Published in final edited form as:

Nat Neurosci. 2015 September ; 18(9): 1272–1280. doi:10.1038/nn.4078.

Naturalistic stimulation drives opposing heterosynaptic plasticity at two inputs to songbird cortex

W. Hamish Mehaffey and Allison J. Doupe

Center for Integrative Neuroscience, University of California San Francisco, San Francisco, CA, 94143

Abstract

Songbirds learn precisely sequenced motor skills (songs) subserved by distinct brain areas, including the premotor cortical analogue HVC, essential for producing learned song, and a ‘cortical’-basal-ganglia loop required for song plasticity. Inputs from these nuclei converge in RA (Robust Nucleus of the Arcopallium), making it a likely locus for song learning. However, activity-dependent synaptic plasticity has never been described in either input. Here, using a slice preparation, we show that stimulation patterns based on singing-related activity could drive opposing changes in the strength of RA’s inputs: when one input was potentiated, the other was depressed, with the direction and magnitude of changes depending on the relative timing of stimulation of the inputs. Moreover, pharmacological manipulations that blocked synaptic plasticity *in vitro* also prevented reinforcement-driven changes to song *in vivo*. Together, these findings highlight the importance of precise timing in basal ganglia–motor cortical interactions subserving adaptive motor skills.

Keywords

songbird; basal ganglia; LTP; LTD; heterosynaptic; latency

Introduction

The song system has provided numerous insights into the neural basis of complex learned motor skills, especially learned vocalizations. As juveniles, zebra finches (*Taeniopygia guttata*) learn from an adult tutor to produce a precise song consisting of a number of distinct elements (‘syllables’) organized into repeating sequences (‘motifs’). Once adult, birds sing stable song, but this song remains modifiable in response to altered auditory feedback^{1–6}.

Well-defined, specialized brain nuclei underly song learning and production, greatly simplifying the study of song production. In particular, the song ‘motor’ pathway (Fig. 1a) includes a premotor cortical analogue, HVC, which sends inputs to a motor cortical analogue (the robust nucleus of the arcopallium, RA). RA in turn drives brainstem nuclei coordinating vocal and respiratory musculature. HVC and its connections to RA are

essential for singing learned song throughout life, suggesting that this circuitry contains key elements of the learned motor program for song^{7,8}. RA motor neurons also receive a second major input from the prefrontal cortical-like nucleus LMAN (lateral nucleus of the medial nidopallium), the outflow of a basal ganglia-thalamo-cortical circuit specialized for song (the anterior forebrain pathway, AFP; Fig. 1a). The AFP originates from a population of HVC neurons distinct from those that project to RA, and although not essential for learned song production in adults, the AFP has a well-established role in song plasticity in both juvenile and adult birds. In early stages of song learning, lesions of LMAN prematurely crystallize song, preventing normal song acquisition^{9,10}. In older birds singing stable song, ablation or inactivation of LMAN does not dramatically affect song, but reduces vocal variability and prevents vocal plasticity^{2,10–12}. These findings suggest a shift in the influence between the two major pathways driving activity in RA, allowing their balance to shape the production of learned song. As RA activity is correlated directly with multiple song features¹³, it has been widely assumed that the summation of HVC and LMAN inputs driving RA is major contributor to learned aspects of song, and that in adult birds, increases or decreases in the strengths of inputs to RA from HVC and LMAN may account for song plasticity. Indeed, when HVC inputs are weakened, LMAN activity becomes more influential and disrupts song significantly^{7,14}. Taken together, these findings point to RA and the interaction of its two major inputs as a nexus for song learning, suggesting a model in which aspects of learned song are stored in HVC-RA synapses but require LMAN-RA synapses to change.

Despite this wealth of knowledge about the roles of specific nuclei in song production and learning, surprisingly little is known about how song changes are reflected in synaptic dynamics. Although there is anatomical evidence of a developmental shift in synaptic innervation of RA from LMAN to HVC and concomitant changes in synaptic strength^{15–17}, neither activity dependent synaptic potentiation nor depression has been described at either of these synapses.

We examined activity dependent synaptic plasticity *in vitro* in slices from late juvenile–adult birds. We stimulated LMAN and HVC inputs concurrently using patterns based on the high frequency bursts that occur during singing *in vivo*^{7,18,19}. We found that such naturalistic stimulation caused changes in synaptic strength at both input pathways, and pharmacological manipulations that blocked synaptic plasticity in the slice also prevented behavioral plasticity during adult reinforcement learning. The plasticity we observed has unique characteristics. The co-activated pathways changed in opposing directions, representing a shift in the balance of influence between the two circuits. Moreover, the direction of the changes in synaptic strength depended on the relative timing between the two inputs, suggesting that the timing of information coming through cortical-basal ganglia circuitry contains important signals for shaping motor learning.

Results

We prepared coronal slices (Fig. 1b) from birds in the late juvenile phase of sensorimotor learning (70–100 d) or older (including greater than 1 year old). We then made whole-cell patch clamp recordings from neurons within RA, whose borders are clearly identifiable

under transillumination (Fig. 1c). Neurons were visible within RA and could be patched using conventional DIC-IR (Fig. 1d), and we recorded from RA projection neurons (PNs), which are characterized by their relatively slow firing rates in response to depolarizing current injection, their sag response to hyperpolarizing current pulses, their spontaneous pacemaking, and/or their low spontaneous EPSC rates²⁰. In the coronal slice, LMAN and HVC inputs enter RA approximately orthogonally and thus can be stimulated separately^{20–22} (Fig. 1b), allowing specific activation of one or both pathways. When we tested inputs from these two pathways, we confirmed that synaptic events evoked from HVC afferents at hyperpolarized potentials (–75 to –80 mV) were weakly sensitive to the n-methyl-d-aspartate (NMDA) receptor antagonist 2-amino-5-phosphonopentanoic acid (APV)^{21,22} (Fig. 1e, $84 \pm 6\%$ of control, $t(4)=-2.70$, $p=0.054$, $n=5$ cells from 4 birds, paired t-test). Although the postsynaptic response to LMAN stimulation is largely mediated by NMDA receptor activation^{21,22}, we were able to observe modest, but reliable EPSCs after stimulation of the LMAN fiber tract, even at hyperpolarized potentials (between –75 to –80 mV, Supplementary Figure 1). Such incomplete hyperpolarization block could reflect the inclusion of Mg^{2+} insensitive NMDAR subunits²³. However, the events evoked by stimulation of LMAN afferents at these potentials were largely blocked by APV (Fig. 1e, $6 \pm 2\%$ of control, $t(7)=-53.95$, $p=0.000000003$, $n=8$ cells from 6 birds, paired t-test), as has been previously described^{20–22}.

Plasticity in RA Requires Paired Burst Stimulation of HVC and LMAN axons

Because neurons in both HVC and LMAN typically burst at very high frequencies in awake, singing birds, we first examined the synaptic currents evoked in RA by trains of high frequency stimulation of HVC and LMAN axons. We chose 4 pulses at 800Hz for HVC stimulation¹⁸, and 8 pulses at 500 Hz for LMAN stimulation based on reported literature^{7,19}. We found that the amplitudes of the compound EPSCs resulting from burst stimulation of either HVC or LMAN axons were significantly greater than those resulting from single stimuli (Fig. 2a, $107 \pm 12\%$ increase in HVC peak synaptic amplitude, $t(12)=4.96$, $p=0.0003$, $n=13$ cells from 9 birds; $188 \pm 38\%$ increase in LMAN peak synaptic amplitude, $t(12)=9.17$, $p=0.0000009$, $n=13$ cells from 9 birds, paired t-tests). This summation suggests that the axons innervating RA PNs from HVC and LMAN are capable of following high frequency stimulus trains, and can translate these high rates of firing into increased release of glutamate observable postsynaptically.

