Noradrenergic Modulation of Activity in a Vocal Control Nucleus In Vitro

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INTRODUCTION

Noradrenergic cells project expansively throughout the brain, giving norepinephrine (NE) the potential to modulate diverse brain functions. NE can strongly modify sensory processing, increasing the salience of sensory responses (Berridge and Waterhouse 2003). In contrast, the effect of NE on motor and sensorimotor systems is less well understood. NE has been linked to increased motor neuron excitability (Fung et al. 1991), sensorimotor gating (Oranje et al. 2004; Sallinen et al. 1998), facilitation of behavioral responses (Clayton et al. 2004), and motor learning (Plewnia et al. 2004). Thus in addition to its better-known sensory role, NE can influence motor function. A recent theory proposes that NE functions to optimize task-related performance, either by facilitating responses within a task or by enabling a switch to a task with greater use to the organism (Aston-Jones and Cohen 2005).

To explore further the role of NE in motor outputs, it is useful to consider the song control system in oscine birds. The song system is the neural circuit controlling song behavior (Fig. 1A) (Nottebohm et al. 1976, 1982), which is a learned motor output composed of stereotyped sequences of vocalizations. The song system is widely innervated by a number of neuromodulatory inputs, including catecholaminergic and cholinergic afferents (Bottjer 1993; Lewis et al. 1981; Mello et al. 1998; Ryan and Arnold 1981; Soha et al. 1995). These neuromodulators potentially mediate the sensitivity of song behavior to social (Collins 2004; Nowicki and Searcy 2004) and seasonal (Brenowitz 2004) factors. In particular, NE modulates auditory responses in the song system (Cardin and Schmidt 2004; Dave et al. 1998). Moreover, NE has been associated with song production: decreases in NE levels decrease singing frequency (Barclay et al. 1996) and singing-related gene expression (Castelino and Ball 2005). The combination of a stereotyped behavior with a discrete neural circuit provides a model system for exploring the diverse roles of NE in the brain, including motor systems.

The robust nucleus of the arcopallium (RA) is a song system nucleus that is a potential site for neuromodulator-induced effects on song behavior. RA is part of the motor pathway, which connects the forebrain to the syrinx, the avian vocal organ, and to premotor areas controlling respiration (Fig. 1A). RA neurons display premotor activity during singing that is notable for its precision (Chi and Margoliash 2001; Leonardo and Fee 2005; McCasland 1987; Yu and Margoliash 1996). Although RA has both auditory and premotor responses, its function is essential for vocal production: lesions effectively mute the bird (Nottebohm et al. 1976; Simpson and Vicario 1990). Activity in RA can be influenced by the anterior forebrain pathway (AFP) (Kao et al. 2005; Ölveczyk et al. 2005), a pathway that is important for song learning but not for production (Bottjer et al. 1984). RA activity is also potentially modified by neuromodulators, including NE. RA receives moderately dense NE terminals (Mello et al. 1998; Sakaguchi and Saito 1989) that originate from the locus coeruleus (LC) (Appeltants et al. 2002), and RA cells have adrenergic receptors (Revilla et al. 1999; Riters and Ball 2002). To begin to understand the influence of NE on RA activity, we studied the effect of NE on spontaneously active RA neurons in vitro. We found that NE markedly reduced spontaneous activity through α2-adrenergic receptors and activated an increase in conductance that did not prevent spikes evoked by afferent activity.

METHODS

Slice preparation

Data were obtained from 38 adult male zebra finches (Taeniopygia guttata) purchased from suppliers. All procedures were in compliance
The storage and recording ACSF was the same as the slicing ACSF, vibrating microtome in ice-cold ACSF and transferred to a storage NaHCO₃, 11-D glucose, and 10 HEPES (osmolarity 275–290 mOsm).

**Electrophysiology**

placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 120 K-methylsulfate, 10 HEPES, 2 CaCl₂, 1 NaH₂PO₄, 16.2 NaHCO₃, 11-D glucose, and 10 HEPES (osmolarity 275–290 mOsm). Parasagittal or coronal slices 350–400 µm thick were cut with a vibrating microtome in ice-cold ACSF and transferred to a storage chamber containing ACSF heated to 35°C. Once slicing was completed, the storage chamber was allowed to cool to room temperature. The storage and recording ACSF was the same as the slicing ACSF, except that the HEPES was replaced with equiosmolar NaHCO₃; this solution is also referred to as “control Ringer.” All solutions were bubbled with 95% O₂-5% CO₂.

