Noradrenergic and GABA<sub>B</sub> Receptor Activation Differentially Modulate Inputs to the Premotor Nucleus RA in Zebra Finches

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Sizemore M, Perkel DJ. Noradrenergic and GABA<sub>B</sub> receptor activation differentially modulate inputs to the premotor nucleus RA in zebra finches. J Neurophysiol 100: 8–18, 2008. First published May 7, 2008; doi:10.1152/jn.01212.2007. Neuromodulators can rapidly modify neural circuits, altering behavior. Songbirds provide an excellent system for studying the role of neuromodulation in modifying circuits that underlie behavior because song learning and production are mediated by a discrete set of interconnected nuclei. We examined the neuromodulatory effects of noradrenergic and GABA<sub>B</sub> receptor activation on synaptic inputs to the premotor robust nucleus of the arcopallium (RA) in zebra finches using whole cell voltage-clamp recording in vitro. In adults, norepinephrine strongly reduced input from the lateral magnocellular nucleus of the anterior nidopallium (LMAN) but only slightly reduced the input from nucleus HVC (proper name), the excitatory input from axon collaterals of other RA neurons, and input from GABAergic interneurons. The effect of norepinephrine was mimicked by the α<sub>2</sub> adrenoceptor agonist UK14,304 and blocked by the α<sub>2</sub> antagonist yohimbine. Conversely, the GABA<sub>B</sub> receptor agonist baclofen strongly decreased HVC, collateral, and GABAergic inputs to RA neurons while causing little reduction in the LMAN input. In juveniles undergoing song learning, norepinephrine reduced the LMAN input, caused only a small reduction in the HVC input, and greatly reduced the collateral and GABAergic inputs. Baclofen caused similar results in juvenile and adult birds, reducing HVC, collateral, and GABAergic inputs significantly more than the LMAN input. Significant increases in paired-pulse ratio accompanied all reductions in synaptic transmission, suggesting a presynaptic locus. The reduction in the LMAN input by norepinephrine may be important for mediating changes in song elicited by different social contexts and is well-placed to play a role in song learning.

INTRODUCTION

Animals must react to changes in their environment. The approach of a predator or the sighting of a potential mate, for example, has to be dealt with swiftly and precisely if an individual is to survive and reproduce. Therefore the neural mechanisms controlling behavior should be able to adapt rapidly. Neuromodulators offer a means of temporally altering the function of existing circuits, allowing an organism to adjust its behavior to different contexts. While much is known about the role of neuromodulators in altering neural activity and behavior in invertebrates and in vertebrate spinal cord and brain stem (see Dickinson 2006; Marder and Bucher 2001; Sillard et al. 1998 for reviews), much less is known about their role in causing rapid changes in forebrain substrates of motor control.

Songbirds have provided a useful model for studying the neural control and modulation of a complex learned behavior (Zeigler and Marler 2004). Juvenile songbirds learn their song by first memorizing the song of a tutor and then practicing, while listening to themselves, until they produce a stereotyped copy of the tutors’ song (Immelmann 1969). There is evidence that social interactions play a role in song learning (King and West 1983; Morrison and Nottebohm 1993) and tutor choice (Roper and Zann 2006). A zebra finch (Taeniopygia guttata) song “crystallizes” when the bird reaches adulthood, and normally he will continue to sing this stereotyped song for the rest of his life; however, under certain circumstances, this song can be modified. For example, the production of adult song in zebra finches can be modified by social context (Sossinka and Böhner 1980). Specifically, a zebra finch sings more often, and its song is more stereotyped and slightly faster, when that song is directed toward a female than when he is singing alone (Hessler and Doupe 1999). These socially driven modifications of behavior suggest a potential role for neuromodulators, such as norepinephrine or dopamine, in the learning and production of song.

A benefit of using songbirds as a model for studying the neural mechanisms underlying behavior is that both the learning and production of song are mediated by a discrete set of brain nuclei, known as the song system (Fig. 1A) (Nottebohm et al. 1976). The song system can be divided into two functional pathways: the motor pathway, which is obligatory for song production, and the anterior forebrain pathway (AFP), which is necessary for song learning but not for singing per se (Bottjer et al. 1984; Scharff and Nottebohm 1991). Although the AFP is not required for singing in adult birds, the pathway is necessary for adult song plasticity. For instance, the degradation of song caused by deafening (Nordeen and Nordeen 1992) is blocked by lesions of the output nucleus of the AFP, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Brainard and Doupe 2000). This evidence suggests that the AFP provides activity or some other element (i.e., trophic factor) necessary for both adult and juvenile song plasticity. Furthermore, lesions or inactivation of LMAN also abolish the context-dependent modulation of song (Kao and Brainard 2005; Olveczky et al. 2005). Thus activity in the AFP likely mediates differences in spectral variability between “directed” and “undirected” song. The premotor robust nucleus of the arcopallium (RA) therefore occupies a pivotal position in the song system as it lies within the motor pathway and...
to, and within, RA provides abundant loci for neuromodulatory effects.

