FAST TRACK
Developmental Regulation of NMDA Receptor 2B Subunit mRNA and Ifenprodil Binding in the Zebra Finch Anterior Forebrain

M. E. Basham,[1] F. Sohrabji,[2] T. D. Singh,[1] E. J. Nordeen,[1] K. W. Nordeen[1]

[1] Neuroscience Program and Department of Brain and Cognitive Sciences, 103 Meliora Hall, University of Rochester, River Campus, Rochester, New York 14627

[2] Department of Medical Anatomy and Neurobiology, Texas A&M University, College Station, Texas 77843

Received 20 October 1998; accepted 11 January 1999

ABSTRACT: **In passerine songbirds, song learning often is restricted to an early sensitive period and requires the participation of several discrete regions within the anterior forebrain. Activation of *N*-methyl-D-aspartate (NMDA) receptors is implicated in song learning and in one forebrain song region, the lateral magnocellular nucleus of the anterior neostriatum (lMAN), NMDA receptors decrease in density, their affinity for the antagonist MK-801 increases, and their currents decay more quickly as young male zebra finches lose the ability to imitate new song elements. These developmental changes in NMDA receptor pharmacology and physiology suggest that the subunit composition of NMDA receptors changes developmentally. Here, we have used *in situ* hybridization and [^{3}H]ifenprodil receptor autoradiography to study the developmental regulation of the NMDA receptor 2B subunit (NR2B) within the anterior forebrain of male zebra finches. NR2B mRNA expression within the lMAN was twice as great in 30-day-old males (early in the sensitive period for song learning) as in adult males, and this developmental decrease in NR2B mRNA expression was mirrored by a decrease in high-affinity (NR2B-associated) [^{3}H]ifenprodil binding within this song region. In another anterior forebrain song region, Area X, NR2B mRNA also declined significantly after 30 days posthatch, but this decline was not accompanied by a significant decrease in [^{3}H]ifenprodil binding. The results are consistent with the hypothesis that developmental changes in NMDA receptor function mediated by regulation of subunit composition contribute to the sensitive period for vocal learning in birds.** © 1999 John Wiley & Sons, Inc. J Neurobiol 39: 155–167, 1999

Keywords: **NMDA receptors; NR2B; ifenprodil; zebra finch; song learning; sensitive periods**

N-Methyl-D-aspartate (NMDA) receptors mediate experience-dependent synaptic reorganization during the development of various neural systems (Bear et al., 1990; Bear, 1996; Cline et al., 1987; Fox et al., 1996; Hahm et al., 1991; Scherer and Udin, 1989; Schlaggar et al., 1993; Schnupp et al., 1995) and participate in several forms of learning in both young and adult animals (Aamodt et al., 1996; Brennan, 1994; Burchuladze and Rose, 1992; Davis et al., 1992; Feldman and Knudsen, 1998; Kim and McGaugh, 1992; Liang et al., 1994; McCabe et al., 1992; Steele and Stewart, 1993). These receptors appear to function as detectors of correlated activity, and their activation leads to elevations in intracellular Ca^{2+} that can activate a variety of biochemical cascades impor-

Correspondence to: K. W. Nordeen

Contract grant sponsor: NIMH; contract grant number: MH45096

 CCC 0022-3034/99/020155-13

tant for learning and memory (Bliss and Collingridge, 1993; Fields et al., 1991; Morris, 1989). Importantly, developmental changes in the physiology of NMDA receptors occur in the hippocampus, cerebral cortex, and midbrain (Ben-Ari et al., 1988; Burgard and Hablitz, 1993; Hestrin, 1992; Kirson and Yaari, 1996) and these changes may alter the propensity for synaptic plasticity. For example, closure of the sensitive period for activity-dependent fine-tuning of connections within the visual system coincides with a decrease in the duration of NMDA receptor-mediated postsynaptic currents (Carmignoto and Vicini, 1992; Hestrin, 1992). Because NMDA receptor activation leads to elevations in intracellular Ca^{2+} (Mayer and Westbrook, 1987), faster NMDA receptor currents likely attenuate the probability of initiating Ca^{2+}-dependent processes presumed to be important for long-term changes in synaptic strength.

Recently, NMDA receptors have been implicated in avian song learning, another case of behavioral plasticity that is age-regulated in many species. Song learning includes a memory acquisition stage (when birds memorize conspecific song material) that is followed by a period of vocal rehearsal. Many songbird species can learn to sing only during restricted developmental or seasonal "sensitive" periods (Marler, 1997). If the NMDA receptor antagonist MK-801 is given systemically during opportunities for memory acquisition, it disrupts normal song learning (Aamodt et al., 1996). Similar although lesser disruptions occur following intracranial infusions of the NMDA receptor antagonist AP5 into the lateral magnocellular nucleus of the anterior neostriatum (lMAN) (Basham et al., 1996). This brain region has been implicated specifically in song learning (Bottjer et al., 1984; Nordeen and Nordeen, 1993; Scharff and Nottebohm, 1991), and closure of the sensitive period for song learning in zebra finches (*Poephila guttata*) is associated with a change in the pharmacology (Aamodt et al., 1995) and physiology (Livingston and Mooney, 1997) of NMDA receptors within the lMAN.

Native NMDA receptors consist of at least one constitutive subunit (NR1) and one or more modulatory subunits (NR2A–2D) (Ishii et al., 1993; Meguro et al., 1992; Monyer et al., 1992; Moriyoshi et al., 1991; Sugihara et al., 1992), and variation in the modulatory subunit composition produces marked variation in NMDA receptor pharmacology and physiology (Buller et al., 1994; Ishii et al., 1993; Kendrick et al., 1996; Kutsuwada et al., 1992; Laurie and Seeburg, 1994; Lynch et al., 1995; Monyer et al., 1992; Priestley et al., 1995; Williams, 1995). In vertebrate forebrain regions, a developmental decrease in the propensity for NMDA receptor–mediated plasticity likely involves loss of NR2B subunits accompanied by an increase in NR2A subunit expression. NR2A and NR2B mRNA and protein are expressed widely throughout the forebrain, whereas NR2C mRNA is found predominately in the cerebellum and NR2D mRNA is generally localized to the diencephalon and brain stem (Ishii et al., 1993; Kutsuwada et al., 1992; Laurie et al., 1997; Monyer et al., 1992; Watanabe et al., 1992, 1993; Zhong et al., 1995). The proportion of neocortical cells expressing NR2A mRNA increases developmentally and individual cells that express NR2A mRNA have shorter NMDA receptor–mediated postsynaptic currents than do cells lacking NR2A transcripts (Flint et al., 1997). Within visual pathways, a developmental decrease in synaptic plasticity coincides with a decrease in NR2B mRNA (Hofer et al., 1994), a decrease in sensitivity to NR2B subunit-specific antagonists (Ramoa and Prusky, 1997), and shorter NMDA receptor–mediated currents (Carmignoto and Vicini, 1992; Crair and Malenka, 1995; Hestrin, 1992; Ramoa and McCormick, 1994). Similarly, in the lMAN of songbirds, the decay time of NMDA receptor–mediated currents becomes shorter as development proceeds (Livingston and Mooney, 1997). Together, these data suggest that a decrease in NR2B subunit expression within the zebra finch anterior forebrain could alter the impact of NMDA receptor activation and decrease the capacity for song learning. To begin investigating mechanisms that regulate NMDA receptor function within the lMAN, we performed a series of experiments designed to identify a developmental change in NR2B subunit expression within this nucleus.