The two inputs to RA both originate in HVC (Fig. 1a). HVC sends a direct, monosynaptic projection to RA. In addition, a separate population of HVC neurons sends a polysynaptic projection via the song cortical-basal ganglia loop, whose outflow gives rise to the LMAN to RA projection. Thus, during singing, RA is likely to receive activity coming from LMAN in close temporal proximity to activity coming from HVC. Because HVC-to-RA activity is required for structured song and LMAN firing is required for song plasticity, plasticity may require co-activation of the two pathways. To test this hypothesis, we stimulated both pathways using high frequency naturalistic burst rates while holding RA PNs in voltage clamp (Fig. 2b, stimulation was paired eight times at 0.5 Hz). After this pairing, we found that the amplitudes of synaptic responses of both input pathways to single test stimuli were altered. LMAN responses following paired stimulation were reliably stronger (Fig. 2d, e,

127% $\pm$ 10% of control, $t(6)=2.86$, $p=0.035$, $n=7$ cells from 6 birds, paired t-test), while the average strength of the HVC synapses decreased in amplitude (Fig. 2c,e, 54% $\pm$ 16% of control, $t(6)=2.76$, $p=0.033$, $n=7$ cells from 7 slices from 6 birds, paired t-test). This change in synaptic strength could be observed for 30 minutes (Supplementary Figure 2). Although inhibition was not blocked in these experiments, in a separate set of experiments, blockade of inhibition did not alter the amplitude or timing of EPSCs, and did not prevent either HVC or LMAN inputs from changing strength (Supplementary Figure 3). Thus, inhibition does not appear to be required for the changes in synaptic strength that we observed.

In adult birds singing well-learned songs, LMAN activity contributes variability to learned features of song, but has little influence on the mean value of song features such as pitch¹¹. However, in a reinforcement learning paradigm in which birds learn to adjust their songs to avoid aversive auditory feedback, LMAN contributes an offset responsible for the early adaptive change during aversive reinforcement^{4,5,24}. An increase in the strength of appropriate LMAN synapses, concurrent with a decrease in HVC synaptic strength could, allow LMAN activity to drive early adaptive changes.

After simultaneous stimulation of HVC and LMAN, the paired pulse ratio (50 ms interval between pulses) of the HVC inputs increased (Fig. 2f, 23% $\pm$ 10% increase, $t(6)=-2.56$, $p=0.043$, $n=7$ cells from 7 slices, from 6 birds, paired t-test), but did not change for the LMAN inputs (Fig. 2f, 0.8% $\pm$ 4% increase, $t(6)=0.09$, $p=0.93$, $n=7$ cells, from 7 slices, from 6 birds, paired t-test). These results suggest a presynaptic locus for the change in HVC synaptic strength, and a postsynaptic locus for changes in LMAN synaptic strength. Furthermore, as these changes could be observed despite the cell being held between -70 and -80 mV during the induction protocol, postsynaptic spiking does not seem to be a necessary component of this plasticity.

Although we found lasting changes in synaptic strength after paired burst stimulation of HVC inputs and LMAN inputs, it was not yet clear whether activation of both pathways was required for plasticity, or whether one pathway was solely responsible for the changes observed in both pathways. Song plasticity is arrested in both juveniles and adults when LMAN is absent^{2,5,9,25,26}, so this plasticity could either depend solely on the activity of LMAN or on the coactivation of both pathways. We therefore tested whether independent burst stimulation of HVC or LMAN was capable of inducing lasting changes in synaptic strength. Burst stimulation of either HVC inputs alone or LMAN inputs alone produced no lasting changes in either the stimulated or unstimulated pathway. (Fig. 3a, HVC stimulation only: LMAN: 99 $\pm$ 0.1% of control, $t(2)=-0.19$, $p=0.87$, $n=3$ cells from 2 birds; HVC: 97 $\pm$ 2% of control, $t(5)=-2.06$, $p=0.09$, $n=6$ cells from 5 birds; Fig. 3b, LMAN stimulation only: LMAN: 97 $\pm$ 2% of control, $t(7)=-1.24$, $p=0.25$, $n=8$ cells from 6 birds; HVC: 94 $\pm$ 5% of control, $t(4)=-1.37$, $p=0.24$, $n=5$ cells from 3 birds, paired t-tests). Thus, under the conditions studied, co-activation of the two pathways was critical for the sustained changes in synaptic strength we observed previously.

In adults, singing-related activity of HVC neurons is dominated by sparse high frequency bursts^{18,27}, and prior work has suggested the burst firing in LMAN is important for song plasticity⁶. LMAN bursts occur during the conditions in which song plasticity has been

elicited^{4,19,24,28}, and manipulations that eliminate burst firing in LMAN also eliminate song plasticity⁶. However, LMAN neurons can switch between bursting and single spikes depending on behavioral context¹⁹, while HVC-X neurons do not switch firing modes²⁷. To examine whether LMAN bursts are required for synaptic plasticity *in vitro*, we paired burst stimulation of HVC axons with single stimulation of LMAN axons. Pairing bursts of activity in HVC axons with single stimulation of LMAN axons caused a transient increase in both HVC and LMAN inputs, but did not result in lasting changes in the strength of either pathway (Fig. 3c, HVC: $98 \pm 3\%$ of control, $t(3) = -0.72$, $p = 0.53$, LMAN: $97 \pm 4\%$ of control, $t(3) = -0.83$, $p = 0.47$, $n = 4$ cells from 4 slices from 4 birds, paired t-tests), demonstrating that burst stimulation of LMAN was required for changes in the strength of the two pathways. Further, the high frequency bursting observed *in vivo* seemed to be necessary, as stimulation of both pathways with slower stimulation frequencies (100Hz), failed to elicit long-lasting changes in synaptic strength (Fig. 3d, HVC: $99 \pm 10\%$ of control, $t(3) = -0.09$, $p = 0.93$, LMAN $107 \pm 8\%$ of control, $t(3) = 0.87$, $p = 0.45$, $n = 4$ cells from 2 birds, paired t-tests).

Latency Between Pathways Controls Strength and Valence of Plasticity

Many forms of plasticity depend on timing²⁹, and as birdsong is a carefully coordinated sequence of precisely timed motor gestures, such timing-dependent neural plasticity has been a key component of many models of song learning^{30,31}. We next examined whether altering the timing of the HVC stimulation relative to LMAN stimulation could influence the synaptic plasticity we observed. We applied HVC burst stimulation either before (negative lags, $t < 0$ ms) or after LMAN stimulation (positive lags, $t > 0$ ms), at separations of up to 500 ms (Fig. 4a). The resulting plasticity was bidirectional, with a striking dependence on latency between the two stimulations. Little change in the strength of either pathway was observed when LMAN stimulation led HVC stimulation by > 50 ms (Fig. 4b,e). However, in experiments where HVC and LMAN were activated near simultaneously (Fig. 4b,d), or where HVC stimulation led LMAN activation by 50 ms (Fig. 4b), the amplitude of HVC inputs to RA decreased, while the amplitude of the LMAN inputs increased. The magnitude of plasticity in both pathways decreased when HVC led LMAN by a brief time interval (~ 75 ms). Further increases in this interval (HVC leading LMAN by ~ 100 ms, Fig. 4b,c) reversed the direction of plasticity, such that HVC inputs increased in amplitude, while the amplitude of LMAN inputs to RA decreased in amplitude, (at -100 ms lag, HVC: $135 \pm 12\%$ of control, $t(4) = 2.91$, $p = 0.044$; LMAN: $46 \pm 16\%$ of control, $t(4) = -3.34$, $p = 0.029$, $n = 5$ cells from 5 birds). Finally, when HVC led LMAN by more than 150 ms, synaptic strengths were again unchanged (Fig. 4b). Thus, the relative timing of inputs to RA determines both the strength and direction of changes in synaptic inputs. Moreover, the magnitude of change in each pathway was negatively correlated with the magnitude of change in the other pathway ($R^2 = 0.318$, $p = 0.00000073$), suggesting an approximately homeostatic principle helping conserve excitatory drive to RA.