In mammals, noradrenergic modulation can affect a variety of cognitive and motor functions including regulation of rhythmic activity in the hypoglossal nucleus (Selvaratnam et al. 1998) and olfactory memory in mice (Brennan et al. 1990) as well as behavioral state and decision-making in primates (reviewed in Aston-Jones and Cohen 2005). In songbirds, histological assays have found noradrenergic terminals throughout much of the telencephalon, including moderate levels in RA (Mello et al. 1998) that appear to arise from the locus coeruleus (LC) and subcoeruleus (Fig. 1B) (Appeltants et al. 2002).

Norepinephrine (NE) release in the nucleus interfacialis of the nidopallium (NII) gates auditory responses to HVC (Cardin and Schmidt 2004), a nucleus that likely drives auditory selectivity in many song system nuclei (Doupe 1997; Doupe and Konishi 1991). In RA, application of NE reduces the spontaneous firing of putative projection neurons (Solis and Perkel 2005); however, little is known about the action of NE on synaptic physiology in RA.

Another neurotransmitter thought to play a role in balancing converging inputs, such as those found in RA, is GABA, which can act on the metabotropic GABA<sub>B</sub> receptor as well as on the ionotropic GABA<sub>A</sub> receptor. GABA<sub>B</sub> receptors are present at many excitatory and inhibitory synapses in the forebrain (Bowery 1993) and often play a modulatory role in synaptic transmission. Activation of these receptors on presynaptic terminals decreases synaptic transmission by inhibiting calcium entry through voltage-gated calcium channels whereas postsynaptic GABA<sub>B</sub> receptors hyperpolarize the cell by activating potassium channels (Nicoll et al. 1990). GABA<sub>B</sub> receptor activation can affect such varied functions as pain processing, cognition, plasticity, and spatial memory (Bowery 1993, 2006; McNamara and Skelton 1996). Because RA contains GABAergic interneurons (Spiro et al. 1999), GABA<sub>B</sub> receptor activation may modulate synaptic transmission in the nucleus. In particular, differential effects of GABA<sub>B</sub> receptor activation in RA could lead to one set of inputs being favored over another.

Understanding how the activation of neuromodulatory receptors can affect synaptic activity in RA will provide a foundation for greater comprehension of the neural control of song behavior. Because the AFP has been directly implicated in developmental, contextual, and adult song plasticity, it will therefore be particularly interesting to know how this input to RA, via LMAN, can be affected by neuromodulation. Furthermore, because RA integrates input from the AFP with motor commands from HVC, differential modulation of inputs to RA may have a profound effect on both the learning and production of song behavior. We tested whether noradrenergic or GABA<sub>B</sub> receptor activation differentially affects synaptic inputs to RA projection neurons and asked whether these effects change over the course of song development in zebra finches.

**METHODS**

Adult male zebra finches were obtained from a supplier and housed in group cages, while juvenile birds were bred in our colony. The juveniles remained in a cage with their parents and siblings until the day of the experiment. All birds were housed in the same room and kept on a 14:10 light/dark cycle. All procedures were performed in...
acquaintance with a protocol approved by the University of Washington Institutional Animal Care and Use Committee.

Slice preparation

Experiments were performed using an acute brain slice preparation. Slices were prepared as described previously (Stark and Perkel 1999). Briefly, birds were anesthetized with isofluorane and decapitated, and their brains were removed and placed in ice-cold, oxygenated solution consisting of (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1 NaH2PO4, 16.2 NaHCO3, 11 p-glucose, and 10 HEPES (osmolality: 275-290 mosM). A vibrating microtome was used to make 300- to 400-μm coronal or horizontal sections containing RA. Slices were then transferred to 37°C artificial cerebrospinal fluid (ACSF), containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl2, 1 NaH2PO4, 26.2 NaHCO3, and 11 p-glucose (osmolality: 275-290 mosM) and allowed to sit for ≥45 min, during which time the solution was allowed to cool to room temperature.