MATERIALS AND METHODS

NR2B *In Situ* Hybridization

Zebra finches were raised in our breeding aviaries and maintained on a 14:10 h light/dark cycle. Thirty-day-old ($n = 4$) and adult (>120 days old; $n = 4$) male zebra finches were rapidly decapitated and the brains were removed and frozen with dry ice. These ages were chosen because 30-day-old males can memorize and reproduce new song material, but adults cannot (Eales, 1985; Immelmann, 1969). Also, NMDA receptor pharmacology (Aamodt et al., 1995) and physiology (Livingston and Mooney, 1997) changes between 30 days and adulthood. Coronal sections (15 μm) through the anterior forebrain were cut on a cryostat and mounted on Vectabond-coated slides. These sections included the lMAN, as well as Area X, another forebrain region necessary for normal song learning (Scharff and Nottebohm, 1991; Sohrabji et al., 1990). Several sections through posterior levels of the brain were also collected to

qualitatively compare regional patterns of hybridization both to the mammalian pattern and to previous NMDA receptor binding studies. The sections were desiccated for 1–3 h at 4°C and stored desiccated at −20°C for up to 2 weeks.

To localize the NR2B mRNA, we used a 45mer oligonucleotide probe (NR2B-oligo1: CTGCT GATGG AGAAG ACCAT GCCAG GCTTG CCAGA ACAGA CACCC) complementary to bases 273–317 of the human NR2B mRNA sequence (Mandich et al., 1994), bases 2610–2654 of rat NR2B mRNA sequence (Monyer et al., 1992), and bases 2553–2597 of mouse NR2B mRNA sequence (Kutsuwada et al., 1992). The most similar non-NR2B sequence in the Genebank database is a NR2A sequence, which is 77% similar to the targeted sequence. The oligonucleotide probe was purified by polyacrylamide gel electrophoresis (Genosys Biotechnologies) and radiolabeled using terminal deoxynucleotidyl transferase (tdt). Five picomoles of probe were incubated at 37°C for 60 min with 50 pmol of [^{35}S]dATP (>1000 Ci/mmol) and 50 U of tdt in tdt buffer with 5 m*M* cobalt chloride. Following incubation, 25 μg of yeast tRNA was added as a carrier and the probe was precipitated in 4 *M* ammonium acetate and ethanol. The probe then was dissolved in 10 m*M* Tris-HCL (pH 8.0), 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 50 m*M* dithiothretial (DTT), and stored overnight at −20°C.

On the day of hybridization slides were warmed to room temperature, fixed in 4% paraformaldehyde for 5 min on ice, washed in phosphate-buffered saline (PBS), acetylated with 0.25% acetic anhydride in 0.1 *M* triethanolamine saline, and dehydrated in graded ethanols. After air-drying briefly, each slide was covered with 150–250 μL of hybridization buffer containing 1×10^7 cpm/mL purified labeled probe, 50% deionized formamide, 4× saline-sodium citrate (SSC), 1× Denhardt's solution, 100 μg/mL denatured salmon sperm DNA, 250 μg/mL yeast tRNA, 100 μg/mL poly A, 5 μg/mL poly dA, 50 m*M* DTT, and 10% dextran sulfate. The sections were covered with plastic coverslips and incubated in a 37°C chamber humidified with 50% deionized formamide/4× SSC for 17–20 h. Sections then were washed for 5 min in 1× SSC at room temperature, 30 min in 1× SSC at 60°C, 1 min each at room temperature in 1× SSC, 0.5× SSC, 70% ethanol, 95% ethanol, and 100% ethanol, and allowed to air-dry. (Condition-setting experiments in which hybridization temperature was raised to 42°C and sections were washed for 30 min in 0.5× SSC at 60°C revealed that these higher-stringency conditions produced no noticeable change in the regional distribution of the hybridization signal, but did result in significant loss of signal overall.) Slides containing hybridized sections and standards were apposed to Hyperfilm-βmax film for 3–5 days and then counterstained with thionin. In each experiment, tissue from an equal number of 30-day-old and adult males was processed together and apposed to the same piece of Hyperfilm.

Films were developed (D-19), fixed, and then analyzed using an image analysis system (Image; NIH). Measurements of optical density were calibrated by reference to known values on ^{14}C standards (Amersham Microscales) using tissue equivalent values for intact gray matter (nCi/g). Autoradiograms were aligned with their corresponding Nissl-stained sections to determine the location of the lMAN and Area X. For each of these areas, at least five optical density measurements per subject were taken in the central portion of the nucleus to determine an average optical density for each area of each subject. The size of the region measured was standardized across animals, but varied across region so as to maximize the proportion of the nucleus measured. Care was taken to avoid measuring binding in the pericellular region that immediately surrounds the magnocellular core of lMAN. On the same sections, optical densities were measured in the lobus parolfactorius (LPO) medial to Area X and in the neostriatum midway between the lMAN and midline. Separate two-way analyses of variance (ANOVAs) (Age × Location) were used to compare NR2B transcript expression within the neostriatum and the LPO.

The specificity of the *in situ* hybridization was confirmed by (a) preincubating sections in 50 μg/mL RNAse-A to digest RNA in the sections prior to hybridization, (b) adding unlabeled probe (0.35 μM) to the hybridization buffer to competitively inhibit specific hybridization, (c) substituting ^{35}S-labeled sense for antisense probe in the hybridization buffer, and (d) comparison to hybridization of a second oligonucleotide probe (NR2B-oligo2) targeted to a separate region of the NR2B mRNA (see Results).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

To confirm that the NR2B-oligo1 probe would recognize zebra finch NR2B mRNA, the following strategy was employed. Rodent-specific PCR primers were designed to reverse-transcribe and amplify a 445-bp sequence of the NR2B mRNA that completely contained the 45-base NR2B oligo-1 sequence as well as (approximately) 200 bases immediately flanking its 5′ and 3′ portions. Using a commercially available kit (GeneAmp; Perkin Elmer) and manufacturer's instructions, total RNA (1 μg) from zebra finch forebrain and adult rat hippocampus (positive control) was reverse-transcribed with a 24-base primer complementary to nucleotides 2856–2879 of the rat NR2B receptor (Genebank Ref. M91562). An aliquot of reverse-transcribed cDNA was then amplified by PCR (95°C for 30 s, 60°C for 30 s, 72°C for 1 min; 30 cycles) with the addition of a 5′ primer homologous to nucleotides 2435–2458 of the rodent NR2B receptor and Taq DNA polymerase. The amplified product was size-fractionated on a 2% agarose gel containing ethidium bromide and photographed under ultraviolet (UV) illumination. The PCR product obtained from zebra finch RNA was then excised from the gel, purified (GeneClean; Bio101), and sequenced (Gene Technologies Laboratory, Texas A&M University). Finally, sequence analysis was confirmed by a partial restriction map. Rodent and zebra finch PCR products were incubated with Msp1 (2 U) at 37°C for 2 h and the digested product was size-fraction-

ated on a 2% agarose gel with ethidium bromide and DNA size markers and photographed under UV illumination.

Ifenprodil Binding

We used the NMDA receptor antagonist ifenprodil to localize expression of NR2B subunit-containing receptors within the zebra finch anterior forebrain. Ifenprodil binds with high affinity to NMDA receptors containing the NR2B subunit (Gallagher et al., 1996; Kutsuwada et al., 1992; Williams, 1993; Williams et al., 1993). This ligand also interacts with several other binding sites; thus, we used a pharmacological competition strategy (as described by Nicolas and Carter, 1994) to exclude all but the NR2B subunit-specific ifenprodil binding in our tissue sections. Trifluoperazine was used to block a low-affinity ifenprodil binding site that is not specific for the NR2B subunit (Nicolas and Carter, 1994; Williams, 1993). Also, because both sigma receptors and piperazine acceptor sites bind ifenprodil (Contreras et al., 1990; Hashimoto and London, 1993; Schoemaker et al., 1990), (+)-3-PPP and GBR-12909 were used to mask these binding sites (Nicolas and Carter, 1994).