Pharmacology of Timing and Burst Dependent Plasticity in RA

We next focused on what mechanisms might underlie the changes in synaptic strength we observed. We began by examining the plasticity induced by simultaneous activation of the two input pathways (0 ms lag, Fig. 5a). As the response seen after LMAN stimulation is predominantly mediated by NMDA receptors^{21,22}, we first examined whether activation of

NMDA receptors was a necessary component for the changes in synaptic strength that we elicited. As APV blocked the synaptic currents evoked by LMAN stimulation, these currents could of course no longer be measured. However, in the presence of APV, paired simultaneous stimulation of LMAN and HVC resulted in a lasting depression of the HVC pathway, similar to that observed when the two pathways were stimulated in the absence of NMDA antagonists (Fig. 5b, $56 \pm 10\%$ of control, $t(3)=-4.28$, $p=0.023$, $n=4$ cells from 3 birds, paired t-test). This suggests that despite LMAN burst stimulation being a necessary component for the plasticity observed in the HVC-RA synapses (see Fig 2c,e, and 3a,c,d), activation of the postsynaptic NMDA receptors that mediate LMAN inputs does not appear to be required for the depression of HVC synaptic inputs that resulted from such paired stimulation.

As NMDA activity was not necessary for the plasticity we observed, we next examined the calcium dependence of the plasticity more generally. We stimulated both pathways after intracellular diffusion of the calcium chelator 1,2-bis(o-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA, 10 mM) in order to block changes in intracellular calcium in the postsynaptic cells. Under these conditions, synaptic strength in either pathway showed no lasting changes, suggesting that a rise in intracellular calcium is a key component in the bidirectional heterosynaptic plasticity we observed (Fig. 5c, calcium (HVC: $98 \pm 23\%$ of control, $t(3)=-0.10$, $p=0.92$, LMAN: $107 \pm 10\%$ of control, $t(3)=0.68$, $p=0.55$, $n=4$ cells from 3 birds, paired t-test). Taken together, these findings imply that calcium increases were required, but did not occur via NMDA receptor mediated mechanisms. Much or all of this increase in intracellular calcium appears to be mediated by release from intracellular calcium stores, as blockade of calcium release from the sarcoplasmic and endoplasmic reticulum with thapsigargin (1 μ M) prevented synaptic plasticity (Fig. 5d, HVC: $98 \pm 7\%$ of control, $t(3)=0.30$, $p=0.85$, LMAN: $101 \pm \%$ of control, $t(3)=0.20$, $p=0.79$, LMAN: $n=4$ cells from 2 birds).

The requirement for high frequency burst activation of both HVC and LMAN (Fig. 3) suggested that the observed plasticity may depend on coordinated release of glutamate after a burst of presynaptic activity, although not necessarily on the activation of NMDA receptors. We thus considered candidates for other glutamate receptors that could be specifically activated by burst stimulation, including receptors activated by spillover or synaptic crosstalk that can occur during burst stimulation of axons³². These include metabotropic glutamate receptors (mGluRs), which are often found extrasynaptically³³. In other systems, group II mGluRs (mGluR2/mGluR3) have been implicated in a presynaptic form of depression in cortical and hippocampal synapses³⁴, and can potentiate NMDA receptors³⁵. In songbirds, Group II mGluRs in particular show high levels of mRNA expression in HVC, LMAN and RA³⁶, making them potential candidates for the plasticity we elicited. When we bath applied the group II mGluR antagonist LY341495 and paired burst stimulation of the two pathways, we found no lasting changes in synaptic strength in either pathway (Fig. 5e, HVC: $96 \pm 5\%$ of control, $t(3)=-0.73$, $p=0.52$, LMAN: $103 \pm 10\%$ of control, $t(3)=0.24$, $p=0.82$, $n=4$ cells from 4 birds, paired t-test). Further, bath application of a group II mGluR agonist (LY354740) mimicked the simultaneous pairing of HVC and LMAN stimulation by causing decreased HVC synaptic strength ($87 \pm 6\%$, $t(5)=3.54$, $p=0.016$, $n=6$ cells from 5 birds, paired t-test) in combination with an increased paired pulse

ratio (PPR, pre: $0.98 \pm 6\%$, post: $1.29 \pm 13\%$, $t(5)=-2.91$, $p=0.033$, $n=6$ cells from 5 birds, paired t-test), while increasing LMAN synaptic strength ($125\% \pm 8\%$ of control, $t(5)=-3.11$, $p=0.026$, $n=6$ cells from 5 birds, paired t-test) without affecting PPR (pre: 1.01 ± 0.05 , post: 1.02 ± 0.08 , $t(5)=-0.12$, $p=0.91$, $n=6$ cells from 5 birds, paired t-tests, Supplementary Figure 4). In contrast, bath application of the group II mGluR antagonist LY341495 to slices had no effect on amplitude or PPR in either pathway (HVC: $117 \pm 16\%$ of control amplitude, $t(7)=1.08$, $p=0.31$, $n=8$ cells from 6 birds, PPR: ctrl: $1.18 \pm 10\%$, LY341495: $1.15 \pm 6\%$, $t(5)=0.43$, $p=0.68$, $n=6$ cells from 4 birds; LMAN: $96 \pm 5\%$ of control amplitude, $t(4)=-0.53$, $p=0.62$, $n=5$ cells from 4 birds, PPRs: ctrl: $1.21 \pm 14\%$, LY341495: $1.24 \pm 13\%$, $t(3)=-0.32$, $p=0.77$, $n=4$ cells from 3 birds, paired t-tests), suggesting little if any constitutive activation of mGluR2/3 in the slice. Taken together, these data show that the form of plasticity we elicited was NMDA independent, but required both mGluR2/3 activation and an increase in post-synaptic calcium through release from intracellular calcium stores. Thus, one interpretation of the requirement for high-frequency burst stimulation is that spillover of glutamate from LMAN axon terminals may activate extrasynaptic mGluR2/3 receptors.

We next examined whether the mechanisms that underlie synaptic plasticity were similar regardless of the latency between HVC and LMAN stimulation or the valence of plasticity (Fig. 5f–j). The pattern of paired pulse depression at -100ms lag was opposite to that found when stimulation were paired at 0ms lag in that LMAN showed significant potentiation of the PPR, while the HVC PPR was unchanged (HVC pre: 1.07 ± 0.10 , post 1.00 ± 0.09 , $t(4)=-1.08$, $p=0.34$, $n=5$ cells from 3 birds; LMAN pre: 0.91 ± 0.12 , post: 1.21 ± 0.05 , $t(4)=0.03$, $p=0.028$, $n=5$ cells from 3 birds, paired t-tests). Pairing stimulation at -100ms lag, we found that the activation of NMDA receptors was not required for changes in the strength of HVC inputs (Fig. 5g, $153 \pm 16\%$ of control, $t(3)=3.28$, $p=0.047$, $n=4$ cells from 3 birds, paired t-tests), but an increase in intracellular calcium (Fig. 5h, HVC: $105 \pm 11\%$ of control, $t(4)=0.43$, $p=0.69$; LMAN $103 \pm 12\%$ of control, $t(4)=0.29$, $p=0.78$, $n=5$ cells from 3 birds, paired t-tests) from intracellular stores (Fig. 5i, HVC: $104 \pm 7\%$ of control, $t(2)=0.30$, $p=0.79$, LMAN $109 \pm 9\%$ of control after thapsigargin, $t(2)=1.15$, $p=0.36$, $n=3$ cells from 2 birds, paired t-tests) was still required, as was activation of group II mGluR receptors (LY341495, Fig. 5j, $87 \pm 6\%$ of control, $t(5)=2.54$, $p=0.06$, LMAN $108 \pm 13\%$ of control, $t(5)=0.63$, $p=0.056$, $n=6$ cells from 4 birds, paired t-tests). Taken together, these results indicate that activation of mGluR2/3 receptors and a resulting increase in intracellular calcium is a prerequisite for both directions of plasticity.

