases within area X during song development; (2) NR2B mRNA decreases markedly within lMAN neurons early in song development; (3) early isolation from conspecific song delays the developmental decline in NR2B, but not NR1, mRNA within lMAN; (4) early T treatment accelerates the developmental decline of NR2B, but not NR1, mRNA within lMAN. These observations support work in other systems showing that early experience has more profound effects on expression of NMDAR modulatory subunits than on the NR1 constitutive subunit (Gordon et al., 1991; Reynolds and Bear, 1991; Carmignoto and Vicini, 1992; Catalano et al., 1997; Quinlan et al., 1999).

At least in lMAN, developmental changes in NR1 and NR2B transcripts likely translate into important

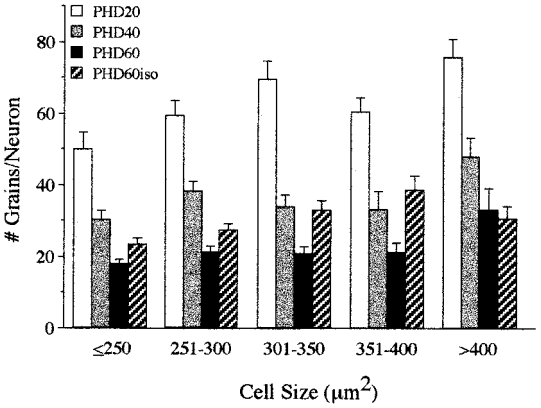

Figure 6 Expression of NR2B mRNA decreases during development among all cell size classes in lMAN. NR2B hybridization levels were measured over individual lMAN neurons (see Materials and Methods) and then all lMAN neurons sampled within each age group were pooled and sorted according to their somal size (bin width = 50 μm^2). All size classes exhibited a decrease in NR2B mRNA labeling intensity between PHD20 and 60 that was most prominent between PHD20 and 40. Early isolation from conspecific song increased NR2B mRNA expression at PHD60 in all neuronal size classes but the very largest. The effect of isolation was particularly evident among medium sized lMAN neurons.

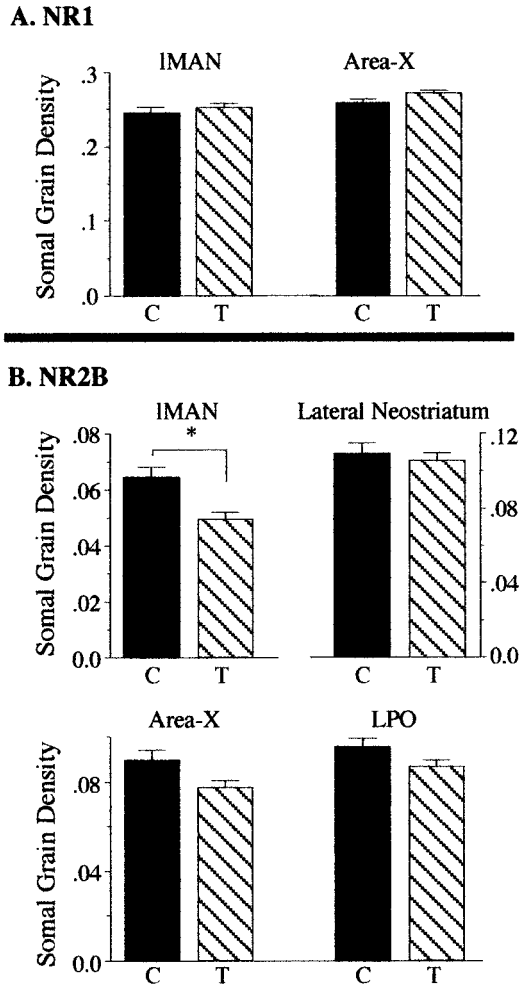

Figure 7 Effects of early testosterone treatment on NR1 and NR2B mRNA expression within lMAN and area X. Filled and hatched bars represent control (C) and testosterone-implanted (T) birds, respectively. Testosterone implants given on PHD20 did not alter NR1 mRNA expression within either lMAN or Area X at PHD35 (A). In contrast, this same hormonal manipulation decreased significantly the expression of NR2B mRNA in lMAN but not in the neostriatum lateral to lMAN, nor in area X or the medial LPO (B). $*p < .02$.