In each of two replications, 30-day-old (n = 3/replication) and adult (>120 days old; n = 3/replication) male zebra finches were decapitated, and the brains were removed and frozen with dry ice. Coronal sections (25 μm) were cut on a cryostat and serial sections through the anterior forebrain were mounted on alternate Vectabond-coated slides. The slides then were desiccated for 1–3 h at 4°C and stored overnight desiccated at −20°C. Within 24 h after cutting, tissue sections were warmed to room temperature and every other section was incubated for 3 h on ice at 4°C in Tris-HCl buffer (pH 7.4) with 40 n*M* [^{3}H]ifenprodil, 3 μM (+)-3-PPP and 30 μM GBR-12909, and 1 m*M* trifluoperazine. Nonspecific binding was defined in alternate sections by incubating the slides as above with the addition of 10 μM excess unlabeled ifenprodil. Following incubation, sections were washed 3 × 5 min in ice-cold Tris-HCl buffer (pH 7.4) and dried with warm air. Slides and ^{3}H standards were apposed to Hyperfilm-^{3}H film at 4°C for 5 weeks.

Films were developed, fixed, and then analyzed using Image software as described for the *in situ* hybridization experiments. At least five measurements that spanned the rostro-caudal extent of each nucleus were made per region per subject, and measurements of optical densities were calibrated by ^{3}H standards using tissue equivalent values (nCi/mg tissue). Specific binding was calculated as the difference between total and nonspecific binding measured from comparable regions of adjacent sections. For each area of each subject, estimates of specific binding were averaged. For the neostriatum (within and medial to the lMAN) and the LPO (within and medial to Area X), separate two-way ANOVAs (Age × Region) were used to evaluate group differences.

In a separate group of animals, we performed slice-wiping studies to evaluate whether the characteristics of ifenprodil binding in zebra finch brain are similar to those reported in rodent brain. Forebrain sections from 30-day-old (n = 3) and adult (n = 3) males were cut, and half of the sections were processed for total binding as described above while the other half were incubated in the absence of masking agents. Immediately after the final washes in cold buffer, sections were wiped from the slides into plastic scintillation vials. The tissue was solubilized overnight in 500 μL of protosol tissue solubilizer, and 3.0 mL of scintillation fluid (Ecoscint-A) was added to each vial the following day. Forty-eight hours later, radioactivity of the tissue was assessed through liquid scintillation counting. Both total and nonspecific [^{3}H]ifenprodil binding were significantly lower in forebrain sections incubated in the presence of pharmacological blockers compared to adjacent sections incubated in the absence of blockers (total binding: $t = -9.4$, df = 5, $p < .001$; nonspecific binding: $t = -4.2$, df = 5, $p < .001$). We also performed a slice-wiping experiment to assess whether the addition of 1 m*M* trifluoperazine isolates the high-affinity ifenprodil site in zebra finch as it does in rodent. In adult rat cerebellum, NR2B subunits are not detectable (Takahashi et al., 1996; Wang et al., 1995) and within this region specific binding of [^{3}H]ifenprodil in the presence of 3 μM (+)-3-PPP and 30 μM GBR-12909 reflects only low-affinity (non-NR2B) NMDA receptor binding that is completely inhibited by 1 m*M* trifluoperazine (Nicolas and Carter, 1994). Coronal sections of zebra finch cerebellum were processed as described above except that half of the sections were incubated with all masking agents and half were incubated in the absence of trifluoperazine. As in rat, specific binding in zebra finch cerebellum was evident only when 1 m*M* trifluoperazine was omitted from the incubation solution. Together, these results suggest that the inclusion of all masking agents in the ifenprodil binding protocol selectively labels high-affinity, trifluoperazine-insensitive (NR2B-related) binding sites in zebra finches as it does in rat.

RESULTS

In Situ Hybridization and RT-PCR

Our NR2B-oligo1 probe, based on a conserved portion of the cDNA sequence for the NR2B subunit in mouse, rat, and human, selectively hybridized to NR2B mRNA in tissue from zebra finches. Pretreatment of sections with RNAse-A eliminated specific hybridization as did competition of ^{35}S-labeled antisense probe with excess unlabeled antisense probe. There also was no specific hybridization in sections incubated with ^{35}S-labeled sense NR2B probe.

The NR2B-oligo1 antisense probe generated high levels of hybridization throughout most of the zebra finch telencephalon. In both 30-day-old and adult males, NR2B mRNA expression was robust within the LPO, neostriatum, hyperstriatum, and dorsal thalamus. In contrast, hybridization was markedly lower in the archistriatum, optic tectum, and ventral thalamus. Virtually no

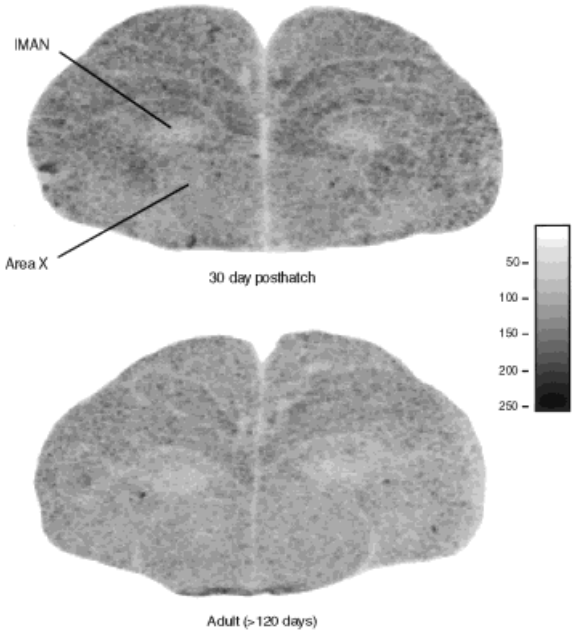

Figure 1 Representative autoradiogram of a coronal section through the anterior forebrain showing NR2B mRNA expression localized by *in situ* hybridization in a 30-day-old (top) and adult (bottom) male zebra finch. At both ages, the lMAN is distinct due to lower hybridization in this region relative to surrounding neostriatum. Hybridization levels within the LPO are homogeneous, and therefore the boundaries of Area X are not distinct. Calibration bar shows relative optical density (gray level) of hybridization.

NR2B mRNA expression was detected within the cerebellum, hypothalamus, and brain stem (outside the optic tectum). Thus, the regional distribution of NR2B transcript expression was remarkably similar to that seen in mammalian brain (Monyer et al., 1992), and somewhat more restricted than the distribution of NMDA receptors in avian brain as visualized by MK-801 binding (Aamodt et al., 1992).

Visual inspection of the autoradiograms revealed striking regional heterogeneity of NR2B transcript expression within the anterior neostriatum (Fig. 1). In both 30-day-old and adult male zebra finches, hybridization levels within the song nucleus lMAN were starkly lower than the relatively high levels of NR2B expression evident throughout the anterior forebrain. In every animal, this song nucleus was clearly visible in the autoradiograms. In contrast, Area X was not distinguishable in the autoradiograms at either age. Area X is clearly defined in Nissl-stained material from both 30-day-old and adult males; yet, NR2B mRNA was homogeneously expressed at high levels throughout the LPO.