Contribution of mGluR2/3 Receptors to Adult Pitch Shift Learning

As the group II mGluR blocker LY341495 was able to prevent lasting changes in synaptic strength in slices, we next examined whether or not group II mGluRs could influence behavioral changes induced by negative reinforcement learning. Previous work has shown that playback of a loud burst of white noise (WN) contingent on the frequency of a targeted syllable can drive adaptive changes in frequency in order to avoid the aversive stimulus^{4,28,25} (e.g., Fig. 6a–c). We used this paradigm to investigate whether the mGluRs required for synaptic plasticity *in vitro* were also required for adaptive behavioral plasticity. During aversive training experiments, we infused either phosphate buffered saline (PBS) or the mGluR2/3 antagonist LY341495 through microdialysis cannulae implanted bilaterally in

RA. A target syllable was then selected (Fig 6a), and an aversive stimulus (white noise, WN) was delivered when the bird sang above or below a target pitch (e.g. Fig. 6b). As reported previously, in control conditions during saline infusion, birds shift the fundamental frequency (FF) of the targeted syllable in order to avoid WN. During PBS infusion in RA, aversive training led to an adaptive shift in the FF (mean $1.8 \pm 0.32\%$ shift in FF, Fig. 6d). However, the adaptive shift achieved during training was significantly reduced after infusion of the mGluR2/3 antagonist LY341495 (Fig. 6d, $0.11 \pm 0.07\%$, $t(9)=5.14$, $p=0.0006$, $n=10$ experiments in 5 birds). The absence of a pitch shift was not due to a reduction in behavioral variability as LY341495 did not alter pitch variability of the targeted syllables (CV pre: 0.017 ± 0.003 , CV in LY341495: 0.016 ± 0.004 , $t(4)=0.89$, $p=0.42$, $n=5$ syllables in 5 birds). These results suggest that LMAN inputs continue to modulate RA activity and song output via NMDAR. Moreover, LY341495 did not alter synaptic responses to LMAN inputs in the *in vitro* preparation. Thus, the lack of behavioral plasticity during LY341495 infusions is not likely to reflect “masking” of LMAN inputs as in a previous study⁵. The blockade of pitch shift occurred whether the pitch was shifted up ($n=6/10$ experiments in 4 birds) or down ($n=4/10$ experiments in 4 birds). At the end of experiments, muscimol-BODIPY (1mM) was infused, and brains were fixed for histology. Muscimol-BODIPY showed the drug spread to encompass RA (Fig. 6e), confirming the targeting of our microdialysis probes. These results show that blockade of the receptors required for synaptic plasticity in the slice was also able to prevent adaptive changes in song in response to aversive stimulation.

Discussion

We have shown a form of synaptic plasticity in which excitatory inputs to a motor nucleus from two separate pathways with distinct functional roles in song production, HVC and LMAN, are altered in opposing directions. This plasticity depends on concurrent burst activation in both LMAN and HVC axons, activation of group II metabotropic glutamate receptors (mGluR2/3), and increases in intracellular calcium from intracellular stores. Furthermore, the relative timing between stimulation of bursting in the two pathways determined the strength and direction of plasticity.

Plasticity rules depending on the timing of activity between a synaptic input and an evoked spike (spike-timing dependent plasticity, STDP) have been described in many systems and can be modulated by both the frequency and timing of synaptic inputs^{37,38}. Moreover, depending on the neuronal class, identical timings can result in Hebbian or anti-Hebbian learning rules^{37,38}. The plasticity described here depends not on spiking but rather on timed activation of different input pathways (termed Input Timing Dependent Plasticity, ITDP)^{39,40}. The heterosynaptic ITDP we elicited exhibits some novel properties. We found that burst activation of both inputs is required, but unlike other examples of ITDP^{39,40} both inputs change, and do so in opposing directions, helping to maintain an overall balance of inputs. Strikingly, the direction of plasticity can be inverted by changing the relative timing between activation of the two pathways. Plasticity of the two inputs is symmetrical not around zero offset between stimulation of the two inputs, but instead is centered around a temporal offset (~ -75 ms, HVC leading LMAN) that is near the lag observed in anaesthetized recordings⁴¹ and near the *in vivo* estimate for vocal-motor mirroring in LMAN⁴².

Synaptic and Song Plasticity

Prior work has suggested that song learning is encoded in RA, particularly in the HVC-RA synapses, while LMAN synapses are required for plasticity. However, LMAN-dependent synaptic plasticity has not been reported previously in adult birds. Here, we show for the first time plasticity of RA inputs. Moreover, this plasticity involves opposing changes in the synaptic strengths of the two inputs. Learned song requires synaptic inputs from HVC, as lesions of HVC-RA inputs cause song to revert to a juvenile-like state⁷, and firing rates of RA neurons can be correlated with multiple aspects of song structure, including pitch¹³. In contrast, in adult finches, LMAN is not required for singing crystallized song^{2,9}, although it contributes to song variability¹¹ and is required for multiple forms of juvenile and adult plasticity. When LMAN is silenced in juveniles, song learning abruptly stops^{9,10,12}, and birds develop more robust HVC synaptic inputs compared to birds with LMAN intact¹⁶. Direct microstimulation of LMAN can also alter song pitch and amplitude⁴³, suggesting that such LMAN-mediated influences on RA spiking may underlie the pitch shifts observed during LMAN-dependent reinforcement training. Interestingly, the influence of the two inputs to RA also shifts during this reinforcement learning paradigm: initially both HVC and LMAN inputs contribute to the learned pitch shift, and this is then followed by consolidation, where the direct motor pathway from HVC to RA maintains the adaptive pitch shift even in the absence of LMAN activity^{4,24}. The plasticity observed in our slice preparation presents a possible mechanism whereby appropriately timed activity can increase or decrease the strength of the HVC motor pathway, with a coordinated change in the influence of the AFP.

Ionotropic and Metabotropic Glutamate Receptors in the Song System

The two excitatory inputs to RA are largely segregated by their ionotropic receptors: the direct pathway from HVC is predominantly mediated by AMPA receptors, while the inputs from LMAN are almost exclusively made up of NMDA receptors^{21,22}. Here, we demonstrate an NMDA-independent form of synaptic plasticity which required activation of both RA input paths (HVC and LMAN), mGluR2/3 receptor activation, and intracellular calcium changes.

Metabotropic glutamate receptors have been mapped previously in the song system³⁶ but have rarely been examined otherwise. *In situ* localization of mRNA for these receptors shows strong expression in RA, HVC and LMAN³⁶, suggesting potential expression postsynaptically in RA neurons, and/or presynaptically in HVC and LMAN axon terminals. In other systems, these receptors have been implicated in timing-dependent depression of glutamatergic synapses³⁴, and can cause calcium-dependent increases in NMDA mediated currents³⁵. To test the contributions of metabotropic receptors, we used selective mGluR2/3 receptor agonists and antagonists to demonstrate that activation of these receptors contributes to the bidirectional heterosynaptic plasticity we observed in slices. Moreover, we found a link between mGluR-dependent synaptic plasticity in RA and behavioral plasticity: adult birds trained to shift their pitch through aversive reinforcement did not shift their pitch when mGluR2/3 antagonists were infused into RA *in vivo*.