Electrophysiology

“Blind” electrophysiological recordings (Blanton et al. 1989) were performed at 30 ± 1°C in oxygenated ACSF. For experiments examining excitatory synaptic inputs, 150 μM picrotoxin was added to the ACSF to block fast GABAergic inhibitory post-synaptic currents (IPSCs). Whole cell voltage-clamp recordings of RA projection neurons were made with electrodes pulled from borosilicate glass (Garner Glass, Claremont, CA) on a horizontal Flaming/Brown micropipette puller (Model P-97, Sutter Instruments, Novato, CA). Electrodes had a resistance of 4–7 MΩ when filled with an internal solution containing (in mM) 120 CsCH3SO3, 10 HEPES, 1 NaCl, 0.2 EGTA, 2 MgCl2, 10 phosphocreatine, 5 QX-314, 2 ATP, and 0.3 GTP. Excitatory post-synaptic currents (EPSCs) from HVC and LMAN were elicited by orthodromic electrical stimulation (100-μs duration) of the fiber tracts leading into RA using either a bipolar stainless steel or a platinum/iridium concentric bipolar electrode (FHC, Bowdoinham, ME). RA collateral inputs were activated by antidromic electrical stimulation (100-μs duration) of RA output fibers using a bipolar stainless steel electrode. Cells were held at +50 mV for the duration of the recording to remove the Mg2+ block of N-methyl-D-aspartate (NMDA) receptors.

The given large number of intrinsic neuronal excitatory synapses in RA (Hermann and Arnold 1991) and the spontaneous activity of RA projection neurons in vitro (Meitzen et al. 2007; Mooney 1992; Solis and Perkel 2006), the following steps were taken to ensure that the EPSCs studied here were monosynaptic in nature: 1) the cation concentration of the bath solution was raised to 4 mM Ca2+ and 4 mM Mg2+, using CaCl2 and MgCl2, to reduce spontaneous activity and overall neuronal excitability; 2) recordings with EPSC onset (measured as the point where the EPSC rose above noise) latencies that varied >0.4 ms (total range) at −70 mV were excluded; 3) EPSCs that did not smoothly rise to peak were excluded; and 4) EPSCs with variations in amplitude exceeding 50% between one trace and the next (not including failures) during the baseline period were excluded.

For experiments examining GABAergic transmission, a different internal solution was used that contained (in mM) 120 CsCl, 10 HEPES, 8 NaCl, 0.2 EGTA, 2 MgCl2, 10 phosphocreatine, 5 QX-314, 2 ATP, and 0.3 GTP. For these experiments, 1 mM kynurenic acid was added to the ACSF to block glutamatergic transmission and picrotoxin was omitted. Cells were held at −70 mV for the duration of the recording. Otherwise all conditions were identical to those described in the preceding text. IPSCs were elicited by electrical stimulation (100-μs duration) with a bipolar stainless steel electrode placed within the boundaries of RA.

Current signals were amplified and low-pass filtered at 10 kHz with an AxoPatch 1D (Axon Instruments, Foster City, CA). Signals were then digitized at 50 kHz with a Digidata 1322A (Axon Instruments) and stored on a PC using pClamp software (Axon Instruments). Data from both HVC and LMAN inputs were collected either interleaved with 10 s separating stimulations of an individual pathway or individually with 5 s between stimuli. Data from collateral and GABAergic inputs were collected individually with 5 s between stimuli. Occasionally collateral data were collected in conjunction with data from the LMAN input (Fig. 2D) in which case, sweeps were interleaved as described above. There were no differences between data collected with the two protocols so all data for a given input were pooled.

Drugs used in these experiments include: picrotoxin (PTX, Sigma), kynurenic acid (Kyn, Sigma), norepinephrine (NE, Sigma), baclofen (BAC, Sigma), yohimbine (Yoh, Sigma), UK 14,304 (UK, Sigma), bicuculline methiodide (BMI, Sigma), 6-cyano-7-nitroquinolinoxaline-2,3-dione (CNQX, Tocris), 2-amino-5-phosphonovaleric acid (APV, Tocris), and QX-314 (Tocris). All drugs were applied to the bath solution unless otherwise stated. The start of drug application was marked as the first sweep recorded in the presence of the drug. The duration of drug application varied by cell and experiment (range: 2–7 min).

Histology

All cells were filled with biocytin (Sigma) during recording. When an experiment was terminated, the slice was immersion-fixed in paraformaldehyde (4% in 0.1 M phosphate buffer) and kept at 4°C at least overnight. Slices were subsequently cryoprotected in a sucrose solution (30% in 0.1 M phosphate buffer). Slices were sectioned to 50 μm thickness with a freezing microtome and processed for visualization with an avidin-biotin horseradish peroxidase complex kit, (Vector ABC Elite Kit, Vector Laboratories, Burlingame, CA) using diaminobenzidine as the peroxidase substrate. Slices were then mounted on glass slides and analyzed under a light microscope to confirm cell type. Cells were categorized as projection neurons if they displayed spiny dendrites (Sapiro et al. 1999).