changes in NMDAR protein expression. First, the decline in NR1 mRNA within the lMAN mirrors a decline in NMDAR density with both being particularly pronounced prior to PHD60 (Aamodt et al., 1995). Also, it has been shown previously that the developmental decline in NR2B mRNA within lMAN coincides with a decline in NR2B protein as measured by ifenprodil binding (Basham et al., 1999). Beyond these quantitative changes in NMDARs, it is likely that the regulation of NR2B transcript levels also produces qualitative changes in receptor phenotype. The fact that the steepest decrease in NR2B mRNA within the lMAN corresponds so closely to the developmental decrease in NMDAR current duration in this region (Livingston and Mooney, 1997; White et al., 1999a) suggests that the reduction in NR2B gene transcription contributes to qualitative changes in the receptor that affect its function. Of course, these data do not exclude the possibility that a developmental increase in NR2A expression also contributes to the decrease in NMDAR current duration (Flint et al., 1997). Nonetheless, the ability of early isolation and early T treatment to effect NR2B, but not NR1 transcript levels, indicates that gene transcripts for these two receptor subunits can be regulated independently and predicts changes in receptor composition consistent with isolation-induced prolongation and T-induced shortening of NMDA-EPSC decay times (see White et al., 1999a; Livingston et al., 2000).

In contrast to the patterns observed within the lMAN, developmental regulation of NR1 and NR2B mRNA within area X is not necessarily reflected in NMDAR protein expression. Here, NR1 mRNA expression increases dramatically during development, whereas MK-801 binding does not (Aamodt et al., 1992), and the decrease in NR2B mRNA is not accompanied by decreased ifenprodil binding (Basham et al., 1999). Intriguingly, in adults, the elevated expression of NR1 mRNA in area X relative to the surrounding LPO is reflected in the pattern of MK-801 binding in some, but not all, birds (Aamodt et al., 1992). This suggests that the reservoir of area X NR1 transcripts in adulthood enables a rapid increase in NMDARs under specific conditions that are not yet defined. Attempts to identify the stimuli responsible for posttranscriptional regulation of NMDARs in area X will provide important insights into the function of this region in adult birds.

It has been suggested that age-related decreases in the duration of NMDAR currents, resulting from declining NR2B:2A ratios, reduce neural and behavioral plasticity (Harris and Teyler, 1984; Carmignoto and Vicini, 1992; Hestrin, 1992; Tang et al., 1999). However, in ferret visual cortex NMDAR currents shorten just when ocular dominance plasticity begins, suggesting that faster NMDAR kinetics may contribute to the onset, rather than the closure of sensitive periods (Roberts and Ramoa, 1999). Similarly, we find that NR2B expression (as well as NMDAR-EPSC decay time; Livingston and Mooney, 1997) declines most precipitously in the lMAN near the beginning, rather than the end, of the sensitive period for song learning in zebra finches. Although young males of this species can memorize songs prior to PHD35 (Bohner, 1990), peak sensitivity for acquisition occurs between PHD35 and 65 (Eales, 1989; Slater et al., 1991; Jones

et al., 1996) and thus coincides with shorter NMDA EPSC durations and lower NR2B transcript expression. We speculate that the early expression of longer NMDAR currents in lMAN prior to PHD35 may achieve an optimal balance between maintaining synaptic abundance (facilitated by long intervals over which NMDARs detect inputs as coincident), while still enabling development of coarse topography within the afferent inputs (see also Ramoa and Prusky, 1997; Scheetz and Constantine-Paton, 1994). Indeed, an initially exuberant set of topographically organized neuronal connections are present in the lMAN up until about PHD35, after which elimination and refinement of connections ensues (Johnson and Bottjer, 1992; Nixdorf-Bergweiler et al., 1995; Iyengar et al., 1999). However, relatively slow NMDAR currents may not be optimal for encoding the rich temporal and acoustic structure of song. Rather, a decrease in the NR2B:2A ratio may facilitate learning and enhance competition among inputs because it ensures that only precisely correlated activity patterns will trigger NMDAR-mediated synaptic strengthening. According to this model, the early decline in NR2B mRNA expression reported here would contribute to a decrease in NMDAR decay times, thus initiating a period of rapid synapse elimination that is the hallmark of sensitive periods. Moreover, transition from slow to faster currents may also promote closure of the sensitive period because neural plasticity will be sharply curtailed as the pool of excess synaptic connections is exhausted.

