Silent Synapses in a Thalamo-Cortical Circuit Necessary for Song Learning in Zebra Finches

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INTRODUCTION

Highly precise synaptic connections underlying age-limited types of learning are established during sensitive periods guided by patterns of neural activity based on experience (Zheng and Knudsen 1999). In the neural system that controls vocal learning and behavior in songbirds, experience-dependent synaptic rearrangements in song-control circuits occur during early phases of the sensitive period for vocal learning (Iyengar and Bottjer 2002a) and may underlie the emergence of specific vocal patterns. This study examined the maturation of synaptic transmission in a thalamo-cortical connection in the song control system of zebra finches as an initial step in testing relationships between developmentally regulated learning and synaptic modification.

Juvenile male zebra finches learn to produce a mature stereotyped song from a male tutor. Initially a song model is acquired from a tutor starting at ~20 days of age up until 40–60 days (Böhner 1990). Starting ~30 days of age, juveniles begin to produce song-related vocalizations that have variable acoustic structure and bear little resemblance to the tutor’s song or to the individual’s own mature song. Juvenile song behavior gradually acquires structure and temporal order characteristic of the tutor’s song and becomes a stereotyped vocal pattern by the onset of adulthood (~90 days). The brain regions necessary for vocal learning in zebra finches during the sensitive period include a pathway linking cortex, basal ganglia, and thalamus (Fig. 1). The cortical nucleus lateral magnocellular nucleus of the anterior nidopallium (LMAN) provides the output of this pathway to motor circuitry and is necessary for normal song behavior during early stages of vocal learning but not for stereotyped song production in older juvenile or adult birds (Bottjer 2002; Bottjer and Arnold 1997). Furthermore, blockade of N-methyl-D-aspartate receptors (NMDARs) within LMAN during tutoring prevents normal song development, suggesting LMAN as a neural locus for vocal learning (Basham et al. 1996).

Postsynaptically “silent” synapses containing only functional NMDARs provide a substrate for some forms of neural plasticity (Durand et al. 1996; Liao et al. 1995; Wu et al. 1996). Due to the pure NMDAR constituency of silent synapses and the voltage-dependent Mg2+ block of NMDARs (Mayer et al. 1984; Monyer et al. 1994), transmitter release at silent synapses goes undetected by the postsynaptic membrane at resting, hyperpolarized potentials. Under depolarizing conditions that remove the Mg2+ blockade, transmitter release activates NMDARs resulting in detectable responses. Thus silent synapses can act as powerful nonlinear amplifiers because their activation requires highly correlated patterns of activity. In addition, activation of silent synapses using LTP induction protocols can result in rapid recruitment of AMPA receptor (AMPA) responses, effectively converting silent synapses to active synapses at resting, hyperpolarized potentials (Isaac et al. 1995; Liao et al. 1995). In this way, synapses with coincidence of pre- and postsynaptic activity are able to strengthen their connections and potentially sculpt neural circuitry that support learned behaviors.

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In vitro whole cell recordings were used to examine synaptic properties of LMAN neurons in response to stimulation of thalamic afferents during and after song learning. Neurons from juvenile birds exhibited a significantly higher incidence of silent synapses than did neurons of adults. However, the ratio of NMDAR to AMPAR-mediated current at DLM→LMAN synapses did not change over the course of song learning, and NMDAR-mediated currents accounted for 95% of the total charge transfer at depolarized holding potentials in both juveniles and adults. These results suggest a role for silent synapses in guiding one or more aspects of vocal learning during the sensitive period.

**METHODS**

**Slice preparation**

Brain slices were prepared from adult (mean = 96 days; range: 89–101) and juvenile (mean = 20 days, range: 19–22, or mean = 40 days, range: 38–42) male zebra finches raised in our breeding colony. Recordings of 19 cells from 16 20-day birds, 10 cells from 8 40-day birds, and 15 cells from 12 adults were included in this study. Birds were anesthetized with 0.05 ml of the barbiturate anesthetic Equithesin (a mixture of pentobarbital and chloral hydrate) and then rapidly decapitated in accordance with protocols approved by the Animal Care and Use Committee at the University of Southern California. Brains were dissected out into an ice-cold, oxygenated (95% O₂–5% CO₂) mixture of artificial cerebrospinal fluid (ACSF) and sucrose solution (50:50). A razor blade was used to block the lateral pole of one hemisphere of the brain, which was then glued with cyanoacrylate to a cutting stage and sliced sagittally in ice-cold oxygenated ACSF/sucrose solution at a thickness of 400 μm using a vibratome. Slices containing LMAN were placed in a holding chamber submerged in oxygenated ACSF that had been warmed to 37°C and then transferred to room temperature just before the slices were added. Slices were allowed to recover in the holding chamber for 1 h and equilibrate to room temperature before recordings were made. Sucrose solution consisted of (in mM) 248 sucrose, 5 KCl, 28 NaHCO₃, 10 glucose, 1.3 MgSO₄·7H₂O, and 1.26 NaH₂PO₄·H₂O. Standard ACSF consisted of (in mM) 125 NaCl, 25 NaHCO₃, 1.27 NaH₂PO₄·H₂O, 2.5 KCl, 1.2 MgSO₄·7H₂O, 2 CaCl₂, and 25 glucose and adjusted with sucrose to a final osmolality of 350 mosM.