Data analysis

Recordings were excluded from analysis if the series resistance changed by >20% between the baseline period and drug application. Furthermore, only recordings from morphologically identified RA projection neurons were included for analysis. Measurements of PSC amplitude were made with Clampfit software (Axon Instruments) and then imported into Excel (Microsoft, Redmond, WA). Amplitude measurements were taken by averaging the peak amplitude of ten consecutive traces either just prior to drug application or immediately before drug washout to give the baseline and “drug” amplitudes, respectively. Paired-pulse ratios were calculated by applying two electrical stimuli (as described in the preceding text) separated by 40 ms and then dividing the peak amplitude of the response to the second stimulus by the peak amplitude of the response to the first (Supplementary Fig. S1). The numbers reported are the mean of the peak amplitude ratios of 6–10 traces from the period of maximal change in the EPSC amplitude during drug application (drug) or 10 traces immediately preceding drug application (baseline). Data were graphed using either Igor (Wavemetrics, Lake Oswego, OR) or Prism (Graphpad Software, San Diego, CA). Data are presented as the means ± SE unless otherwise stated.

Statistics

Statistical analysis was performed with Prism software. Unpaired t-tests were used to compare NE versus BAC modulation of IPSCs and NE modulation of the EPSCHVC versus NE + Yoh modulation of the EPSCLMAN. Paired t-tests were used to compare pre- vs. post-drug paired-pulse ratios. One-sample t-tests were used to compare the

1 The online version of this article contains supplemental data.
effects of each drug with respect to baseline for all inputs. All other comparisons were made using ANOVA with Tukey’s post hoc test. Differences were considered significant when $P < 0.05$.

**RESULTS**

We recorded from a total of 51 neurons from 31 adult zebra finches and 56 neurons from 27 juvenile (30–50 days post-hatch) zebra finches. All cells were confirmed to be RA projection neurons with post hoc histology (see METHODS).

*Norepinephrine preferentially reduces the LMAN input in adults*

To determine the modulatory effects of NE on excitatory inputs to RA, we bath applied NE to acute brain slices while recording from RA projection neurons. NE dramatically reduced the peak amplitude of the EPSC evoked by electrical stimulation of LMAN afferents (EPSC\textsubscript{LMAN}), while only slightly reducing the EPSC generated by stimulation of the HVC afferents (EPSC\textsubscript{HVC}; Fig. 2, A and B). Bath application of 10 µM NE reduced the EPSC\textsubscript{LMAN} to 20.0% of baseline (SE 3.3; Fig. 3A, $n = 19$). On the other hand, NE caused a much smaller reduction of the EPSC\textsubscript{HVC}, to 86.1 ± 4.5% of baseline (mean ± SE; Fig. 3A, $n = 13$). Similarly, the EPSC generated by stimulation of collateral synapses between RA projection neurons (EPSC\textsubscript{COLL}) in adult birds was only weakly affected by NE application (Fig. 2, C and D), which reduced the EPSC\textsubscript{COLL} to 81.4 ± 6.3% of baseline (Fig. 3A, $n = 9$). While NE caused a significant reduction from baseline in all three inputs ($P < 0.01$), the effect of NE on the EPSC\textsubscript{LMAN} was substantially greater than on the EPSC\textsubscript{HVC} or the EPSC\textsubscript{COLL} ($P < 0.001$). The effects of NE on EPSC\textsubscript{HVC} and EPSC\textsubscript{COLL} were not significantly different from each other in adult birds ($P > 0.05$).

*NE reduces both LMAN and collateral inputs in juvenile birds*

As in adult tissue, NE application reduced the EPSC\textsubscript{LMAN} to 18.6 ± 3.6% of baseline in RA from juvenile birds (Fig. 3B, $n = 19$) while only reducing the EPSC\textsubscript{HVC} to 68.8 ± 6.1% of baseline (Fig. 3B, $n = 16$). The effect of NE on both LMAN and HVC inputs in juveniles was not significantly different from that in adult birds ($P > 0.05$). However, NE application also reduced the EPSC\textsubscript{COLL} to 27.8 ± 6.7% of baseline in juveniles (Figs. 2, E and F, and 3A, $n = 11$), indicating a significantly greater effect of NE on the EPSC\textsubscript{COLL} than in adult birds ($P < 0.001$). The effect of NE on the EPSC\textsubscript{COLL}...
was not significantly different from the effect of NE on the EPSC$_{LMAN}$ in juvenile birds ($P > 0.05$), and both were significantly greater than the effect of NE on the EPSC$_{HVC}$ ($P < 0.001$). NE nonetheless significantly reduced the EPSC$_{HVC}$ from baseline ($P < 0.001$).