Burst Timing, Sources of Timing Differences, and Implications for Song Production

In vitro, we found that LMAN burst stimulation was required for synaptic plasticity in RA and that the relative timing between burst stimulation of HVC and LMAN axons determined both the magnitude and direction of plasticity. Prior work suggests that naturally varying differences in the arrival times of the inputs from the two pathways do occur during singing. While HVC neurons projecting to RA fire stereotyped, precisely timed high frequency bursts of activity in every rendition¹⁸, LMAN neurons fire variably timed bursts both during juvenile song acquisition⁷ and in the solitary song conditions in which most adult plasticity experiments have been performed¹⁹. Although individual LMAN neurons burst preferentially within certain temporal windows relative to song, bursts may or may not occur during any individual rendition, and their precise timing within these windows varies from rendition to rendition. Thus, the relative timing of events from the direct HVC-RA motor pathway and the AFP-mediated LMAN-RA pathway varies naturally from rendition to rendition during normal song production.

There are a number of possible sources of timing differences between HVC-RA and LMAN-RA inputs. First, the architecture of HVC is such that HVC-RA neurons drive HVC-X neurons as well as inhibitory interneurons (HVC-INT), and HVC-INT primarily influence HVC-X neurons⁴⁴. Such functional connectivity suggests that HVC-X neurons provide a readout of the activity of HVC-RA, such that timing differences between HVC-RA and LMAN-RA inputs could emerge during the propagation of activity through the multiple nuclei of the AFP. Although strong electrical stimulation of HVC can evoke activity in LMAN as quickly as 10–30 ms⁴⁵, simultaneous recordings of spontaneous and BOS-evoked activity in RA and LMAN in anaesthetized animals show that RA and LMAN spiking is coherent even at late time points, with a peak near 65 ms (ranging from 10–150ms) due to common input from HVC⁴¹. Second, propagation of activity may be modulated in the AFP. Recordings from behaving birds have shown that during singing there is precisely timed activity in HVC-X projection neurons, but variably timed events are apparent in Area X²⁷ and LMAN¹⁹, and prior studies have shown that manipulation of dopamine can alter propagation times through the circuit⁴⁶. Third, auditory-vocal mirroring experiments have raised the possibility that some HVC-X neurons fire identically timed activity during song production and auditory playback^{47,48}, suggesting they may be temporally offset from the direct HVC-RA motor commands. In contrast, LMAN neurons have a longer vocal-motor mirroring latency⁴². These provide yet another possible source of temporal offsets between HVC-RA and LMAN-RA drive.

Regardless of their origins, variable delays between HVC and LMAN bursts *in vivo* have direct implications for how LMAN activity modulates the HVC-RA direct motor signals to change song. LMAN is known to be a key source of motor variability^{7,11,12}, and drives much of the initial pitch shift during reinforcement learning^{4,5,24}. In order for LMAN to bias the direct motor command from HVC driving song, LMAN firing must occur within some time window in order to be integrated with the HVC-RA motor command it biases. The synaptic learning rule we found *in vitro* suggests that such closely timed pairings will lead to an increase in the influence of the LMAN pathway and a concomitant decrease in the

influence in the HVC pathway, which could contribute to the expression of adult reinforcement learning^{4,24}.

In contrast, if HVC-RA inputs drive some song element before LMAN-RA inputs arrive, no acute LMAN-mediated biasing is possible for that element. Nonetheless, we observed that at longer lags between HVC-RA and LMAN-RA inputs (e.g. –100 ms), stimulation still altered synaptic strength *in vitro*, potentially allowing changes in these inputs in the absence of acute perturbation of the song. Future studies that elucidate the relative timing between HVC-RA events and LMAN-RA events could help reconcile how LMAN can contribute to the many roles it has been suggested to play, including propagation of mirror neuron-like efference copies⁴⁸, the implementation of a ‘forward model’ for reinforcement learning^{49,50}, generating variability in motor output^{12,43}, and actively biasing song production^{4,24,43}.

The *in vitro* plasticity rule described here is consistent with many observed aspects of song learning, including a greater influence of LMAN initially during learning, the gradual shift towards control by the direct motor pathway from HVC^{4,5,24}, the requirement of LMAN for changes in learned aspects of syllable structure in adults^{4,24}, and the requirement for timed LMAN bursts in adult vocal motor plasticity⁶. In addition, the learning rule seen *in vitro* in the inputs to the song motor cortex analogue RA may have very general implications for motor learning and its dependence on cortical-basal ganglia interactions and their timing.

Methods

Subjects

Subjects were male zebra finches (*Taeniopygia guttata*). Their care and treatment were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Slice Preparation

Brain slices were prepared from late juvenile (70–90) and adult (90+) birds raised in our breeding colony, or purchased from an outside supplier. Bird were anaesthetized with isoflurane, and rapidly decapitated according to protocols approved by the Animal Care and Use Committee of the University of California San Francisco. Brains were rapidly dissected in an ice cold cutting solution consisting of (in mM): 125 C₅H₁₄ClNO, 3 MgSO₄, 0.5 CaCl₂, 2.5 KCl, 25 NaHCO₃, and 35 glucose. In some solutions, 0.3 mM ascorbate was added. Approximately coronal sections were cut on a Leica VT1000 microtome, and incubated at 36 °C in recording ACSF (consisting of (in mM): 125 NaCl, 1 MgSO₄, 2 CaCl₂, 2.5 KCl, 25 NaHCO₃, and 35 glucose) for 30–60 minutes. Slices were then maintained at room temperature in well-oxygenated (95% O₂-5% CO₂) holding chambers until transferred to the recording chamber.

Electrophysiology

All recordings were made in oxygenated solution which consisted of standard ACSF, heated to near-physiological temperature (35–38 °C, TC-324B, Warner Instruments). Glass electrodes (3–8 MΩ) were filled with intracellular solution containing (in mM) 120 KGluc,

0.1 EGTA, 40 HEPES, 5 KCl, 0.3 MgCl₂, 2 K₂ATP, 0.5 Tris-GTP, and 10 Na-Creatinine. When noted, KGluc was replaced with K-Methylsulfonate, and D-890 (1μM) and QX-314 (1μM) were added to the intracellular solution to inhibit K⁺, Ca²⁺ and Na⁺ currents, respectively. In some cases 0.5% biocytin, or Alexa-594 was added to the pipette solution for identification of neuronal morphology after recording. Targeted recordings were made under DIC-IR visualization using an Olympus BX-51 microscope and a camera (DAGE-MTI). The borders of RA are easily identifiable in the slice. Recordings were made within these borders from apparent projection neurons (projecting to NXIIIs, nucleus retroambigualis or paraambigualis), which could be identified physiologically by their spontaneous firing *in vitro*, slow firing and adaptation in response to depolarizing currents steps, and prominent sag current²⁰. Unless otherwise stated, cells were held at -75 to -80 mV for the duration of the experiments, including during synaptic stimulation, in order to keep the cell at the Cl⁻ reversal potential to prevent GABA_A mediated currents from contaminating the traces. This was sufficient to prevent the high frequency IPSCs in RA PNs from being apparent, and further application of Gabazine (SR-95531), had only a small effect on baseline holding currents. Further, Gabazine did not affect the amplitude of EPSPs, or the potentiation and depression we observed (Supplementary Figure 3). Recordings were made with a Multiclamp 700B (Molecular Devices), controlled by PClamp 10.0 software or by custom acquisition software written in Matlab. Current and voltage clamp data was acquired at 10 kHz, and filtered at 3kHz.