Initial attempts to obtain healthy tissue led to the discovery that zebra finch plasma osmolarity is higher than the osmolarity of most standard extracellular ACSF solutions. Using a vapor pressure osmometer (Wescor) the average zebra finch plasma osmolarity in this study measured 350 ± 0.9 (SE) mosM. Plasma osmolarity of juveniles (342 ± 2.8; n = 9) was significantly lower than that of adults (356 ± 2.6; n = 10; t₁₇ = 3.73, P = 0.002). Raising the osmolarity of our ACSF to 350 mosM with sucrose facilitated our ability to obtain healthy tissue.

**Electrophysiology**

All recordings were made in room temperature in oxygenated, high-divalent solution which consisted of standard ACSF with the addition of (in mM) 0.1 picrotoxin, 2.0 CaCl₂, and 1.2 MgSO₄·7H₂O. Glass electrodes (3–6 MΩ) were filled with (in mM) 120 CsGlu, 17.5 CsCl, 10 TEA-Cl, 10 HEPES, 5 QX-314, 2 Mg-ATP, and 0.3 GTP and adjusted to pH 7.3 with CsOH. In some cases 0.5% biocytin (Molecular Probes) was included in the internal solution. Lateral MAN consists of a core and surrounding shell region (Iyengar et al. 1999; Johnson and Bottjer 1992). Under low magnification (×4), we were able to distinguish between LMANcore and LMANshell. Recordings were made from cells in LMANcore and this was confirmed in a portion of cells that were filled with biocytin and counterstained with thionin to delineate LMANcore boundaries. At high magnification (×60) using DIC-IR optics, large (15–20 μm diam) projection neurons in LMANcore were distinguished from smaller interneurons and recorded from selectively.

Whole cell currents were recorded in voltage-clamp mode under visual guidance using an Axopatch 200B amplifier, sampled at 10 kHz and filtered at 1–2 kHz. Data were acquired and analyzed with pCLAMP software (Axon Instruments, Foster City, CA). The measured liquid junction potential of 12 mV was corrected for. Prior to compensation of series resistance and cellular capacitance, series resistance was measured from the current response to a 10-mV pulse and found not to differ between juveniles [14 ± 1.14 (SE) MΩ] and adults (18 ± 1.56 MΩ; P > 0.05). Cells with access resistance >30 MΩ or with changes in compensated transients of >25% were not included in analyses. Cells with input resistance <100 MΩ were considered unhealthy and weren’t included in analyses. The average synaptic reversal potential was 9 ± 3.0 mV across juvenile and adult cells (n = 11). A twisted, bipolar stimulating electrode (0.0035 in Teflon-coated silver wire; California Fine Wire, Grover City, CA) was placed caudal to Area X in the pathway of DLM axons that project to LMAN to generate EPSCs in LMAN cells. The stimulation frequency for all experiments was 0.2 Hz.

**Analysis of NMDAR-to-AMPAR ratios and NMDAR kinetics**

To examine the proportion of synaptic current carried by NMDA versus AMPA receptors in 20-day and adult birds, the relative NMDA:AMPAR (N:A) contribution to reversed EPSCs was calculated. The membrane potential was initially held at −75 mV, and the stimulation intensity was adjusted to elicit ~200 pA synaptic responses at a frequency of 0.2 Hz (with no synaptic failures). After collecting 20 trials at −75 mV, the holding potential was switched to +50 mV and stimulation was resumed at the same intensity and frequency. Isolation of reversed NMDAR- or AMPAR-mediated cur-
rents was obtained by application of selective antagonists of NMDARs, D,L-2-amino-5-phosphonovalerate (APV, Sigma; 100 µM) or AMPARs, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzox [1]quinoxaline-7-sulfonamide (NBQX, Sigma; 10 µM). A control baseline response at +50 mV was established followed by bath application of NBQX or DL-APV (cf. Stark and Perkel 1999). EPSC amplitude was measured by subtracting the peak value of the EPSC from the baseline value just prior to stimulus onset. The relative contribution of functional NMDARs to AMPARs was calculated as the ratio of the peak NMDAR- to AMPAR-mediated current at +50 mV for each cell. The peak NMDAR-mediated EPSC was measured directly with NBQX in the bath while the peak AMPAR-mediated EPSC was determined by subtracting the EPSC recorded in the presence of NBQX from the baseline EPSC. Likewise, with APV in the bath, the peak AMPAR-mediated response was measured directly while the peak NMDAR-mediated current was obtained by subtracting the EPSC recorded in the presence of APV from the baseline EPSC. Ten to 20 trials for each cell were averaged for analysis.