**Effect of NE on the EPSC$_{LMAN}$ is mediated by the $\alpha_2$ adrenergic receptor**

To determine which receptor subtype mediates the effect of NE, we pharmacologically manipulated the $\alpha_2$ adrenergic receptor. The specific $\alpha_2$ adrenergic receptor agonist UK 14,304 (10 $\mu$M) reduced the EPSC$_{LMAN}$ to $20.1 \pm 3.3\%$ of baseline (Fig. 4, $n = 6$) and reduced the EPSC$_{HVC}$ to $58.7 \pm 7.4\%$ of baseline (Fig. 4, $n = 6$). These values were not significantly different from the effect of NE on the EPSC$_{LMAN}$ or the EPSC$_{HVC}$ ($P > 0.05$). The specific $\alpha_2$ adrenergic receptor antagonist yohimbine (10 $\mu$M), applied 2 min prior to, and throughout, the application of NE, entirely blocked the effect of NE on the EPSC$_{LMAN}$ ($83.3 \pm 7.8\%$ of baseline; $P > 0.05$ compared with baseline; Fig. 4, $n = 5$). The findings that an $\alpha_2$ agonist mimicked the effect of NE and an $\alpha_2$ antagonist blocked the effect of NE indicate that the effect of NE on the EPSC$_{LMAN}$ is likely mediated by $\alpha_2$ receptors.

**GABA$_B$ receptor agonist baclofen preferentially reduces HVC and collateral inputs**

In contrast to the effects of NE, GABA$_B$ receptor activation at all ages dramatically reduced the EPSC$_{HVC}$ and the EPSC$_{COLL}$, while leaving the EPSC$_{LMAN}$ largely intact (Fig. 5, A–D) Bath application of the GABA$_B$ receptor agonist baclofen (30 $\mu$M) reduced the EPSC$_{HVC}$ to $21.5 \pm 4.5\%$ of baseline (Fig. 6A, $n = 14$). Baclofen exerted a similar effect on the EPSC$_{COLL}$, reducing the amplitude of the EPSC$_{COLL}$ to $25.7 \pm 4.7\%$ of baseline in adults (Fig. 6A, $n = 8$). The effect of baclofen on these two inputs was not significantly different ($P > 0.05$). Interestingly, the EPSC$_{LMAN}$ was much less affected by GABA$_B$ receptor activation. Baclofen only reduced the EPSC$_{LMAN}$ to $79.8 \pm 6.2\%$ of baseline in adults (Fig. 6A, $n = 14$), which, although a significant reduction ($P < 0.01$), was substantially smaller than the effect of baclofen on HVC or collateral inputs ($P < 0.001$).

In recordings from juvenile birds, baclofen reduced the EPSC$_{HVC}$ to $12.7 \pm 2.2\%$ of the baseline response (Fig. 6B, $n = 20$). Similarly, bath application of baclofen reduced the EPSC$_{COLL}$ to $11.2 \pm 3.3\%$ of baseline in juveniles (Fig. 6B, $n = 8$). There were no significant differences between the effects of baclofen on HVC and collateral inputs ($P > 0.05$) nor were there any differences between the results obtained from juveniles and adults at either of these synapses ($P > 0.05$). The EPSC$_{LMAN}$, on the other hand, was reduced to $52.5 \pm 5.0\%$ of baseline in juveniles (Fig. 6B, $n = 13$). The difference between the effect of baclofen on LMAN and HVC, or collateral, inputs was significant ($P < 0.001$). Baclofen also exerted a significantly greater effect on the EPSC$_{LMAN}$ in juveniles than in adults ($P < 0.001$).

**NE modulates GABAergic transmission in RA from juvenile, but not adult, birds**

To determine the modulatory effects of NE on inhibitory synaptic transmission in RA, we blocked glutamatergic trans-
mission and recorded inhibitory postsynaptic currents (IPSC) elicited by stimulation within RA (see METHODS). Evoked IPSCs were reversibly blocked by bicuculline methiodide (BMI, Fig. 7, A and B), confirming earlier reports that they are mediated by \( \gamma \)-aminobutyric acid (GABA) acting on GABA\(_\text{A} \) receptors (Mooney 1992; Spiro et al. 1999). In adult birds, bath application of NE reduced the IPSC to 80.1 \( \pm \) 3.2 on October 8, 2016 http://jn.physiology.org/ Downloaded from

GABA\(_\text{ergic} \) transmission is modulated by baclofen

In slices from adult birds, the GABA\(_\text{B} \) antagonist baclofen reduced the IPSC to 20.6 \( \pm \) 6.4% of baseline (Figs. 7, C and D, and 8A, \( n = 8 \)). Similarly, in juvenile tissue baclofen reduced the IPSC to 10.2 \( \pm \) 3.8% of baseline (Figs. 7, E and F, and 8B, \( n = 7 \)). The effects of baclofen in adults and juveniles were not significantly different (\( P > 0.05 \)), and at both ages, the IPSC was significantly reduced from baseline (\( P < 0.0001 \)).