Stimulation

Concentric bipolar or matrix (100μm) stimulating electrodes (FHC) were placed in the afferent tracts from HVC and LMAN (Fig. 1b). HVC and LMAN showed comparable pharmacology to previous studies, where LMAN inputs were blocked by APV, and HVC neurons were less APV-sensitive^{21,22}. A smaller, but measureable LMAN mediated EPSC was observed even at hyperpolarized potentials (-75 to -80mV, Supplementary Figure 1), allowing recording of both pathways at identical input voltages. When applying burst stimulation, HVC was driven at 800 Hz four times¹⁸, and LMAN was driven at 500 Hz eight times^{7,19}. Bursts were presented eight times at 0.5 Hz, while holding the cell in voltage clamp at -75 to -80mV. Although LMAN neurons can display significant variability in their burst patterns and timing *in vivo*, in these experiments these parameters were fixed. Burst stimulation of either pathway was presented either individually, or by pairing activation of both pathways at various latencies. Cells were then monitored for at least 20 minutes in order to be included in the data set, and were excluded if series resistance changed by > 20%. After a stable baseline of 3–5 minutes was obtained, induction protocols were performed, and the two input pathways were stimulated alternately every minute, or else stimulated five times (0.5 Hz) at five minute intervals. Results were grouped into five minute time periods so that the protocols were comparable. Cells had to be stable for at least 15 minutes after stimulation in order to be included in the data set, but effects could be seen out to longer periods (Supplementary Figure 2). Significance for potentiation experiments was tested with a paired t-test between the average of the control period and the final 5 minutes time window to test whether a lasting change was observed. Slices were discarded after pharmacological or plasticity experiments. Data distribution was assumed to be normal but this was not formally tested. Data collection and analysis were not performed blind to

the conditions of the experiments. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications^{21,51,52}. All t-tests reported and two-tailed. Animals were not grouped, and therefore no randomization was performed. When mentioned, drugs were bath applied at the following concentrations: LY341495 (20nM), APV (25μM), Gabazine (10μM), thapsigargin (1μM). All drugs were obtained from Sigma-Aldrich or abcam Biochemicals.

Histology

Birds were deeply anesthetized with isoflurane before being transcardially perfused with 0.9% saline, followed by 3.7% formaldehyde in 0.025 M phosphate buffer (PB). Brains were postfixed for 4–24 h, then cryoprotected in 30% sucrose. Forty micrometer sagittal sections were cut on a freezing microtome.

Retrodialysis for In Vivo Behavior Experiments

For *in vivo* microdialysis experiments to examine behavioral plasticity, cannulae were targeted to RA bilaterally with stereotaxic coordinates as described previously⁵³, and phosphate-buffered saline or the mGluR2/3 antagonist LY341495 (100–200 nM) was infused into RA through microdialysis probes (CMA-7, Harvard Apparatus). After the final experiment, birds were infused with fluorescent muscimol-BODIPY (Life Technologies, 1 mM) to confirm targeting of the cannulae and drug spread to RA.

Pitch Shift Training

Birds were kept in sound isolating chambers (Acoustic Systems), and provided with food and water *ad libitum*. Brief bursts of white noise were played back in response to detection of specific syllables with custom software written in LabView (National Instruments). As previously described²⁵ these techniques reveal a form of adult song plasticity mediated by adaptive changes in the pitch of the fundamental frequency of targeted syllables. 10–20% trials were ‘catch’ trials, where white noise was not played regardless of the fundamental frequency of the syllable. Only syllables within these trials were analyzed in order to examine learning trajectories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Michael Brainard, Michael Stryker, Kevin Bender, Fernando Fernandez, Roger Nicoll, and members of the Doupe, Brainard and Sabes laboratories for discussions and comments on the manuscript, in particular the exceptional patience of Mimi Kao. This work was supported by NIH grant MH55987 and a NARSAD Distinguished Investigator award (AJD), and Canadian Institute of Health Research (CIHR) and Grass Foundation Fellowships (WHM).

References

1. Nordeen KW, Nordeen EJ. Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. *Behav Neural Biol.* 1992; 57:58–66. [PubMed: 1567334]

2. Brainard MS, Doupe AJ. Interruption of a basal ganglia-forebrain circuit prevents plasticity of learned vocalizations. *Nature*. 2000; 404:762–6. [PubMed: 10783889]
3. Leonardo A, Konishi M. Decrystallization of adult birdsong by perturbation of auditory feedback. *Nature*. 1999; 399:466–70. [PubMed: 10365958]
4. Andalman AS, Fee MS. A basal ganglia-forebrain circuit in the songbird biases motor output to avoid vocal errors. *Proc Natl Acad Sci U S A*. 2009; 106:12518–23. [PubMed: 19597157]
5. Charlesworth JD, Warren TL, Brainard MS. Covert skill learning in a cortical-basal ganglia circuit. *Nature*. 2012; 486:251–5. [PubMed: 22699618]
6. Kojima S, Kao MH, Doupe AJ. Task-related ‘cortical’ bursting depends critically on basal ganglia input and is linked to vocal plasticity. *Proc Natl Acad Sci U S A*. 2013; 110:4756–61. [PubMed: 23449880]
7. Aronov D, Andalman AS, Fee MS. A specialized forebrain circuit for vocal babbling in the juvenile songbird. *Science*. 2008; 320:630–4. [PubMed: 18451295]
8. Nottebohm F, Stokes TM, Leonard CM. Central control of song in the canary, *Serinus canarius*. *J Comp Neurol*. 1976; 165:457–86. [PubMed: 1262540]
9. Bottjer SW, Miesner EA, Arnold AP. Forebrain lesions disrupt development but not maintenance of song in passerine birds. *Science*. 1984; 224:901–3. [PubMed: 6719123]
10. Scharff C, Nottebohm F. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. *J Neurosci*. 1991; 11:2896–913. [PubMed: 1880555]
11. Kao MH, Brainard MS. Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability. *J Neurophysiol*. 2006; 96:1441–55. [PubMed: 16723412]
12. Olveczky BP, Andalman AS, Fee MS. Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. *PLoS Biol*. 2005; 3:e153. [PubMed: 15826219]
13. Sober SJ, Wohlgemuth MJ, Brainard MS. Central contributions to acoustic variation in birdsong. *J Neurosci*. 2008; 28:10370–9. [PubMed: 18842896]
14. Thompson JA, Wu W, Bertram R, Johnson F. Auditory-dependent vocal recovery in adult male zebra finches is facilitated by lesion of a forebrain pathway that includes the basal ganglia. *J Neurosci*. 2007; 27:12308–20. [PubMed: 17989295]
15. Herrmann K, Arnold A. The development of afferent projections to the robust archistriatal nucleus in male zebra finches: a quantitative electron microscopic study. *J Neurosci*. 1991; 11:2063–2074. [PubMed: 2066775]
16. Kittelberger JM, Mooney R. Lesions of an avian forebrain nucleus that disrupt song development alter synaptic connectivity and transmission in the vocal premotor pathway. *J Neurosci*. 1999; 19:9385–98. [PubMed: 10531443]
17. Iyengar S, Bottjer SW. The role of auditory experience in the formation of neural circuits underlying vocal learning in zebra finches. *J Neurosci*. 2002; 22:946–58. [PubMed: 11826123]
18. Hahnloser RHR, Kozhevnikov AA, Fee MS. An ultra-sparse code underlies the generation of neural sequences in a songbird. *Nature*. 2002; 419:65–70. [PubMed: 12214232]
19. Kao MH, Wright BD, Doupe AJ. Neurons in a forebrain nucleus required for vocal plasticity rapidly switch between precise firing and variable bursting depending on social context. *J Neurosci*. 2008; 28:13232–47. [PubMed: 19052215]
20. Spiro JE, Dalva MB, Mooney R. Long-range inhibition within the zebra finch song nucleus RA can coordinate the firing of multiple projection neurons. *J Neurophysiol*. 1999; 81:3007–20. [PubMed: 10368416]
21. Mooney R, Konishi M. Two distinct inputs to an avian song nucleus activate different glutamate receptor subtypes on individual neurons. *Proc Natl Acad Sci U S A*. 1991; 88:4075–9. [PubMed: 11607180]
22. Stark LL, Perkel DJ. Two-stage, input-specific synaptic maturation in a nucleus essential for vocal production in the zebra finch. *J Neurosci*. 1999; 19:9107–16. [PubMed: 10516328]
23. Cavara NA, Hollmann M. Shuffling the deck anew: how NR3 tweaks NMDA receptor function. *Mol Neurobiol*. 2008; 38:16–26. [PubMed: 18654865]