In 6 of 12 cells, NBQX produced a partial blockade of the later component of the synaptic response, indicating a nonspecific blockade of NMDARs (cf. Stark and Perkel 1999). In these cases, the EPSC recorded in the presence of NBQX was scaled up by the proportion of the response that was blocked at a point ~50 ms after the stimulus artifact. AMPAR-mediated currents had decayed to <5% of their peak value by 50 ms after the stimulus artifact. In such cases where the later, NMDAR-mediated component of the reversed EPSC was partially blocked by NBQX the peak AMPAR response was calculated in two ways; once by subtracting the scaled NBQX response from the control trace at +50 mV and again by subtracting the unscaled NBQX responses from the control trace at +50 mV. The resulting values for N:A ratios did not differ for these two methods and did not affect comparisons between juveniles and adults (see RESULTS).

Isolated NMDAR decay kinetics were quantified by measuring e-fold decay and by exponential fitting. The e-fold decay of reversed EPSCs was measured as the time required for currents to decay to 1/e of the peak current amplitude (Crair and Malenka 1995; Livingston and Mooney 1997). Isolated NMDAR decay kinetics were also quantified by fitting with a two-termed exponential (\( y = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \)) constrained to baseline. This equation provided us with \( \tau_1 \) and \( \tau_2 \), slow and fast time constants, and allowed us to calculate the proportion of the fit attributed to the slow component \( [A_1/(A_1 + A_2)] \times 100 \). A weighted time constant \( \tau_w = \tau_1 * A_1/(A_1 + A_2) + \tau_2 * A_2/(A_1 + A_2) \) was also calculated as a measure of overall decay. NMDAR EPSC rise times were measured as the time between 10 and 90% of the peak amplitude. All values are reported as mean ± SD unless otherwise stated.

Analysis of silent synapses

To examine the incidence of silent synapses, low stimulation intensities were used to examine synaptic failure rates in slices from 20-day, 40-day, and adult birds. Stimulation intensity was adjusted to low levels that produced detectable responses with a projected 50% failure rate (0.08–1.4 mA) while holding the cell at −70 to −75 mV. Epochs of 15–60 trials (55 trials in most cases) were recorded at −75 mV, and then the holding potential was changed to +50 mV and the stimulation protocol was repeated. An important aspect of this experiment is that the same proportion of inputs is being stimulated at each holding potential. As a test of this assumption, the failure rate for the first half versus the second half of all trials at each holding potential was calculated (e.g., trials 1–27 vs. trials 28–55 for a block of 55 trials at −70 mV). If the failure rate during the second half changed by >15% of the first half, that cell was discarded.

The synaptic failure rate was estimated by visual inspection using four criteria (onset latency, onset kinetics, amplitude relative to noise, and decay kinetics) to unambiguously discriminate synaptic failures from successes. A synaptic response had to meet both of the first two criteria and a total of at least three of four criteria to be judged as a success. Thus a cell that met all four criteria had a monosynaptic onset latency (~1.5–4.5 ms), a rapid rise in slope of onset, an amplitude that was unambiguously different from baseline noise, and appropriate decay kinetics (rapid at −75 mV and slow at +50 mV). Use of these criteria avoided using data from any cells that did not have a monosynaptic response; in addition cells that met all criteria but that included a delayed disynaptic response were not included. All judgments of synaptic failures were made by an observer with no knowledge of the age of the bird. The observer made two independent judgments of synaptic successes versus failures for a subset of 10 cells separated by ≥1 min, and the agreement in average measurements for these 10 cells was >95% at both −75 and +50 mV.