A relatively widespread, age-related decline in hybridization signal was apparent in the autoradiograms. Within the anterior forebrain, this impression was confirmed quantitatively (Fig. 2). A two-way ANOVA of optical density measurements within the lMAN and adjacent medial neostriatum revealed a significant effect of age ($F = 8.75$, $p < .02$), a weak effect of region ($F = 3.47$, $p = .09$), and no interaction. Thus, between 30 days posthatch and adulthood, hybridization levels declined by approximately 65% within the lMAN and 60% within the adjacent neostriatum. Because the effect of region was most appropriately evaluated using pairwise comparisons, we probed the ANOVA using post-hoc paired *t* tests

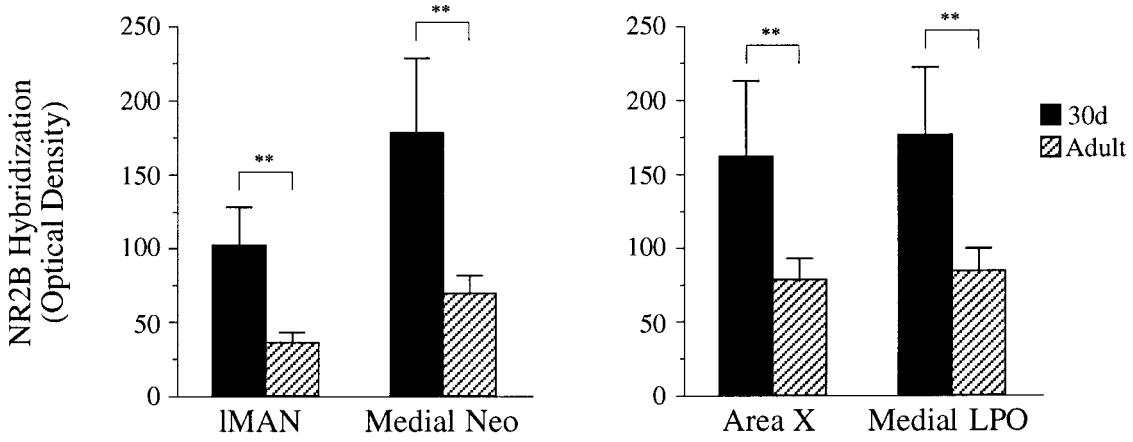

Figure 2 Quantitation of NR2B transcript levels in the anterior forebrain of male zebra finches at 30 days posthatch and in adulthood. Within the neostriatum (left), hybridization levels both within the lMAN and in neostriatum medial to the lMAN declined significantly between 30 days posthatch and adulthood. Also, hybridization within the lMAN was significantly less than in the adjacent medial neostriatum in adults ($p < .05$). Within the LPO (right), NR2B mRNA expression declined significantly both within Area X and in the surrounding medial LPO between 30 days and adulthood. Data shown are means ± S.E.M. **30 days versus adult, $p < .05$.

(Bonferoni adjustment = 2). Although NR2B transcript expression was lower in the lMAN than in the adjacent medial neostriatum in every 30-day-old as well as adult male, posthoc tests revealed a significant regional difference in hybridization only in the adults ($t = 4.81$, $p < .05$). The lack of statistical significance in the 30-day-old group was most likely due to inter-experiment variability in the overall level of hybridization: In one film that contained one juvenile and one adult bird, the autoradiograms were noticeably lighter than those of other films. Because hybridization in both lMAN and the medial neostriatum was higher in the juvenile group than in adults, the unusually low levels of hybridization in this one film created significantly more variability in the juvenile than in the adult group.

Within the LPO, quantitative analysis confirmed that NR2B mRNA expression also is developmentally regulated in this region (Fig. 2). A two-way ANOVA on measures of optical density within Area X and the medial LPO revealed a significant effect of age ($F = 6.01$, $p = .05$) but not region. The interaction term also was not significant. Thus, between 30 days post-hatch and adulthood, hybridization levels declined by approximately 50% within both Area X and in the surrounding medial LPO.

Figure 3 Reverse-transcriptase–polymerase chain reaction product from rodent and zebra finch mRNA amplified by rodent-specific NR2B primers. Rodent-specific NR2B primers amplified a similar sized (~445 bp) cDNA product from both rodent (Lane 1) and zebra finch (Lane 3) mRNA. While cDNAs from both species were digested into two fragments by *Msp1* (Lane 2: rodent; Lane 4: zebra finch), the size of the resulting fragments differed, indicating the presence of a single *Msp1* consensus sequence located at different sites in the avian and rodent NR2B gene.

The RT-PCR results confirmed a high degree of similarity between zebra finch and mammalian NR2B message. The rodent-specific NR2B primers amplified an approximately 445-bp cDNA product from both rodent and zebra finch RNA (Fig. 3). Sequence analysis indicated that the zebra finch cDNA shared 85% nucleotide homology to the rat, mouse, and human NR2B receptor (GenBank; NIH) across the amplified region (Fig. 4). The putative protein sequence (based on nucleotide analysis; Swiss Prot) was 99% homologous to the rat, mouse and human NR2B subunit. In the rodent NR2B nucleotide sequence, the region amplified by the primers contained a single *Msp1* site (CCGG; nucleotides 2595–2598). A single base substitution in the zebra finch sequence at (rodent) nucleotide 2595 results in a loss of the rodent-specific *Msp1* site, while another base substitution corresponding to (rodent) nucleotide 2631 creates a novel zebra finch-specific *Msp1* site. The presence of a single *Msp1* site was confirmed by restriction digestion with the enzyme, which resulted in two fragments in both zebra finch and rodent cDNA (Fig. 3). The disparity in the size of the zebra finch- and rodent-digested fragments further confirmed the presence of an atypical *Msp1* site in the avian NR2B.

Within the 45-bp region specific to NR2B-oligo1, the zebra finch sequence was 91% similar to the human, rat, and mouse sequence. The four bases that differed among these species were dispersed through the internal portion of the oligonucleotide probe. Moreover, the pattern of hybridization observed in zebra finches using NR2B-oligo1 did not differ from that observed with a second probe, NR2B-oligo2 that was 100% complementary to the zebra finch NR2B mRNA pulled out from our RT-PCR experiments (Fig. 4). The NR2B-oligo2 probe (GGGCT TGCCA GAGCA GACAC CCATG AAACA GTGGC GGAAT TGCCA) overlapped 21 bases that were in the NR2B-oligo1 probe and was 89% complementary to bases 2587–2630 of rat NR2B mRNA sequence (Monyer et al., 1992). NR2B-oligo2 was 62% similar to mouse M-cadherin mRNA (the most similar non-NR2B mRNA in Genebank) and was <50% similar to any NR2A mRNA sequences. Tissue from one juvenile and one adult male zebra finch was processed as described in Materials and Methods except that hybridization temperature was increased to 42°C and sections were washed 2 × 30 min at a higher stringency (0.5× SSC at 60°C). Unlike for the NR2B-oligo1 probe, these higher-stringency conditions did not produce a detectable loss of hybridization signal. The regional pattern of hybridization observed with NR2B-oligo2 was identical to that seen using NR2B-oligo1. Moreover, quantitative measures of NR2B-