24. Warren TL, Tumer EC, Charlesworth JD, Brainard MS. Mechanisms and time course of vocal learning and consolidation in the adult songbird. *J Neurophysiol.* 2011; 106:1806–21. [PubMed: 21734110]
25. Tumer EC, Brainard MS. Performance variability enables adaptive plasticity of ‘crystallized’ adult birdsong. *Nature.* 2007; 450:1240–4. [PubMed: 18097411]
26. Scott LL, Nordeen EJ, Nordeen KW. LMAN lesions prevent song degradation after deafening without reducing HVC neuron addition. *Dev Neurobiol.* 2007; 67:1407–18. [PubMed: 17694506]
27. Woolley SC, Rajan R, Joshua M, Doupe AJ. Emergence of context-dependent variability across a basal ganglia network. *Neuron.* 2014; 82:208–23. [PubMed: 24698276]
28. Ali F, et al. The basal ganglia is necessary for learning spectral, but not temporal, features of birdsong. *Neuron.* 2013; 80:494–506. [PubMed: 24075977]
29. Feldman DE. The spike-timing dependence of plasticity. *Neuron.* 2012; 75:556–71. [PubMed: 22920249]
30. Fiete IR, Hahnloser RHR, Fee MS, Seung HS. Temporal sparseness of the premotor drive is important for rapid learning in a neural network model of birdsong. *J Neurophysiol.* 2004; 92:2274–82. [PubMed: 15071087]
31. Troyer TW, Doupe AJ. An associational model of birdsong sensorimotor learning I. Efference copy and the learning of song syllables. *J Neurophysiol.* 2000; 84:1204–23. [PubMed: 10979996]
32. Kullmann DM. Spillover and synaptic cross talk mediated by glutamate and GABA in the mammalian brain. *Prog Brain Res.* 2000; 125:339–51. [PubMed: 11098670]
33. Tamaru Y, Nomura S, Mizuno N, Shigemoto R. Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: differential location relative to pre- and postsynaptic sites. *Neuroscience.* 2001; 106:481–503. [PubMed: 11591452]
34. Egger V, Feldmeyer D, Sakmann B. Coincidence detection and changes of synaptic efficacy in spiny stellate neurons in rat barrel cortex. *Nat Neurosci.* 1999; 2:1098–105. [PubMed: 10570487]
35. Tyszkiewicz JP, Gu Z, Wang X, Cai X, Yan Z. Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-dependent mechanism in pyramidal neurones of rat prefrontal cortex. *J Physiol.* 2004; 554:765–77. [PubMed: 14645456]
36. Wada K, Sakaguchi H, Jarvis ED, Hagiwara M. Differential expression of glutamate receptors in avian neural pathways for learned vocalization. *J Comp Neurol.* 2004; 476:44–64. [PubMed: 15236466]
37. Bell CC, Han VZ, Sugawara Y, Grant K. Synaptic plasticity in a cerebellum-like structure depends on temporal order. *Nature.* 1997; 387:278–81. [PubMed: 9153391]
38. Tzounopoulos T, Kim Y, Oertel D, Trussell LO. Cell-specific, spike timing-dependent plasticities in the dorsal cochlear nucleus. *Nat Neurosci.* 2004; 7:719–25. [PubMed: 15208632]
39. Humeau Y, Shaban H, Bissière S, Lüthi A. Presynaptic induction of heterosynaptic associative plasticity in the mammalian brain. *Nature.* 2003; 426:841–5. [PubMed: 14685239]
40. Cho JH, et al. Coactivation of thalamic and cortical pathways induces input timing-dependent plasticity in amygdala. *Nat Neurosci.* 2012; 15:113–22.
41. Kimpo RR, Theunissen FE, Doupe AJ. Propagation of correlated activity through multiple stages of a neural circuit. *J Neurosci.* 2003; 23:5750–61. [PubMed: 12843279]
42. Giret N, Kornfeld J, Ganguli S, Hahnloser RHR. Evidence for a causal inverse model in an avian cortico-basal ganglia circuit. *Proc Natl Acad Sci.* 2014; 111:6063–6068. [PubMed: 24711417]
43. Kao MH, Doupe AJ, Brainard MS. Contributions of an avian basal ganglia-forebrain circuit to real-time modulation of song. *Nature.* 2005; 433:638–43. [PubMed: 15703748]
44. Mooney R. Different subthreshold mechanisms underlie song selectivity in identified HVC neurons of the zebra finch. *J Neurosci.* 2000; 20:5420–36. [PubMed: 10884326]
45. Leblois A, Bodor AL, Person AL, Perkel DJ. Millisecond timescale disinhibition mediates fast information transmission through an avian basal ganglia loop. *J Neurosci.* 2009; 29:15420–33. [PubMed: 20007467]
46. Leblois A, Wendel BJ, Perkel DJ. Striatal dopamine modulates basal ganglia output and regulates social context-dependent behavioral variability through D1 receptors. *J Neurosci.* 2010; 30:5730–43. [PubMed: 20410125]

47. Fujimoto H, Hasegawa T, Watanabe D. Neural coding of syntactic structure in learned vocalizations in the songbird. *J Neurosci.* 2011; 31:10023–33. [PubMed: 21734294]
48. Prather JF, Peters S, Nowicki S, Mooney R. Precise auditory-vocal mirroring in neurons for learned vocal communication. *Nature.* 2008; 451:305–10. [PubMed: 18202651]
49. Amador A, Perl YS, Mindlin GB, Margoliash D. Elemental gesture dynamics are encoded by song premotor cortical neurons. *Nature.* 2013; 495:59–64. [PubMed: 23446354]
50. Hanuschkin A, Ganguli S, Hahnloser RHR. A Hebbian learning rule gives rise to mirror neurons and links them to control theoretic inverse models. *Front Neural Circuits.* 2013; 7:106. [PubMed: 23801941]
51. Murugan M, Harward S, Scharff C, Mooney R. Diminished FoxP2 Levels Affect Dopaminergic Modulation of Corticostriatal Signaling Important to Song Variability. *Neuron.* 2013
52. Sizemore M, Perkel DJ. Premotor synaptic plasticity limited to the critical period for song learning. *Proc Natl Acad Sci U S A.* 2011; 108:17492–7. [PubMed: 21969574]
53. Stepanek L, Doupe AJ. Activity in a cortical-basal ganglia circuit for song is required for social context-dependent vocal variability. *J Neurophysiol.* 2010; 104:2474–86. [PubMed: 20884763]

Figure 1. Schematic of the song system and slice preparation

a) Diagram of the major nuclei involved in song generation. The motor region RA receives inputs primarily from two sources- HVC (used as a proper name) and LMAN, the output of a cortical-basal ganglia loop. RA in turn directly drives brainstem motor neurons which control the vocal musculature. The inputs from HVC and LMAN are anatomically distinct (**b**). RA has well defined borders (**c**), and RA neurons are easily visualized under transillumination (**d**). **e)** The two pathways innervating RA PN's are pharmacologically distinct: HVC is weakly blocked by APV ($84 \pm 6\%$ of control, $t(4) = -2.70$, $p = 0.054$, $n=5$ cells from 4 birds, paired t-test), while LMAN gives rise to APV-sensitive NMDA receptor mediated currents^{21,22} ($6\% \pm 2\%$ of control, $t(7) = -53.95$, $p < 0.0001$, $n=8$ cells from 6 birds, paired t-test) Scale bar in (**c**), 100 μm, in (**d**) 20 μm. All values are mean $\pm$ s.e.m.