To test the degree to which these subjective judgments discriminated unambiguously between failures and successes, the distribution of all EPSC amplitudes along with the corresponding distribution of noise amplitudes (at each holding potential) was plotted for each cell that was characterized as having silent synapses (see following text, Fig. 6). EPSC amplitudes at −70 mV were calculated as the mean current during a 2-ms window during the peak EPSC minus the mean current during a similar window just prior to the stimulus artifact. EPSC amplitudes at +50 mV were measured the same way except that a 5-ms window encompassing the peak of the EPSC was used. Noise amplitudes were measured as the difference between the mean current during the 2-ms baseline window just prior to stimulus artifact and a second 2-ms window 10 ms prior to the stimulus artifact. Comparisons between the degree of overlap between successes versus failures (as judged by an independent observer) with the noise distribution for each cell revealed that >95% of the trials judged as failures fell within the range of noise, whereas trials judged as successes fell outside the noise distribution. In those cases for which single trials judged as failures did not overlap with the distribution of noise (or for which single trials judged as events did overlap with the distribution of noise), in almost every case, it was clear from the waveform that the original judgment was nevertheless correct.

RESULTS

NMDA and AMPA currents at DLM-LMAN synapses

The relative contribution of NMDA versus AMPA receptor-mediated currents at DLM→LMAN synapses was examined during and after song learning using selective pharmacological blockers of glutamate receptor subtypes. The top panels of Fig. 2 show that blockade of NMDARs with APV reduced the peak amplitude of reversed EPSCs substantially and by equal proportions in both juvenile and adult LMAN neurons. A relatively small, fast EPSC persisted, indicating the presence of a small AMPAR-mediated component at DLM-LMAN synapses. In accord with this pattern, blockade of AMPAR-mediated currents with NBQX resulted in only a modest reduction of the EPSCs of juvenile and adult cells alike, revealing a large and characteristically slow NMDAR-medi-
Developmental acceleration of NMDAR EPSC kinetics

NMDAR kinetics were investigated by examining e-fold decay and two-term exponential fits of reversed NMDAR-mediated currents in 20-day juvenile versus adult birds. An overall acceleration of NMDAR-mediated currents occurred with development. Figure 3 shows representative traces of reversed NMDAR- and AMPAR-mediated EPSCs from juvenile and adult cells illustrating the slower decay time of the NMDAR component in juvenile versus adult LMAN neurons as has been reported previously (cf. Livingston and Mooney 1997; White et al. 1999).

To quantify NMDAR kinetics, the decay phase of reversed NMDAR-mediated currents was fit with a two-term exponential equation to yield time constants for the fast and slow components of the NMDAR response, from which we derived a weighted tau for each cell (τw; see Methods). Comparisons of τw revealed that NMDAR EPSCs in juvenile LMAN cells decay significantly more slowly than in adult cells (180.14 ± 49.69 vs. 62.82 ± 14.25, U = 0, P = 0.004; Fig. 4). NMDAR EPSC decay kinetics were also measured as the time required for EPSCs to decay to 1/e of their peak value. Comparisons of the e-fold decay also indicated a significantly slower decay time of NMDAR EPSCs in LMAN cells of juvenile zebra finches (129.13 ± 20.89) than in adult cells (50.66 ± 9.13, U = 0, P = 0.004; Fig. 4). The changes we observed in

![FIG. 2. Representative traces of reversed excitatory postsynaptic currents (EPSCs) in LMAN neurons elicited by DLM afferent stimulation at a holding potential of +50 mV show a substantial contribution of NMDARs in both juvenile and adult cells. Furthermore, the relative contribution of NMDAR vs. AMPAR receptors does not change during the sensitive period for song learning. Top: reversed EPSCs from juvenile (20-day) and adult cells in normal artificial cerebrospinal fluid (ACSF; control) and with APV in the bath to block the NMDAR-mediated component. Application of APV reduced the EPSC peak substantially, revealing a fast AMPAR-mediated component. Bottom: the addition of NBQX to block the AMPAR-mediated component produced a small decrease in the control EPSC in both juveniles and adults as would be expected if NMDARs were the principal conductors of synaptic current at depolarized potentials. The faster decay kinetics of adult vs. juvenile NMDAR currents can be seen by comparing the left and right bottom.](http://jn.physiology.org/)

![FIG. 3. Representative traces of reversed EPSCs at +50 mV showing NMDAR- and AMPAR-mediated currents in 20-day (top) and adult (bottom) LMAN neurons. The NMDAR:AMPAR (N:A) ratio for the juvenile cell was 4.26 and that for the adult cell was 2.90. The duration of the isolated NMDAR-mediated component decreased substantially between 20-day and adult birds, indicating faster kinetics in adult neurons at DLM→LMAN synapses.](http://jn.physiology.org/)
NMDAR EPSC kinetics are not ancillary to changes in input resistance of LMAN cells (321 ± 25 vs. 306 ± 61 MΩ in 20-day vs. adult cells, respectively), indicating that changes in intrinsic membrane properties are not responsible for the differences we observed in kinetics.