On the basis of our results, we propose further that early isolation from conspecific song both delays and extends the sensitive period for song learning (Eales, 1987; Aamodt et al., 1995) in part, by retarding the developmental decline of NR2B mRNA in lMAN. This would slow the transition to fast NMDAR-EPSCs in lMAN (see Livingston et al., 2000) and postpone the pruning of dendritic spines on lMAN neurons (see Wallhäusser-Franke et al., 1995). As noted above, early dark rearing similarly prolongs expression of slow NMDA-EPSCs (Carmignoto and Vicini, 1992) within primary visual cortex and delays closure of the sensitive period for monocular deprivation. But in this case the developmental decline in NR2B mRNA and protein expression are unaffected by early experience, which acts instead to delay a developmental increase in NR2A expression (Quinlan et al., 1999). In subsequent experiments, it will be important to determine if age-related and experience-dependent changes in NR2A gene transcription also contribute to changes in NMDAR phenotype within the avian song system.

Although our results indicate that the developmental decrease in NR2B mRNA levels is facilitated by the presence of an adult male, we have not established which feature(s) of this stimulus regulates transcript levels or by what cellular mechanism(s) regulation is accomplished. In the current study, isolates were selectively deprived of conspecific song, but otherwise experienced a rich auditory environment, including juvenile and adult female vocalizations. Thus, one possibility is that conspecific song in particular elicits patterns of neural activity in the anterior forebrain pathway that promote changes in NMDAR expression. In adult anesthetized birds, lMAN neurons exhibit a preference for complex auditory stimuli (Doupe and Konishi, 1991) and respond more strongly to a combination of syllables than to individual or smaller subsets of syllables (Doupe, 1997). In juveniles (PHD30–45), lMAN neurons also are responsive to song; however, they lack certain features of selectivity present in adult birds (Doupe, 1997), and it is not known whether presentation of conspecific song to awake juveniles would promote patterns of activity within the lMAN that are significantly different from those evoked by conspecific calls.

It is also possible that hormones normally regulate NMDAR expression in lMAN and that early isolation compromises normally occurring endocrine changes. In particular, we show here that androgens can alter the expression of NR2B mRNA in the lMAN. This latter observation is particularly interesting given that many lMAN neurons contain androgen receptors (Arnold et al., 1976; Arnold and Saltiel, 1979), and chronic testosterone treatment disrupts normal song development (Bottjer and Hewer, 1992) and hastens the transition to fast NMDAR-EPSC kinetics within lMAN (White et al., 1999a). Importantly, at PHD45, androgen levels in isolation-reared males are much lower than in normal birds and early androgen exposure can overcome the effects of isolation on NMDAR current duration within lMAN (Livingston et al., 2000). Thus, the effects of early isolation on NMDAR gene expression may be mediated by alterations in the endocrine system. This does not imply, however, that normal age-related changes in NMDAR expression and function are achieved solely through androgen action: in fact, the developmental change in lMAN NMDAR physiology is not prevented by early exposure to flutamide, an androgen receptor antagonist (White et al., 1999a).

Our earlier demonstration that NMDAR activation within lMAN is critical for sensory acquisition, together with research describing synaptic rearrangements within lMAN during song learning, support the widely held view that NMDARs mediate experience-dependent developmental plasticity by selectively

strengthening and eliminating synapses. In other words, acquired songs may be encoded, in part, by NMDAR-mediated changes in the distribution of synapses among lMAN neurons and/or their afferents. On the basis of the present report, we suggest further that developmental changes in NMDAR gene expression alter receptor composition and physiology in ways that optimize their ability to mediate this sculpting of connections. Reductions both in the terminal field of DLM afferents to lMAN (Johnson and Bottjer, 1992) and in dendritic spine density among lMAN neurons (Nixdorf-Bergweiler et al., 1995) occur after PHD35, following the most pronounced decreases in both lMAN NR2B mRNA levels and NMDAR-EPSC duration. Moreover, early isolation from conspecific song delays these changes in NMDAR expression, while also preventing the normal reduction in lMAN spine density (Wallhäusser-Franke et al., 1995). The ability to manipulate developmental changes in lMAN gene expression either with hormones or gene transfer technology will make it possible to determine directly if regional changes in NMDAR function are necessary and/or sufficient to trigger this experience-dependent synaptic reorganization, and thereby define the time course of vocal learning.

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