**Electrophysiology**

Recording began >1 h after slices were made. For recording, a slice was submerged in a small chamber perfused with the HEPES-free ACSF at 2 ml/min. ACSF temperature was maintained at 30°C. RA, HVC, and the axon tract between them are visible in brain slices when trans-illuminated and viewed with a microscope.

Extracellular and perforated patch techniques were used to record RA cells. Both types of electrodes were pulled with a Sutter Instru-

ments (Novato, CA) P-97 micropipette puller. Extracellular recordings were made using glass pipettes pulled to tip widths of 5–10 µm. The pipettes were filled with a 0.9% NaCl solution, and the resulting resistances ranged from 3 to 10 MΩ. Single units were isolated, and their waveforms had a signal-to-noise ratio of ≥3. Voltage signals were initially amplified with an Axoclamp 2B (Axon Instruments, Foster City, CA) and then low-pass filtered (5 kHz) and further amplified with a Brownlee Model 410 amplifier (San Jose, CA). Total signal amplification for extracellular recordings was 1,000 times. The filtered signals were digitized at 10 kHz with a National Instruments (Austin, TX) digitizing board and stored on a PC using customized data acquisition programs written in LabView (National Instruments) by M. Farries (University of Washington) and D. Perkel. Data were collected during 5-s-long trials with 5 s between each trial (except for experiments in which NE was pressure-applied locally). Similar extracellular recording methods in RA slices have been used by Park et al. (2005).

Current-clamp recordings were made with the gramicidin-perforated patch method (Rhee et al. 1994). Glass electrodes were pulled to a tip width <2 µm, and the tip of the pipette was filled with internal solution that consisted of (in mM) 120 K-methylsulfate, 10 HEPES, 2 EGTA, 8 NaCl, 2 ATP, 0.3 GTP, and 1 MgCl₂; pH was 7.3 and osmolarity was 0–5% less than the ACSF osmolarity. The rest of the pipette was filled with the same internal solution supplemented with a gramicidin solution: gramicidin (Sigma, St. Louis, MO) stock solution was made fresh in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) at a concentration of 0.1–0.3 mg/ml; this solution was added to the internal solution to make a final concentration of 0.1–0.3 µg/ml. Final resistance were 5–8 MΩ. Once a gigaohm seal was achieved using the blind patch technique (Blanton et al. 1989), the recorded potential stabilized within 10 min, and the series resistance stabilized at ~200 MΩ within 20 min. Input resistance and resting potential were monitored throughout the experiment, and a cell was not included in the data set if either varied by >20%. In some cases, 10 mM biocytin (Vector Laboratories, Burlingame, CA) was included in the internal solution for histological identification of the cells recorded. To allow access of the biocytin to the cell, at the end of the experiment, the patch was ruptured with gentle negative pressure. Current-clamp voltage signals were amplified 100 times, low-pass filtered at 3 kHz, and digitized at 6 kHz.

For stimulation experiments, a stimulating electrode was placed within HVC or immediately ventral to HVC within the HVC-RA fiber tract. The stimulating electrode was either a stainless steel bipolar electrode or a platinum/rhodium concentric bipolar electrode (FHC, Bowdoinham, ME). Single stimulus pulses to HVC or the HVC-RA fiber tract were delivered until a spike was consistently evoked from an RA cell (recorded extracellularly) within 5 ms; across cells, the latency averaged 3.3 ms (SD) from the stimulus pulse onset and the spike (measured at the maximum negative-going deflection) was 3.3 ± 0.8 ms (SD) at 100 Hz for 20–30 ms (i.e., 3 or 4 pulses per train, respectively); the interval between each train was 1 s. An example of this stimulus is shown in Fig. 1B. Spikes evoked by this stimulation were counted as those occurring within 5 ms of each stimulation pulse. The total number of spikes (evoked or not) was counted during a period circumscribed by the first pulse of the first train and the last pulse of the third train (+5 ms).

Drugs used in these experiments included 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Cookson, Ellisville, MO), 2-amino-5-phosphonovaleric acid (APV; Tocris), kynurenic acid (KA; Sigma), picrotoxin (PTX; Sigma), CdCl₂ (Sigma), NE (Sigma), yohimbine (Sigma), and clonidine (Sigma). Unless otherwise noted, drugs were bath applied. We marked the start of drug application as the first trial...
with any drug present. The duration of bath application of the drug varied between cells (range: 1.4–11.4 min).