We calculated the values of the individual fast and slow time constants as well as the percent contribution of the slow component to the total NMDAR current to determine the source of the difference in decay kinetics between juveniles and adults. The slow time constant was significantly longer in 20-day juveniles compared with adults (308.41 ± 131.60 vs. 109.19 ± 42.64, U = 1, P = 0.008). The value of the fast time constant also declined significantly between juvenile and adult LMAN cells (52.98 ± 14.85 vs. 27.96 ± 11.28, U = 2, P = 0.02). We did not observe a decrease in the percent contribution of the slow component to the total NMDAR current between juveniles and adults (53.6 ± 13.0 vs. 47.4 ± 16.8, U = 4, P = 0.05). Thus decreases in both fast and slow decay time constants contributed to the decreased duration of NMDAR-mediated currents at DLM-LMAN synapses.

To complete our quantitative analysis of NMDAR EPSC kinetics, we compared the rise times of juvenile and adult LMAN neurons, which revealed that isolated NMDAR EPSCs tended to rise at a slower rate in juveniles than in adults (5.47 ± 1.19 vs. 3.90 ± 1.34 ms, U = 5.5, P = 0.052). However, the mean peak amplitude of NMDAR EPSCs was slightly larger in juveniles than in adults (321.7 ± 150.4 vs. 247.6 ± 199.5, U = 12, P = 0.33). When the rise time was normalized to peak amplitude, the differences between juveniles and adults was not significant (P = 0.50).

Silent synapses in the song control system

Synaptic failure rates were examined at depolarized versus hyperpolarized potentials at DLM-LMAN synapses to assess the incidence of silent synapses in birds at different stages of vocal development: 20 days, 40 days, and adult (>90 days). Figure 5 shows examples of synaptic events and failures recorded at −75 and +50 mV, respectively, in a cell from a 20-day bird. The failure rate for this cell was 84% at −75 mV but decreased to 31% at +50 mV, indicating the presence of a population of NMDAR-only synapses that are nonfunctional at hyperpolarized potentials but are unmasked at depolarized potentials.

Figure 6 shows a plot of EPSC amplitude versus trial number at each holding potential for a cell from a 40-day bird. The failure rate for this cell was 91% at −75 mV, indicated by the high incidence of events of zero amplitude, and 0% at +50 mV (no events at 0 amplitude). The mean EPSC amplitude for synaptic successes was smaller at −75 mV (~80 pA) than at +50 mV (~60 pA; Fig. 6, top right). A substantial increase in the amplitude of synaptic successes at +50 mV relative to −75 mV was observed in the majority of cells, and presumably reflects the preponderance of NMDAR-mediated currents at these synapses (i.e., high N:ΔA ratios), as well as a large proportion of silent synapses in individual cells. Figure 6, bottom, shows the distribution of EPSC amplitudes for all trials along with the corresponding distribution of noise amplitudes at each holding potential (see METHODS). The distribution of trials judged as synaptic failures at −75 mV overlapped the amplitude distribution of the recording noise, whereas trials judged as synaptic successes (n = 5) fell outside of the noise distribution. Thus an objective index of successes versus failures (as judged by overlap with noise) corresponded very closely to judgments of an independent observer, demonstrating that failures and successes could be discriminated unambiguously.

Figure 7 shows a summary of the differences in synaptic failure rates at hyperpolarized versus depolarized potentials for all cells as a function of age. In 20-day birds, the average failure rate was 79 versus 57% at hyperpolarized and depolarized potentials, respectively (mean difference score = 22.0 ± 31.7). In 40-day birds, the average failure rate was 73% at hyperpolarized potentials and 42% at depolarized potentials (mean difference score = 30.9 ± 32.1). In adults, the average failure rate was 66% at hyperpolarized potentials versus 49% at depolarized potentials (mean difference score = 16.2 ± 35.5), indicating a decreased incidence of NMDAR-only synapses in adult neurons. Wilcoxon sign tests revealed a significant difference between the proportion of synaptic failures at depolarized versus hyperpolarized potentials in both 20- and 40-day

**FIG. 4.** NMDAR decay kinetics are slower in juvenile (○) than in adult (●) cells (mean ± SEMs). Measurements of NMDAR decay kinetics at DLM–LMAN synapses using a weighted Tau value or e-fold decay (see text) indicated a greater than 2-fold decrease in the decay time of NMDAR-mediated currents between juveniles and adults.