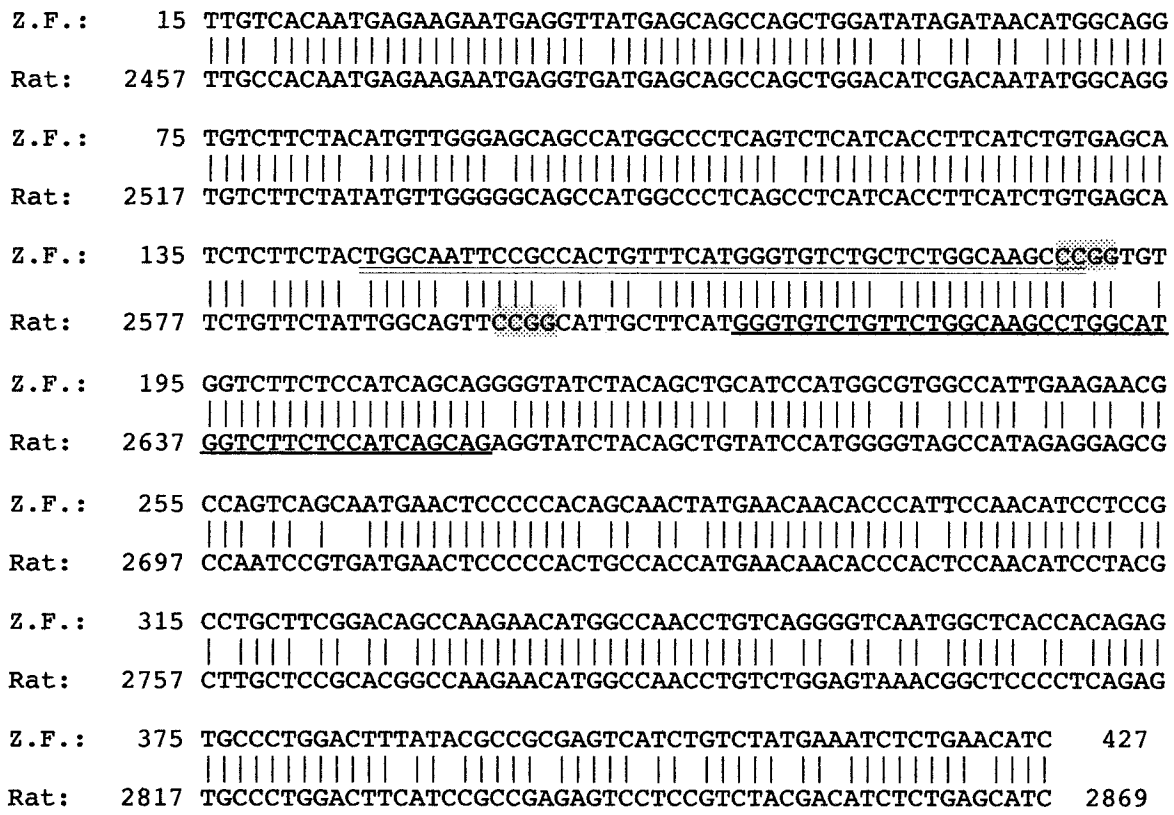

Figure 4 Comparison of a partial nucleotide sequence (413 bp) for NR2B mRNA of zebra finch and rat. The single underline indicates the portion of the rat sequence that was used as a template for design of the oligonucleotide probe (NR2B-oligo1) used in quantitative *in situ* hybridization experiments. The double underline indicates the portion of the zebra finch that was used as a template for design of NR2B-oligo2 (see Results). Shaded sequences shows *Msp1* restriction sites.

oligo2 hybridization in the juvenile and adult anterior forebrain confirmed the developmental decline in NR2B mRNA expression within the anterior forebrain. Relative to the juvenile, hybridization in the adult was 76% lower within the lMAN (162 ± 7 vs. 38 ± 2), 68% lower in the neostriatum medial to the lMAN (159 ± 4 vs. 51 ± 3), 51% lower within the Area X (135 ± 3 vs. 66 ± 5), and 55% lower in the LPO medial to Area X (128 ± 3 vs. 58 ± 6).

Ifenprodil Binding

In both 30-day-old and adult male zebra finches, medium to high levels of specific [^{3}H]ifenprodil binding were evident through most of the LPO, neostriatum, and hyperstriatum. Specific [^{3}H]ifenprodil binding within the archistriatum and dorsal thalamus was low to moderate. The ventral thalamus and brainstem (including the optic tectum) exhibited the lowest levels of binding except for the hypothalamus and cerebellum, where specific binding was notably absent. These relative levels of binding were virtually identical to the pattern of NR2B mRNA expression described above, and similar to the regional variations in high-affinity, NR2B-related ifenprodil binding described in rodent (Nicolas and Carter, 1994).

Although most of the anterior forebrain expressed high levels of specific [^{3}H]ifenprodil binding, markedly reduced binding was evident within the forebrain song regions lMAN and Area X. In both 30-day-old and adult males, these regions were clearly visible in the autoradiograms owing to their relatively low levels of [^{3}H]ifenprodil binding (Fig. 5). Thus, in the anterior neostriatum [^{3}H]ifenprodil binding was similar to NR2B mRNA expression in that both were obviously lower in the lMAN than in the surrounding neostriatum (compare Figs. 1 and 5). In contrast, whereas [^{3}H]ifenprodil binding clearly distinguished Area X owing to lower levels of binding as compared to the rest of the LPO (Fig. 5), NR2B mRNA expression was relatively high throughout the LPO, and

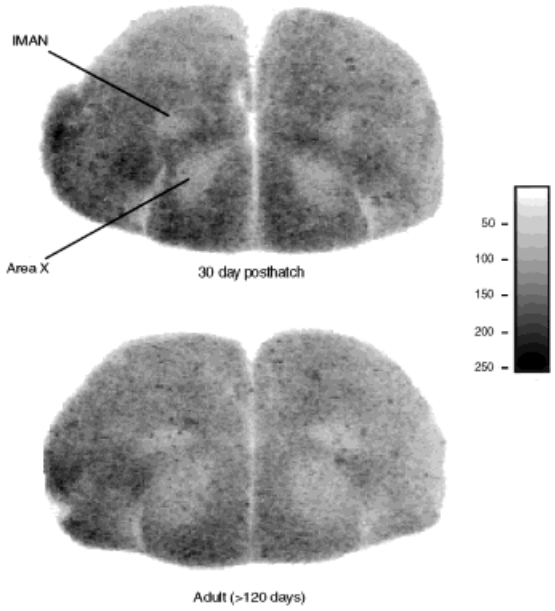

Figure 5 Representative autoradiogram of a coronal section showing high-affinity [^{3}H]ifenprodil binding in the anterior forebrain of a 30-day-old (top) and adult (bottom) male zebra finch. The lMAN and Area X are both distinct owing to lower levels of [^{3}H]ifenprodil binding relative to the surrounding regions. Calibration bar shows relative optical density (gray level) of total binding.

Area X was not distinguishable in the NR2B autoradiograms (Fig. 1).

When comparing [^{3}H]ifenprodil binding within the lMAN to specific binding within the surrounding neostriatum, an ANOVA revealed a significant effect of region ($F = 13.47$, $p < .01$) and age ($F = 4.38$, $p < .05$) with no interaction. As shown in Figure 6 (left), there was a 65% decrease in specific binding within the lMAN between 30 days after hatching (0.0662 ± 0.0143 pmol/mg tissue) and adulthood (0.0238 ± 0.0074 pmol/mg tissue). The age-related decrease in specific binding within neostriatal tissue medial to the lMAN was somewhat more modest (0.1347 ± 0.0279 pmol/mg at 30 days posthatch vs. 0.0965 ± 0.0210 pmol/mg in adulthood). Within Area X and the surrounding LPO (Fig. 6, right), an ANOVA revealed a significant effect of region ($F = 10.51$, $p < .01$) but not age. These results indicate that in addition to the obviously lower levels of binding in the lMAN and Area X compared to surrounding areas at both ages, there was an overall developmental decline in [^{3}H]ifenprodil binding in the lMAN and surrounding anterior neostriatum, but not in the LPO.