Modulation of both glutamatergic and GABA\(_\text{ergic} \) transmission is presynaptic

As a first step toward establishing the locus of the modulatory effects of noradrenergic and GABA\(_\text{B} \) receptor agonists in RA, we calculated the paired-pulse ratio (PPR) for the baseline period and during drug treatments (see METHODS). Increases in the PPR are thought to indicate a reduction in release probability, suggestive of a presynaptic locus (Manabe et al. 1993; Zucker and Regehr 2002). Table 1 lists the PPRs for both glutamatergic and GABA\(_\text{ergic} \) inputs to RA projection neurons. Overall these data suggest that both NE and baclofen act presynaptically. NE significantly increased the PPR for LMAN inputs in both juvenile (\( P < 0.01 \)) and adult (\( P < 0.03 \)) birds without affecting HVC inputs (\( P > 0.05 \)). The PPR during UK application was significantly increased over baseline for the EPSC\(_\text{LMAN} \) (\( P < 0.01 \)) but not for the EPSC\(_\text{HVC} \) (\( P > 0.05 \)), which suggests that the \( \alpha_2 \) adrenoceptors mediating the modulatory effect of NE on inputs from LMAN are located presynaptically. Collateral synapses in juvenile birds, which are also modulated by NE, showed a significant increase in PPR in the presence of the drug (\( P < 0.03 \)), suggesting that NE acts presynaptically at collateral inputs. Conversely, baclofen significantly increased the PPR for HVC inputs in both age groups (juvenile: \( P < 0.01 \); adult: \( P < 0.015 \), while leaving the PPR for LMAN inputs unaffected (\( P > 0.05 \)). Baclofen also significantly increased the PPR for collateral inputs in juveniles (\( P < 0.005 \)). Finally, for GABA\(_\text{ergic} \) inputs in adults, baclofen caused a significant increase in the PPR (\( P < 0.02 \)) but NE did not (\( P > 0.05 \)), consistent with a presynaptic locus of GABA\(_\text{B} \) modulation and a lack of effect of NE. In juveniles a significant increase in the PPR of GABA\(_\text{ergic} \) inputs was caused by both baclofen (\( P < 0.02 \)) and NE (\( P < 0.032 \)) application. These data suggest that NE acts presynaptically to reduce the LMAN inputs to RA in both age groups and collateral and GABA\(_\text{ergic} \) inputs in juveniles and that baclofen reduces the HVC, collateral, and GABA\(_\text{ergic} \) inputs through presynaptic action as well.

Discussion

We have tested the effects of noradrenergic or GABA\(_\text{B} \) receptor activation on synaptic inputs to projection neurons in nucleus RA in both adult zebra finches and juveniles in the process of song learning. We find that bath application of
NE causes a large, reversible decrease in the amplitude of the EPSC_LMAN but has only a small effect on the EPSC_HVC or the EPSC_COLL in adult birds. The reduction of the EPSC_LMAN by NE in adults was mediated by α2 adrenoceptors, which are likely located on the presynaptic LMAN terminals. In juvenile birds, NE reduces both the EPSC_LMAN and the EPSC_COLL but had significantly less of an effect on the EPSC_HVC. NE showed no effect on the adult GABAergic IPSC but significantly reduced IPSC amplitude in juvenile birds, also with a presynaptic locus.

The GABA_B receptor agonist baclofen, on the other hand, caused a dramatic reduction in the amplitude of the EPSC_LMAN but has only a small effect on the EPSC_HVC or the EPSC_COLL in adult birds. The reduction of the EPSC_LMAN by NE in adults was mediated by α2 adrenoceptors, which are likely located on the presynaptic LMAN terminals. In juvenile birds, NE reduces both the EPSC_LMAN and the EPSC_COLL but had significantly less of an effect on the EPSC_HVC. NE showed no effect on the adult GABAergic IPSC but significantly reduced IPSC amplitude in juvenile birds, also with a presynaptic locus.