**FIG. 5.** Different failure rates of DLM–LMAN EPSCs at hyperpolarized vs. depolarized membrane potentials indicate the existence of silent synapses in a cell from a 20-day bird. Left: individual traces show 4 synaptic failures and 1 evoked response (2nd trace from bottom) at −75 mV. Right: individual traces show 1 synaptic failure (top) and 4 evoked responses at +50 mV. The failure rate for this cell was 84% at hyperpolarized potentials and 31% at depolarized potentials. A block of 55 trials was given at each holding potential, and traces above were selected so as to reflect the percentage of synaptic failures from each block of trials. At a holding potential of +50 mV, EPSCs are dominated by the NMDAR-mediated component, which is blocked by Mg2+ at −75 mV holding potential, leading to purely AMPAR-mediated EPSCs.
birds (T = 16, P < 0.05 at 20 days; T = 3.0, P < 0.02 at 40 days), but not in adults (T = 11, P > 0.05).

We tested the difference in synaptic failure rate at −75 versus +50 mV for individual cells in each age group by calculating z scores. Individual cells with z values >1.5 were arbitrarily designated as having a substantial number of silent synapses. According to this criterion, 7 of 13 cells from 20-day birds had a decreased failure rate at depolarized potentials, 8 of 10 cells from 40-day birds had fewer failures at depolarized potentials, and 3 of 9 cells from adult birds had fewer failures at depolarized potentials. This analysis also indicates that LMAN neurons in 20- and 40-day birds have a higher incidence of silent synapses than do those from adult birds.

One alternative explanation of silent synapses has been the possibility that glutamate spillover from adjacent synapses produces a low concentration of glutamate in the cleft of putative silent synapses, a situation that may selectively activate NMDARs due to their high affinity for glutamate (Kullmann et al. 1992). Because we observed a trend toward a developmental decrease in the mean rise time of NMDAR EPSCs, the rise time of which is strongly dependent on glutamate concentration (Clements and Westbrook 1991), it might be argued that our results are consistent with such an explanation. To evaluate this idea, the rise times of NMDA EPSCs for both juvenile and adult cells (at +50 mV) were compared with the difference in failure rate between de- and hyperpolarized potentials (i.e., large difference scores correspond to cells with a large proportion of silent synapses). These two variables were completely independent for both juveniles and adults. For example, the mean rise time for 20-day cells with few or no silent synapses was 7.09 ± 1.25 ms (n = 6, z scores <1.5), and was 7.92 ± 3.0 ms for cells with the highest incidence of silent synapses (n = 7, z scores >1.5). The peak amplitude of EPSCs for cells with few versus many silent synapses was 58.5 and 59.8 pA, respectively. The absence of systematic variation in rise times of cells with many versus few silent synapses indicates that glutamate concentration was comparable at both functional and silent synapses in this situation.

DISCUSSION

The present study characterizes transmission at DLM→LMAN synapses in juvenile zebra finches during the sensitive period for vocal learning and in adult birds that have completed the acquisition of a stereotyped vocal pattern. The data show that the relative contribution of NMDAR- and AMPAR-mediated currents is constant across song learning and that the NMDAR component constitutes the vast majority of the total synaptic current at DLM→LMAN synapses at depolarized potentials. The results also indicated that kinetics of NMDAR-mediated
responses speed up during development, as reported previously (Livingston and Mooney 1997; White et al. 1999). The proportion of total charge transferred via AMPA receptors at depolarized potentials is <5% at both ages, suggesting that a significant portion of excitation may normally be mediated through NMDA receptors in this thalamo-cortical pathway in vivo (cf. Daw et al. 1993; Di Prisco et al. 1997; Nelson and Sur 1992). Although a previous study reported that NMDAR blockade in brain slices produced only a modest decrement of evoked responses at DLM→LMAN synapses (Boettiger and Doupe 1998), the use of in vitro current-clamp recordings at hyperpolarized membrane potentials in that study may have resulted in an underestimate of the potential contribution of NMDA receptors.

The present results also demonstrate fewer synaptic failures at depolarized than at hyperpolarized potentials in 20- and 40-day cells compared with adult cells, indicating that LMAN neurons in juvenile birds during the sensitive period for vocal learning have a high incidence of NMDAR-only synapses that are postsynaptically silent at hyperpolarized potentials. Although alternative mechanisms generating silent synapses such as glutamate spillover from neighboring terminals and changes in the dynamics of vesicular fusion pores have been proposed (Choi et al. 2000; Kullmann et al. 1996), silent synapses have been demonstrated conclusively by both electrophysiological techniques (e.g., Montgomery et al. 2001) and by immunocytochemical studies showing the existence of pure NMDAR synapses (Gomperts et al. 1998; Liao et al. 1999; Petralia et al. 1999). Our demonstration that the rise time of NMDAR EPSC’s does not vary between cells with a high versus low incidence of silent synapses is inconsistent with the idea that glutamate concentration is lower in the synaptic cleft of silent synapses (cf. Rumpel et al. 1998).