DISCUSSION

The results of these experiments suggest that the sensitive period for avian song learning is associated with heightened expression of NMDA receptor 2B subunits in the lMAN and adjacent medial neostriatum. Between 30 days of age, when young male zebra

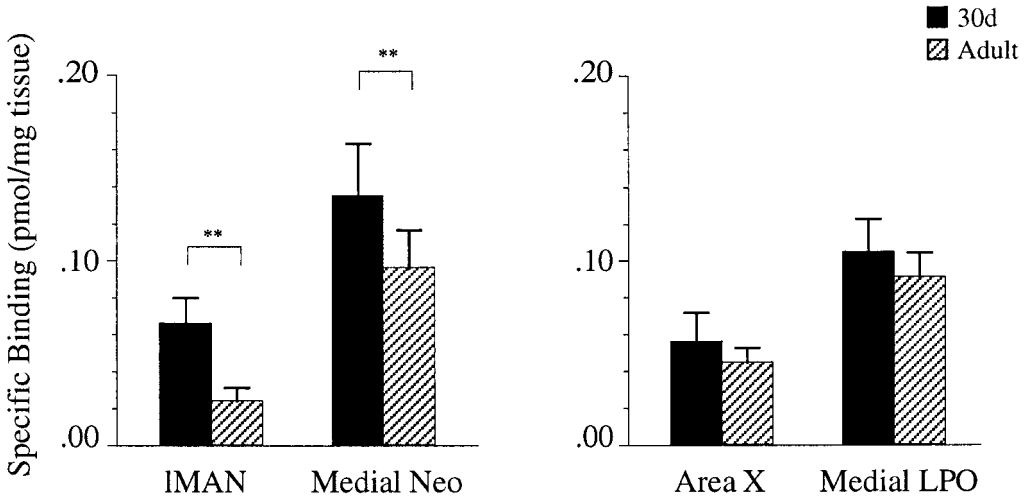

Figure 6 Specific [^{3}H]ifenprodil binding (pmol/mg) in the anterior forebrain of male zebra finches at 30 days posthatch and in adulthood. Within the neostriatum (left), binding both within the lMAN and in neostriatum medial to the lMAN exhibited a significant developmental decrease during the juvenile period. In contrast, there was no significant developmental change in [^{3}H]ifenprodil binding within the LPO (right). At both ages, specific binding within the lMAN and Area X was significantly lower than in the surrounding neostriatum and LPO, respectively (see Results). Data shown are means $\pm$ S.E.M. **30 days versus adult, $p < .05$.

finches are just beginning to memorize song material, and adulthood, when males no longer modify their songs, there is a significant reduction in high-affinity, NR2B-associated ifenprodil binding within these regions. This decline in NR2B subunit expression appears to reflect developmental regulation of NR2B gene expression: Coincident with the decrease in [^{3}H]ifenprodil binding, there is a significant decline in the expression of NR2B transcripts within the lMAN. Thus, the results support the hypothesis that a developmental decrease in the expression of NR2B subunits may constrain when song learning occurs in zebra finches.

The developmental decline in NR2B subunit expression is clearly not restricted to the lMAN. Downregulation of NR2B mRNA and specific [^{3}H]ifenprodil binding is evident throughout much of the forebrain, and we have documented this quantitatively for both the lMAN and the neostriatum medial to this song nucleus. It is likely that our measurements medial to the lMAN included the medial magnocellular nucleus of the anterior neostriatum (mMAN), another region that is presumed to participate in avian song behavior. Because this region is not histologically distinct in Nissl-stained zebra finch brain, we cannot evaluate accurately whether our measurements of the medial anterior neostriatum included this nucleus. The mMAN innervates the HVc, a nucleus critical to song production (Nottebohm et al., 1982, 1976); however, no evidence is available concerning the function of the mMAN.

The decline in ifenprodil binding within the lMAN may be a consequence of the overall decrease in NMDA receptor density known to occur within this region (Aamodt et al., 1995). However two independent observations suggest that a change in the subunit composition of lMAN NMDA receptors may contribute to the observed decline in ifenprodil binding. First, MK-801 binding affinity increases within the lMAN during this same period, suggesting that the makeup of individual receptors may be changing (Aamodt et al., 1995). In general, recombinant receptors containing NR2A subunits have higher affinities for antagonists than those containing NR2B subunits (Buller et al., 1994). Although MK-801 has similar affinity for NR1/NR2A and NR1/NR2B recombinant heteromeric receptors (Laurie and Seeburg, 1994), native NMDA receptors likely contain more than one modulatory subunit (Chazot et al., 1994; Chazot and Stephenson, 1997; Luo et al., 1997; Sheng et al., 1994; Wafford et al., 1993). Thus, MK-801 binding affinity could be affected by allosteric interactions occurring among NR2A and NR2B subunits expressed in native NMDA receptor complexes. Recent physiological data provide additional, and stronger support for the notion that the subunit composition of NMDA receptors within the lMAN changes during song development. Whole-cell voltage-clamp recordings from lMAN neurons show that the slow component of NMDA receptor-mediated currents accounts for a significantly larger fraction of the total NMDA receptor current in 22- to 32-day-old males as compared to 50-day-old or adult males (Livingston and Mooney, 1997). Since expression studies have shown that NMDA receptors containing the NR2B subunit have longer channel open times than do receptors that lack the NR2B subunit (Monyer et al., 1994), a developmental loss of the NR2B subunit in individual receptors within the lMAN could account for these recent physiological data.

If the composition of lMAN NMDA receptors does change during development, this likely involves a replacement of NR2B with NR2A subunits. As stated above, many native forebrain NMDA receptors contain both of these subunits, and a decrease in NR2B incorporation could lead to an increase in receptors containing NR1/NR2A subunits and/or multimeric receptors (NR1/NR2A/NR2B) with a larger NR2A:NR2B ratio. Either of these outcomes would likely result in shorter NMDA receptor currents. In the neocortex, the proportion of cells expressing NR2A mRNA increases developmentally and individual cells that express NR2A mRNA have faster NMDA receptor–mediated postsynaptic currents than do cells lacking NR2A transcripts (Flint et al., 1997). Similarly, recombinant receptors containing a NR2A subunit have faster current deactivation than do those containing only NR1 and NR2B subunits (Kutsuwada et al., 1992; Laurie and Seeburg, 1994; Monyer et al., 1994; Vicini et al., 1998). In recombinant receptors that incorporate both NR2A and NR2B subunits into single complexes, an increase in the NR2A:NR2B ratio leads to faster deactivation times (Vicini et al., 1998). The developmental profile of NR2A subunits within zebra finch song regions has not been determined, and the data reported here do not discriminate between changes in overall receptor number and changes in receptor composition. However, given the age-related changes in lMAN physiology described by Livingston and Mooney (1997), it will be interesting to establish directly whether the relative expression of NR2A and NR2B subunits changes developmentally. Whether or not such relative changes occur, the significant decline in NR2B expression, coupled with our previous demonstration of a decrease in overall NMDA receptor density within the lMAN (Aamodt et al., 1995), suggests that normal song learning in male zebra finches is accompanied by a

decline in NMDA receptor–mediated calcium entry within lMAN neuron.

Comparisons between ifenprodil binding and NR2B mRNA expression within the zebra finch brain suggest that both pre- and posttranscriptional events influence the incorporation of NR2B subunits into functional NMDA receptors. Within the lMAN and medial neostriatum, the parallel developmental decrease in ifenprodil binding and NR2B mRNA expression is consistent with the hypothesis that a down-regulation of transcription leads to a decline in NR2B protein expression. However, in the LPO the significant decline in NR2B transcripts is not accompanied by a significant developmental change in high-affinity ifenprodil binding. This raises the possibility that posttranscriptional processing of NR2B transcripts may be more efficient in adult than in young birds. Also, regional variation in posttranscriptional processing appears to be significant within the LPO, since the obvious heterogeneity in NR2B protein expression as visualized by ifenprodil binding is not evident in the pattern of NR2B mRNA expression. In both juvenile and adult males, ifenprodil binding is significantly lower in Area X than in the surrounding tissue despite high levels of mRNA expression throughout the LPO. Posttranscriptional processing may be either less efficient or in some other way different within Area X compared to other portions of the LPO. Although it is possible that the differential [^{3}H]ifenprodil-specific binding reflects regional variation in affinity characteristics, this seems unlikely because our protocol limits ifenprodil binding to high-affinity sites. Nevertheless, Scatchard analysis of saturation binding curves would provide a direct test of this possibility.