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The differential and specific nature of the effects of NE and baclofen in RA found in this study, combined with data from the literature (Mooney 1992; Solis and Perkel 2006; Spiro et al. 1999; Stark and Perkel 1999), allows the creation of a detailed working model of noradrenergic and GABA_B receptor action in RA (Fig. 9). Two aspects of the model require further explanation. First, the localization of the modulatory receptors to the presynaptic membrane of inputs provides the most parsimonious explanation for the observed changes in PPR, although further experiments are necessary to verify this. Second, the receptor composition of RA collateral synapses has yet to be formally described, but in our recordings, we found that the amplitude of the late phase of the EPSC (latency ≥50 ms) was much greater at depolarized potentials than at −70 mV (data not shown). This suggests that transmission at these glutamatergic synapses is mediated by both NMDA and non-NMDA receptors as depicted in the model (Fig. 9). Localization of modulatory receptors to different presynaptic terminals provides an elegant mechanism by which neuro-modulators may specifically modify individual inputs to an RA projection neuron.

When taken together, the reduction in GABA release by NE in juvenile birds and the effect of GABA_B receptor activation on both the HVC and collateral inputs create a situation in which the release of NE could control the balance of HVC and LMAN inputs to RA projection neurons. With a tonic background level of GABA release, activated presynaptic GABA_B receptors would create a tonic reduction in glutamatergic input from HVC and RA collaterals. The decrease in GABA release caused by NE in juvenile birds would then increase the amplitude of EPSCs from the HVC input by releasing them from the GABA_B receptor induced depression, while simultaneously reducing collateral and LMAN inputs directly. This mechanism provides a means by which one signal, NE, could alter the efficacy of excitatory inputs to RA in young birds within the critical period for song learning.

NE release may also create conditions in which the motor program underlying song production can be more efficiently executed. The results of Cardin and Schmidt (2004), that NE blocks input from NIf to HVC, and our finding that NE blocks LMAN input to RA lead to a situation in which NE release could effectively isolate the motor pathway from two of its major inputs: auditory information from NIf and AFP input from LMAN. Furthermore, Solis and Perkel (2006) proposed that NE may increase the signal-to-noise ratio of the HVC input to RA. Taken together, these results suggest that NE release in the song system may create a situation in which the bird would be able to enact a learned motor program without interference. This is particularly interesting in light of NE’s role in mediating arousal (Berridge 2006; Berridge and Waterhouse 2003) in that it suggests a means by which a stimulus (such as the presentation of a female) could cause the neural mechanisms controlling song production to become isolated from outside influences. This switching of modes, from “learning” to “recall,” may allow the bird to produce the best possible version of song that he is currently capable of singing without losing the capability to modify song later.

The reduction of input from LMAN to RA by NE in particular may have direct consequences for the contextual modulation of song production. In adult zebra finches, song sung alone (undirected) is slower and less stereotyped than song that is directed at a female (Sossinka and Böhner 1980). LMAN inputs into RA have been hypothesized to mediate this variability. Lesions or pharmacological inactivation of LMAN...
cause song in the undirected context to be as highly stereotyped as song directed at a female, suggesting that activity in LMAN increases variability of the undirected song (Kao and Brainard 2006; Ölveczky et al. 2005). Furthermore, electrical stimulation of LMAN can induce real-time changes in the fundamental frequency of a syllable in a bird’s song (Kao et al. 2005). One way that the song variability induced by LMAN activity could be reduced is by the blockade of LMAN inputs to RA by NE, which we have shown to affect predominantly this input to RA in adult birds. Given the role of NE in mediating arousal and sexual behavior in other species (Berridge 2006; Devidze et al. 2006), presentation of a female may cause NE release, which, among other actions, would lead to a blockade of LMAN input to RA, which in turn causes song to become more stereotyped. This hypothesis does not preclude the participation of other mechanisms for reducing LMAN-induced song variability, such as a decrease in LMAN multunit activity during directed song (Hessler and Doupe 1999; Kao et al. 2005) or changes in dopamine levels in Area X, which lies upstream from LMAN (Sasaki et al. 2006). These mechanisms could work synergistically to increase the stereotypy of song in the presence of a female. It will be interesting to determine whether infusion of noradrenergic receptor blockers, particularly of the α2 receptor subtype, into RA can reduce or prevent the contextual change in song initiated by the presentation of a female.

Our data show that the effect of NE on both the collateral and GABAergic inputs to RA projection neurons changes over the course of development, during the critical period in which the bird is also learning to sing. Although our experiments do not address how changes in modulation by NE may affect song learning, Aston-Jones and Cohen (2005) have suggested a role for the LC in the learning of cued behavior in primates. It therefore would be interesting to explore a potential role for LC activity and NE release in RA during song learning.