Relative contribution of NMDARs and AMPARs

Significant declines in NMDAR-mediated synaptic transmission relative to the AMPAR component occur commonly during development (Bottjer 2002; Crain and Malenka 1995; Hestrin 1992; Stark and Perkel 1999; Wu et al. 1996 for review). It is therefore unusual that the N:A ratio at DLM→LMAN synapses does not change over the course of song development. Furthermore, the constancy of the N:A ratio between juveniles and adults is maintained despite a significantly higher proportion of silent synapses in juvenile cells.

N:A ratios were estimated using the ratio of peak synaptic currents with and without selective glutamate receptor antagonists. If individual receptor conductances do not differ between juveniles and adults, then EPSC peak amplitudes should be directly proportional to the number of glutamate receptors. Hence, constant N:A ratios would indicate that relative numbers of functional NMDA and AMPA receptors are present in juveniles and adults over the population of DLM→LMAN synapses. This conclusion appears to conflict with the finding of Aamodt et al. (1992, 1995) that MK-801 binding declines substantially between 20 days and adulthood in LMAN. However, the methods used by Aamodt et al. do not discriminate between declines in NMDAR abundance at functional synapses versus extrasynaptic NMDARs. A loss of extrasynaptic NMDARs during neural maturation could account for the difference Aamodt et al. observed. In addition, there is a large population of intrinsic LMAN synapses (Boettiger and Doupe 1998), at which NMDARs could decrease independently of DLM→LMAN synapses. Furthermore, there are no data that address AMPAR abundance in LMAN that could covary with NMDAR abundance, leading to a constant N:A ratio across development (cf. Watt et al. 2000).

![Graph showing synaptic failure rates at holding potentials of -75 vs. +50 mV in individual LMAN neurons for 20-day, 40-day, and adult (95-day) birds. Thick black lines depict mean percent synaptic failures at each membrane potential. The mean difference in failure rate between holding potentials was 22.0 at 20 days, 30.9 at 40 days, and 16.2 in adults; the difference was significant in both groups of juvenile birds, but not in adults, indicating a substantial presence of postsynaptically silent DLM→LMAN synapses at least up until 40 days of age during the period for vocal learning. * P < 0.05; ** P < 0.02.](http://jn.physiology.org/issue/article-pdf/94/8/3704/3692935/3704.Bottjer.pdf)
Assuming that the N:A ratio does reflect a constant ratio of N:A receptors, then the increased incidence of silent synapses in juvenile birds could be explained by a differential distribution of glutamate receptor subtypes at individual synapses in juvenile versus adult cells. For example, cells from juvenile birds could express relatively more AMPARs at synapses where AMPARs are present. This scenario is consistent with patterns of cellular homeostasis that maintain an optimal level of excitatory activity throughout development (Desai et al. 2002; Turrigiano et al. 1998; Watt et al. 2000). As conductance at silent synapses is increased through the addition of AMPARs, cells would experience an increase in levels of activity and compensate by lowering the "gain" at individual synapses with high AMPAR expression. Interestingly, Watt et al. (2000) showed that N:A ratios in cultured cortical neurons remained stable after activity-dependent synaptic scaling and demonstrated directly that both NMDAR- and AMPAR-mediated currents were scaled up and down proportionally through changes in the number of both types of receptors (cf. Carroll and Zukan 2002; Perez-Otano and Ehlers 2005; Song and Huganir 2002). Thus recent data encourage the notion that both NMDA and AMPA receptors are highly regulated, meaning that dynamic changes in each could preserve N:A ratios at DLM→LMAN synapses.

NMDAR kinetics

The current results support previous findings that NMDAR-mediated EPSCs at DLM→LMAN synapses decay more quickly in adult zebra finches compared with juveniles, although developmental declines in both fast and slow components of NMDAR decay kinetics rather than in the slow component only were observed in the present study (cf. Livingston and Mooney 1997; White et al. 1999). Developmental acceleration of NMDAR decay kinetics is attributable to changes in the subunit composition of NMDARs (Flint et al. 1997; Monyer et al. 1994) and the acceleration of NMDAR decay kinetics observed here is consistent with a developmental decline of NR2B mRNA abundance and ifenprodil binding in LMAN (Basham et al. 1999).