The data presented are consistent with a model of avian song learning in which song memorization requires activation of NMDA receptors in the anterior neostriatum and is constrained to an early sensitive period by changes in the expression of NMDA modulatory subunits within this portion of the song system. Normally, song learning occurs during a period of unique NMDA receptor pharmacology within the lMAN. Here we provide evidence that during development, a profound loss of NR2B subunits within this nucleus occurs as birds' ability to learn new song elements declines. This loss of NR2B subunits is likely due to both an overall decrease in the density of NMDA receptors within the lMAN and to a shift from a highly plastic "juvenile" form of the NMDA receptor to a less plastic "adult" form of the NMDA receptor. This developmental profile is consistent with studies from several systems and species showing that changes in the physiology and pharmacology of NMDA receptors and a decline in NR2B subunit expression occur in parallel with changes in the propensity for synaptic plasticity (Brady et al., 1994; Carmignoto and Vicini, 1992; Hestrin, 1992; Hofer and Constantine-Paton, 1994). Also, this model would predict that treatments that extend the sensitive period for song learning will also delay the developmental decrease in ifenprodil binding (and perhaps NR2B mRNA expression) within the lMAN. Experience-dependent regulation of NMDA receptors occurs within visual cortex, where environmental manipulations that prolong the period of developmental cortical plasticity also delay developmental changes in NMDA receptor channel properties (Carmignoto and Vicini, 1992). In songbirds, early isolation from conspecific song delays closure of the sensitive period, and male zebra finches previously denied access to a song model can acquire and imitate new song material first encountered as late as 60–120 days of age (Eales, 1987; Morrison and Nottebohm, 1993). Thus, manipulating early exposure to conspecific song enables us to separate the contributions of experience and maturation to the regulation of NMDA receptor expression. Early isolation does not alter the developmental decrease in overall MK-801 binding within the lMAN (Aamodt et al., 1995); however, if heightened expression of the NR2B subunit is necessary for the acquisition of new song material, late learning during an extended sensitive period should be accompanied by a delay in the developmental down-regulation of the NR2B subunit.

This research was supported by National Institute of Mental Health Grant MH45096. The authors are grateful to Dr. Suzanne Haber, who generously offered the use of her laboratory for the *in situ* hybridization experiments. They also thank Donna Shannon and Joanne Macoretta for their expert technical support.

REFERENCES

Aamodt SA, Kozlowski MR, Nordeen EJ, Nordeen KW. 1992. Distribution and developmental change in [^{3}H]MK-801 binding within zebra finch song nuclei. J Neurobiol 23:997–1005.

Aamodt SM, Nordeen EJ, Nordeen KW. 1995. Early isolation from conspecific song does not affect the normal developmental decline of *N*-methyl-D-aspartate receptor binding in an avian song nucleus. J Neurobiol 27:76–84.

Aamodt SM, Nordeen EJ, Nordeen KW. 1996. Blockade of NMDA receptors during song model exposure impairs song development in juvenile zebra finches. Neurobiol Learn Mem 65:91–98.

Basham ME, Nordeen EJ, Nordeen KW. 1996. Blockade of NMDA receptors in the anterior forebrain impairs sen-

sory acquisition in the zebra finch (*Peophila guttata*). Neurobiol Learn Mem 66:295–304.

Bear M, Kleinschmidt A, Gu Q, Singer WS. 1990. Disruption of experience-dependent modifications in striate cortex by infusion of an NMDA receptor antagonist. J Neurosci 10:909–925.

Bear MF. 1996. NMDA-receptor-dependent synaptic plasticity in the visual cortex. Prog Brain Res 108:205–218.

Ben-Ari Y, Cherubini E, Krnjevic K. 1988. Changes in voltage dependence of NMDA currents during development. Neurosci Lett 94:88–97.

Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361:31–39.

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

Brady RJ, Gorter JA, Monroe MT, Swann JW. 1994. Developmental alterations in the sensitivity of hippocampal NMDA receptors to AP5. Dev Brain Res 83:190–196.

Brennan PA. 1994. The effects of local inhibition of *N*-methyl-D-aspartate and AMPA/kainate receptors in the accessory olfactory bulb on the formation of an olfactory memory in mice. Neuroscience 60:701–708.

Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, Monaghan DT. 1994. The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. J Neurosci 14:5471–5484.

Burchuladze R, Rose SPR. 1992. Memory formation in day-old chicks requires NMDA but not non-NMDA glutamate receptors. Eur J Neurosci 4:535–538.

Burgard EC, Hablitz JJ. 1993. NMDA receptor-mediated components of minature excitatory synaptic currents in developing rat neocortex. J Physiol 70:1841–1852.

Carmignoto G, Vicini S. 1992. Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. Science 258:1007–1011.

Chazot PL, Coleman SK, Cik M, Stephenson FA. 1994. Molecular characterization of *N*-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. J Biol Chem 269:24403–24409.

Chazot PL, Stephenson FA. 1997. Molecular dissection of native mammalian forebrain NMDA receptors containing the NR1 C2 exon: direct demonstration of NMDA receptors comprising NR1, NR2A, and NR2B subunits within the same complex. J Neurochem 69:2138–2144.

Cline HT, Debski E, Constantine-Paton M. 1987. NMDA receptor antagonist desegregates eye specific stripes. Proc Natl Acad Sci USA 84:4342–4345.

Contreras PC, Bremer ME, Gray NM 1990. Ifenprodil and SL 82.0715 potently inhibit binding of [3H](+)-3-PPP to σ binding sites in rat brain. Neurosci Lett 116:190–193.

Crair MC, Malenka RC. 1995. A critical period for long-term potentiation at thalamocortical synapses. Nature 375:325–328.

Davis S, Butcher SP, Morris RGM. 1992. The NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5) impairs spatial learning and LTP *in vivo* at intracerebral concentrations comparable to those that block LTP *in vitro*. J Neurosci 12:21–34.

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

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

Feldman DE, Knudsen EI. 1998. Pharmacological specialization of learned auditory responses in the inferior colliculus of the barn owl. J Neurosci 18:3073–3087.

Fields RD, Yu C, Nelson PG. 1991. Calcium, network activity, and the role of NMDA channels in synaptic plasticity *in vitro*. J Neurosci 11:134–146.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H. 1997. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17:2469–2476.

Fox K, Schlaggar BL, Glazewski S, O'Leary DD. 1996. Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. Proc Natl Acad Sci USA 93:5584–5589.

Gallagher MJ, Hui H, Pritchett DB, Lynch DR. 1996. Interactions between ifenprodil and the NR2B subunit of the *N*-methyl-D-aspartate receptor. J Biochem 271:9603–9611.

Hahm J-O, Langdon RB, Sur M. 1991. Disruption of retinogeniculate afferent segregation by antagonists to NMDA receptors. Nature 351:568–570.

Hashimoto K, London ED. 1993. Further characterization of [3H]ifenprodil binding to σ receptors in rat brain. Eur J Pharmacol 236:159–163.

Hestrin S. 1992. Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. Nature 357:686–689.

Hofer M, Constantine-Paton M. 1994. Regulation of *N*-methyl D-aspartate (NMDA) receptor function during the rearrangement of developing neuronal connections. Prog Brain Res 102:277–285.

Hofer M, Prusky GT, Constantine-Paton M. 1994. Regulation of NMDA receptor mRNA during visual map formation and after receptor blockade. J Neurochem 62: 2300–2307.