Noradrenergic input has effects on other parts of the song system besides RA, and possibly on other song-related brain regions as well. Castelino and Ball (2005) showed that contextual modulation of ZENK expression in Area X disappears after lesioning noradrenergic neurons with systemic DSP-4 treatment. Area X receives noradrenergic input from the locus coeruleus (Castelino et al. 2007), which is therefore likely to be activated during song. Studies in mammals have implicated NE...
release from the LC in the medial preoptic area (POM) in arousal and modification of behavioral state (Berridge 2006). In birds, the POM also receives input from the LC and may be necessary for motivating singing and other sexually motivated behaviors (Alger and Ritters 2006; Riters and Alger 2004; Riters and Ball 1999). It remains to be seen whether NE release in the POM modulates singing or other behaviors associated with arousal.

GABAB receptors can preferentially affect one functional set of inputs over another in the hippocampus (Ault and Nadler 1982; Colbert and Levy 1992), olfactory bulb (Isaacson and Vitten 2003), and cortex (Tang and Hasselmo 1994). It has been hypothesized that the activation of these receptors facilitates the transition between the acquisition and recall functions of these brain regions (Hasselmo and Fehlau 2001). It is possible that a similar process occurs in RA, where GABAB receptor activation decreases the efficacy of HVC and collateral inputs, resulting in increased influence of the input from LMAN.

A key question in the study of the neural mechanisms of learning in songbirds is how the AFP, which is essential for song learning, can lead to changes in song that remain once the AFP has been removed (i.e., adult zebra finches with AFP lesions continue to sing well) (Bottjer et al. 1984; Brainard and Doupe 2001; Scharff and Nottebohm 1991). One hypothesis is that the AFP somehow facilitates a lasting change in the motor pathway via the LMAN-RA synapse. Although no such mechanism has been described to date, the neuromodulatory effects described here may potentially play a role in mediating such changes. Studies in the hippocampus of rodents have suggested that GABAB receptor activation may facilitate the induction of LTP (Mott and Lewis 1991), one model by which this lasting change could be effected in RA. Furthermore, NE has been

FIG. 8. Baclofen reduces IPSCs at all ages, whereas norepinephrine reduces IPSCs only in juvenile birds. A: IPSC amplitude in adult birds measured as a percent of baseline for each drug condition. Each point represents an individual experiment and the horizontal bars depict the mean for each group. B: EPSC amplitude in juvenile birds.

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FIG. 9. Inputs to RA projection neurons are differentially modulated. This working model shows the hypothetical localization of receptors on an RA projection neuron and its afferents based on the data presented here and in the literature (see text).

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TABLE 1. Paired-pulse ratio increases when postsynaptic current amplitude decreases

<table>
<thead>
<tr>
<th>Condition</th>
<th>HVC</th>
<th>LMAN</th>
<th>Collateral</th>
<th>Interneuron</th>
<th>HVC</th>
<th>LMAN</th>
<th>Collateral</th>
<th>Interneuron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.8948</td>
<td>1.103</td>
<td>—</td>
<td>0.8163</td>
<td>1.024</td>
<td>1.132</td>
<td>0.5708</td>
<td>1.013</td>
</tr>
<tr>
<td>30 µM BAC</td>
<td>1.503</td>
<td>1.197</td>
<td>—</td>
<td>1.153</td>
<td>1.668</td>
<td>1.188</td>
<td>0.9714</td>
<td>1.875</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.768</td>
<td>1.097</td>
<td>—</td>
<td>0.7497</td>
<td>1.560</td>
<td>1.120</td>
<td>0.0498</td>
<td>0.9921</td>
</tr>
<tr>
<td>10 µM NE</td>
<td>1.333</td>
<td>1.575</td>
<td>—</td>
<td>0.7900</td>
<td>1.869</td>
<td>1.592</td>
<td>0.6761</td>
<td>2.092</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.8962</td>
<td>1.016</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10 µM UK</td>
<td>0.8843</td>
<td>1.258</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Numbers represent a ratio and therefore have no units. Bold values are significantly increased compared to baseline (P ≤ 0.03) LMAN, lateral magnocellular nucleus of the anterior nidopallium; BAC, baclofen; NE, norepinephrine; UK, UK 14,304.
implicated in the enhancement of certain forms of LTP (Hopkins and Johnston 1988). It therefore becomes important to understand the interactions of these two neuromodulators with the synaptic inputs to RA projection neurons, not only for the effect they might have on song production but also for the role they may play in the process of song learning.

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