Developmental acceleration of NMDAR decay kinetics has been shown to occur at the onset or during early stages of sensitive periods (e.g., Livingston and Mooney 1997; Roberts and Ramoa 1999), and juvenile songbirds can learn new syllables from a tutor after the transition to faster kinetics (Livingston et al. 2000), suggesting that faster NMDAR currents may help to trigger the onset of sensitive periods or be conducive to learning during the sensitive period (but cf. Barth and Malenka 2001; Bottjer 2002; Lu et al. 2001). There is no evidence from any system that faster NMDAR responses are importantly involved in regulating the closure of sensitive period plasticity.

Decline in silent synapses during vocal development

We observed a significant developmental decline between 40 days to adulthood in silent synapses in LMAN, which is part of a basal ganglia-forebrain circuit necessary for song learning in zebra finches (Bottjer 2004). Developmental decreases in silent synapses have also been observed during sensitive periods in somatosensory and visual systems (Chen and Regehr 2000; Isaac et al. 1997; Itami et al. 2003; Rumpel et al. 2004). The decline in DLM→LMAN silent synapses corresponds to functional changes within LMAN that occur as juvenile birds learn their vocalizations. For example, the ability of LMAN lesions to disrupt song production decreases at ~60–70 days of age (Bottjer and Arnold 1986; Bottjer et al. 1984), around the time that birds are beginning to produce stable song patterns and auditory responses of LMAN neurons are becoming selectively tuned to playback of each bird's own song (Rosen and Mooney 2000; Solis and Doupe 1999). Thus the increased incidence of silent synapses in 20- and 40-day birds occurs as birds are learning the auditory and motor-articulatory patterns of song syllables, suggesting that silent synapses underlie one or more aspects of vocal learning. This hypothesis can be tested in future studies that manipulate auditory and social experience so as to modulate the timing of the sensitive period for vocal learning and examine potential effects on the expression of silent synapses.

Substantial morphological changes in DLM axon morphology and LMAN spine abundance also occur during song learning and development. Axon arbors of DLM neurons in LMAN undergo substantial retraction during song development (Iyengar and Bottjer 2002a). Regression of DLM arbors is mirrored by a decline in spine frequency of LMAN dendrites, and the total number and density of synapses within LMAN decrease to approximately half their original levels during the course of song learning (Nixdorf-Bergweiler 2001; Nixdorf-Bergweiler et al. 1995). Raising juvenile birds in acoustic isolation prevents or delays the normative decrease in spine density (Wallhausen-Franke et al. 1995), suggesting that these morphological changes may reflect the establishment of precise synaptic connections that encode learned vocal patterns (cf. Iyengar and Bottjer 2002a).

Because NMDAR-mediated synaptic activity is thought to contribute to stabilization of activated synapses and branch maintenance versus retraction (Cline and Constantine-Paton 1990; O’Rourke et al. 1994), silent synapses may contribute to the overall regression of axonal and dendritic arbors in the DLM→LMAN pathway by contributing to stabilization only at synapses that are activated by highly correlated patterns of song-related activity. For example, NMDARs at silent synapses of LMAN neurons could detect correlated activity of auditory and motor inputs that correspond to the acoustic structure of tutor song and thereby reinforce specifically activated synapses (cf. Cline et al. 1987; Feldman et al. 1996; Hickmott and Constantine-Paton 1997; Lu and Constantine-Paton 2004; Rabacchi et al. 1992).

The finding that the density of synapses decreases substantially in LMAN during development (Nixdorf-Bergweiler 2001) indicates that both NMDA and AMPA receptors may decline. Interestingly, DLM→LMAN synapses show long-term depression (Boettiger and Doupe 1998), suggesting that LTD may contribute to synaptic elimination. If so, a relatively greater loss of silent synapses could contribute to the preservation of constant N:A ratios (see preceding text). Only those silent synapses that preferentially transmit highly correlated song-related inputs that match tutor song may be “protected” from LTD and retraction. Thus a large population of silent synapses during early stages of song learning could serve as a reservoir of structural plasticity from which experience-dependent activation of NMDARs couldselect (cf. Chklovskii et al. 1997).
2004; Marie et al. 2005). Such refinement of synaptic connectivity may represent an important neural signature of learning; remodeling within this pathway may be necessary for the development of auditory selectivity in LMAN neurons and the establishment of stable vocal patterns. Recent work showing that activation of silent synapses can influence patterns of connectivity in neuronal populations is consistent with this idea (Nakayama et al. 2005; Voigt et al. 2005). Furthermore, a decrease in the incidence of silent synapses as vocal learning progresses may reflect one aspect of neuronal maturation that acts to limit plasticity and curtail the sensitive period for song learning.

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References


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