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

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, Nakanishi S. 1993. Molecular characterization of the family of the *N*-methyl D-aspartate receptor subunits. J Biol Chem 268:2836–2843.

Kendrick SJ, Lynch DR, Pritchett DB. 1996. Characterization of glutamate binding sites in receptors assembled from transfected NMDA receptor subunits. J Neurochem 67:608–616.

Kim M, McGaugh JL. 1992. Effects of intra-amygdala injections of NMDA receptor antagonists on acquisition and retention of inhibitory avoidance. Brain Res 585:35–48.

Kirson ED, Yaari Y. 1996. Synaptic NMDA receptors in developing mouse hippocampal neurones: functional properties and sensitivity to ifenprodil. J Physiol 497: 437–455.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, Mishina M. 1992. Molecular diversity of the NMDA receptor channel. Nature 358:36–41.

Laurie DJ, Bartke I, Schoepfer R, Naujoks K, Seeburg PH. 1997. Regional, developmental and interspecies expression of the four NMDAR2 subunits, examined using monoclonal antibodies. Mol Brain Res 51:23–32.

Laurie DJ, Seeburg PH. 1994. Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. Eur J Pharmacol 268:335–345.

Liang KC, Hon W, Davis M. 1994. Pre- and posttraining infusion of *N*-methyl-D-aspartate receptor antagonists into the amygdala impair memory in an inhibitory avoidance task. Behav Neurosci 108:241–253.

Livingston FS, Mooney R. 1997. Development of intrinsic and synaptic properties in a forebrain nucleus essential to avian song learning. J Neurosci 17:8997–9009.

Luo J, Wang YH, Yasuda RP, Dunah A, Wolfe BB. 1997. The majority of *N*-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits. Mol Pharmacol 51:79–86.

Lynch DR, Lawrence JL, Lenz S, Anegawa NJ, Dichter M, Pritchett DB. 1995. Pharmacological characterization of heterodimeric NMDA receptors composed of NR 1a and 2B subunits: differences with receptors formed from NR 1a and 2A. J Neurochem 64:1462–1468.

Mandich P, Schito AM, Bellone E, Antonacci R, Finelli P, Rocchi M, Ajmar F. 1994. Mapping of the human NMDAR2B receptor subunit gene (GRIN2B) to chromosome 12p12. Genomics 22:216–218.

Marler P. 1997. Three models of song learning: evidence from behavior. J Neurobiol 33:501–516.

Mayer ML, Westbrook GL. 1987. The physiology of excitatory amino acids in the vertebrate central nervous system. Prog Neurobiol 28:65–90.

McCabe BJ, Davey JE, Horn G. 1992. Impairment of learning by localized injection of an *N*-methyl-D-aspartate receptor antagonist into the hyperstriatum ventrale of the domestic chick. Behav Neurosci 106:947–953.

Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, Kumanishi T, Arakawa M, Sakimura K, Mishina M. 1992. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 357:70–74.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12:529–540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH. 1992. Heteromeric NMDA receptors: Molecular and functional distinction of subtypes. Science 256:1217–1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. 1991. Molecular cloning and characterization of the rat NMDA receptor. Nature 354:31–37.

Morris RGM. 1989. The biology of memory. Bernried, Germany: F.K. Schattauer Verlag. p 298–318.

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

Nicolas C, Carter C. 1994. Autoradiographic distribution and characteristics of high- and low-affinity polyamine-sensitive [3H]ifenprodil sites in the rat brain: possible relationship to NMDAR2B receptors and calmodulin. J Neurochem 63:2248–2258.

Nordeen KW, Nordeen EJ. 1993. Long-term maintenance of song in adult zebra finches is not affected by lesions of a forebrain region involved in song learning. Behav Neural Biol 59:79–82.

Nottebohm F, Kelley DB, Paton JA. 1982. Connections of vocal control nuclei in the canary telencephalon. J Comp Neurol 207:344–357.

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

Priestley T, Laoughton P, Myers J, Le Bourdelles B, Kerby J, Whiting PJ. 1995. Pharmacological properties of recombinant human *N*-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells. Mol Pharmacol 48:841–848.

Ramoa AS, McCormick DA. 1994. Enhanced activation of NMDA receptor responses at the immature retinogeniculate synapse. J Neurosci 14:2098–2105.

Ramoa AS, Prusky G. 1997. Retinal activity regulates developmental switches in functional properties and ifenprodil sensitivity of NMDA receptors in the lateral geniculate nucleus. Brain Res 101:165–175.

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

Scherer WJ, Udin SB. 1989. *N*-Methyl-D-aspartate antagonists prevent interaction of binocular maps in *Xenopus* tectum. J Neurosci 9:3837–3843.

Schlaggar BL, Fox K, O'Leary DD. 1993. Postsynaptic control of plasticity in developing somatosensory cortex. Nature 364:623–626.

Schnupp JW, King AJ, Smith AL, Thompson ID. 1995. NMDA-receptor antagonists disrupt the formation of the auditory space map in the mammalian superior colliculus. J Neurosci 15:1516–1531.

Schoemaker H, Allen J, Langer SZ. 1990. Binding of [3H]ifenprodil, a novel NMDA antagonist, to a polyamine-sensitive site in the rat cerebral cortex. Eur J Pharmacol 176:249–250.

Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. 1994.

Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. Nature 368: 144–147.

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

Steele RJ, Stewart MG. 1993. 7-Chlorokynurenate, an antagonist of the glycine binding site on the NMDA receptor, inhibits memory formation in day-old chicks (*Gallus domesticus*). Behav Neural Biol 60:89–92.

Sugihara H, Moriyoshi K, Ishii T, Masu M, Nakanishi S. 1992. Structure and properties of seven isoforms of the NMDA receptors generated by alternative splicing. Biochem Biophys Res Commun 185:826–832.

Takahashi T, Feldmeyer D, Suzuki N, Onodera K, Cull-Candy SG, Sakimura K, Mishina M. 1996. Functional correlation of NMDA receptor epsilon subunits expression with the properties of single-channel and synaptic currents in the developing cerebellum. J Neurosci 16: 4376–4382.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR. 1998. Functional and pharmacological differences between recombinant *N*-methyl-D-aspartate receptors. J Neurophysiol 79:555–566.

Wafford KA, Bain CJ, Le Bourdelles B, Whiting PJ, Kemp JA. 1993. Preferential co-assembly of recombinant NMDA receptors composed of three different subunits. Neuroreport 4:1347–1349.

Wang Y-H, Bosy TZ, Yasuda RP, Grayson DR, Vicini S, Pizzorusso T, Wolfe BB. 1995. Characterization of NMDA receptor subunit-specific antibodies: distribution of NR2A and NR2B receptor subunits in rat brain and ontogenic profile in the cerebellum. J Neurochem 65: 176–183.

Watanabe M, Inoue Y, Sakimura K, Mishina M. 1992. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport 3:1138–1140.

Watanabe M, Inoue Y, Sakimura K, Mishina M. 1993. Distinct distributions of five *N*-methyl-D-aspartate receptor channel subunit mRNAs in the forebrain. J Comp Neurol 338:377–390.

Williams K. 1993. Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. Mol Pharmacol 44:–851–859.

Williams K. 1995. Pharmacological properties of recombinant *N*-methyl-D-aspartate (NMDA) receptors containing the epsilon 4 (NR2D) subunit. Neurosci Lett 184:181–184.

Williams K, Russell SL, Shen YM, Molinoff PB. 1993. Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. Neuron 10:267–278.

Zhong J, Carrozza DP, Williams K, Pritchett DB, Molinoff PB. 1995. Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. J Neurochem 64:531–539.