Figure 2. Burst stimulation of both input pathways simultaneously can drive lasting changes in synaptic strength

a) Stimulation of either HVC (black) or LMAN (red) at high frequencies (800 Hz and 500 Hz respectively) drove a significantly larger summed current in comparison to single stimulations, suggesting increased glutamate release after burst stimulation, even at these high frequencies; (HVC: $107 \pm 12\%$ increase, $t(12)=4.96$, $p=0.0003$, $n=13$, LMAN: $188 \pm 38\%$ increase, $p=0.0000009$, $n=13$ cells from 9 birds, paired t-tests). **b)** Schematic of protocol used for plasticity induction- HVC and LMAN inputs were paired 8 times at 0.5 Hz, with HVC driven at 800 Hz and LMAN driven at 500 Hz. After the induction protocol, LMAN inputs showed a modest increase in synaptic strength ($127 \pm 10\%$ of control, $t(6)=2.76$, $p=0.035$, $n=7$ cells/slices from 6 birds, paired t-test), while HVC inputs showed a large decrease in synaptic strength ($54\% \pm 16\%$ of control, $t(6)=2.86$, $p=0.033$, $n=7$ cells/slices from 6 birds, paired t-test). A representative experiment (**c,d**) and summary data (**e**) are illustrated. **f)** The induction protocol increased the paired-pulse ratio of HVC inputs ($23\% \pm 10\%$ increase, $t(6)= -2.56$, $p=0.043$, 7 cells/slices from 6 birds, paired t-test), but did not affect the paired pulse ratio of LMAN inputs ($0.8\% \pm 4\%$, $t(6)=0.09$, $p=0.93$, $n=7$ cells/slices from 6 birds, paired t-test). All values are mean $\pm$ s.e.m.

Figure 3. High frequency bursting in both pathways is required for induction of plasticity
a) Burst stimulation of HVC alone did not cause changes in the strength of inputs from either HVC or LMAN HVC stimulation only: LMAN: $99 \pm 0.1\%$ of control, $t(2) = -0.19$, $p = 0.87$, $n = 3$ cells from 2 birds; HVC: $97 \pm 2\%$ of control, $t(5) = -2.06$, $p = 0.09$, $n = 6$ cells from 5 birds, paired t-test. **b)** Burst stimulation of LMAN alone did not cause changes in the strength of inputs from either HVC or LMAN (LMAN: $97 \pm 2\%$ of control, $t(7) = -1.24$, $p = 0.25$, $n = 8$ cells from 6 birds; HVC: $94 \pm 5\%$ of control, $t(4) = -1.37$, $p = 0.24$, $n = 5$ cells from 3 birds, paired t-tests). **c)** Pairing HVC bursts with single stimulation of LMAN failed to produce lasting changes in synaptic strength (HVC: $98 \pm 3\%$ of control, $t(3) = -0.72$, $p = 0.53$, LMAN: $97 \pm 4\%$ of control, $t(3) = -0.83$, $p = 0.47$, $n = 4$ cells from 4 birds, paired t-tests). **d)** Paired, low frequency burst stimulation failed to produce lasting changes in synaptic strength in either input (HVC: $99 \pm 10\%$ of control, $t(3) = -0.09$, $p = 0.93$, LMAN $107 \pm 8\%$ of control, $t(3) = 0.87$, $p = 0.45$, $n = 4$ cells from 2 birds, paired t-tests). All values are mean $\pm$ s.e.m.

Figure 4. The relative timing of burst stimulation in the two pathways determines the magnitude and valence of synaptic strength changes

a) Illustration of the manipulation of timing of paired burst stimulation. Negative lags indicate when HVC is leading LMAN, positive lags indicate when LMAN is leading HVC.

b) The sign and magnitude of plasticity in both LMAN and HVC inputs depend on the timing between burst stimulation. **c–e)** Representative examples at -100 ms ($n=5$), 0 ms ($n=7$), and 100 ms ($n=3$) lags of the changes in the two pathways. All values are mean $\pm$ s.e.m.

Figure 5. Both valences of plasticity have similar pharmacological profiles

Depression of HVC synapses in response to the 0 ms lag burst induction protocol (diagrammed in **a**) did not require activation of NMDA receptors ($56 \pm 10\%$ of control, $t(3) = -4.28$, $p = 0.023$, $n = 4$ cells from 3 birds, paired t-test) (**b**), but lasting plasticity was blocked by chelating of intracellular calcium (HVC: $98 \pm 23\%$ of control, $t(3) = -0.10$, $p = 0.92$, LMAN: $107 \pm 10\%$ of control, $t(3) = 0.68$, $p = 0.55$, $n = 4$ cells from 3 birds, paired t-test) (**c**), blockade of calcium release from intracellular stores (HVC: $98 \pm 7\%$ of control, $t(3) = 0.30$, $p = 0.78$, LMAN: $101 \pm 3\%$ of control, $t(3) = 0.20$, $p = 0.85$, $n = 4$ cells from 2 birds, paired t-test) (**d**) and blockade of Group II mGluR receptors (HVC: $96 \pm 5\%$ of control, $t(3) = -0.73$, $p = 0.52$, LMAN: $103 \pm 10\%$ of control, $t(3) = 0.24$, $p = 0.82$, $n = 4$ cells from 4 birds, paired t-test) (**e**). When a different lag between stimulations was adopted, the same pattern held (**f–j**). When HVC stimulation led LMAN stimulation by 100ms (**f**), the increase in HVC synaptic strength did not depend on NMDA receptor activation ($153 \pm 16\%$ of control, $t(3) = 3.28$, $p = 0.047$, $n = 4$ cells from 3 birds, paired t-test) (**g**). Buffering intracellular calcium rises (HVC: $105 \pm 11\%$ of control, $t(4) = 0.43$, $p = 0.69$; LMAN $103 \pm 12\%$ of control after BAPTA, $t(4) = 0.29$, $p = 0.78$, $n = 5$ cells from 3 birds, paired t-tests) (**h**) or release from intracellular stores (HVC: $104 \pm 7\%$ of control, $t(2) = 0.30$, $p = 0.79$, LMAN $109 \pm 9\%$ of control after thapsigargin, $t(2) = 1.15$, $p = 0.36$, $n = 3$ cells from 2 birds, paired t-tests) (**i**) prevented changes in either pathway. **j**) Activation of group II mGluRs was again required for changes in both pathways (HVC: $87 \pm 6\%$, $t(5) = 2.54$, $p = 0.06$, LMAN $108 \pm 13\%$, $t(5) = 0.63$, $p = 0.056$, $n = 6$ cells from 4 birds, paired t-tests.) All values are mean $\pm$ s.e.m.

Figure 6. Blockade of mGluR2/3 receptors by LY341495 prevents adult song pitch training

a) Specific syllables (denoted by letters) can be targeted with negative reinforcement (white noise, WN) contingent on the fundamental frequency (expanded view of targeted syllable in **(b)**), which drives changes in the pitch of targeted syllables (**(c)**). Adult birds reliably shifted the fundamental frequency of targeted syllables when PBS was infused through microdialysis cannulae, while infusion of the mGluR2/3 blocker LY341495 into RA prevented learned changes in pitch ($0.11 \pm 0.07\%$ of control, $t(9)=5.14$, $p=0.0006$, $n=10$ experiments in 5 birds). **(c,d)**. Placement of microdialysis probes and drug spread were confirmed by injection of Muscimol-BODIPY. Area circled in **(e)** delineates the borders of RA (scale bar = 500 μ m). All values are mean $\pm$ s.e.m.