Local application of NE was made to four cells using a glass pipette with a 10-μm tip diameter that was filled with 10 mM NE and placed within 100 μm of the recording electrode. The NE solution was pressure applied for brief durations (10–40 ms) at 10–14 PSI using a Pressure System IIe (Toohey Company, Fairfield, NJ). This resulted in pressure applied for brief durations (10 – 40 ms) at 10 – 14 PSI using an average percent change in amplitude was 8.5%/H11021

/h11021

of cells (\(n\) = 0.02 (SD), \(n\) = 0.07 (SD), \(n\) = 0.02 (SD), \(n\) = 0.02 (SD), \(n\) = 0.02 (SD)). The percent changes in frequency values obtained from one bird was compared with a simulated distribution of variances that resulted from random draws from the pool of all percent change in frequency values obtained from all birds. The distribution was constructed from 100 Monte Carlo simulations, which randomly selected \(n\) percent change in frequency values from the entire pool of values, where \(n\) equals the number of cells recorded in each bird. The median of the simulated variance distribution was compared with the variance of the values obtained from the bird, using a one-sample test (1-tailed). If the experimental variance was significantly less than the median variance of the simulated distribution, the values for that bird were considered “clustered.” This procedure was repeated for each bird.

**Histology**

In cases in which cells were filled with biocytin, slices were immersion-fixed in paraformaldehyde (4% in 0.1 M phosphate buffer) and stored at 4°C overnight. Slices were subsequently cryoprotected in a sucrose solution (30% in 0.1 M phosphate buffer) and stored at 4°C overnight. Slices were resectioned to 40 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 dianinobenzidine as the peroxidase substrate. Sections were counterstained with cresyl violet.

**Data analysis**

Spikes were detected using procedures written in IGOR 4.0 (WaveMetrics, Lake Oswego, OR) by M. Solis. Spike detection depended on two parameters set by the user: 1) a minimum height of the differentiated waveform (expressed in SD of average of the entire waveform), which constrained the initial slope of an acceptable spike waveform, and 2) a minimum amplitude of the spike. If a spike met both criteria, the user was prompted to accept or reject the spike. The user could compare the spike’s height and width to previously accepted spikes in making this decision. These parameters were maintained for detecting postsuppression spiking. Thus this off-line spike discrimination could further confirm that the recording came from a well-isolated cell. All cells used in this study had stable spike waveforms during a baseline period of 5 min: their spike amplitudes had a CV of 0.10 or less (mean = 0.07 ± 0.02 (SD), \(n\) = 108).

When spiking resumed after a period of NE-induced suppression, we verified that we were recording from the same cell. For this subset of cells (\(n\) = 53), the same parameters for spike detection were used both before and after NE-induced suppression. In comparing presuppression spike amplitude to postsuppression spike amplitude, the average percent change in amplitude was 8.5 ± 8.1% (SD) (\(n\) = 53). Eighty-nine percent of these cells had a percent change in amplitude <20%; 74% had a percent change in amplitude <10%. For cells with percent changes in amplitude >20%, we verified that they were the same cell based on spike shape, and the lack of other units in the recording. If this subset of cells was eliminated from this study, our findings would not change.

Once spikes were detected, spike frequency and the CV of frequency were measured for each trial. Spike frequency was calculated as the mean of the reciprocals of the individual intervals between spikes (i.e., average instantaneous frequency). The CV was the SD of each frequency measurement divided by the mean. To assay the effect of drugs on these parameters, values were averaged from trials collected during the last minute before drug application and compared with the averaged values from the trials collected during the last minute of drug application. Unpaired t-tests (IGOR) determined whether these average values were significantly different from each other for a single cell. Effects of drug were described as increases or decreases in firing only when this test determined a significant difference from control. The change induced by a drug was calculated as a percent change relative to predrug values: (drug value – predrug value)/predrug value. Positive values indicated increases, negative values indicated decreases, and values near 0 reflected no change relative to control values.

For perforated-patch recordings, we measured input resistance by delivering hyperpolarizing current pulses (1 s long). The steady-state voltage was measured during the last 200 ms of the current pulse and compared with 200 ms of baseline just before current pulse delivery. These values were used to construct I-V plots.

Statistical analyses of cell populations were done with Prism (GraphPad Software, San Diego, CA); tests were two-tailed. Parametric tests were used for distributions that passed the Kolmogorov-Smirnov goodness of fit test for normality; otherwise, nonparametric methods were used.

A cluster analysis was used to determine whether the effects of NE on RA cells obtained from the same bird were more similar than expected by chance. To do this, the variance of the NE-induced percent change in frequency values obtained from one bird was compared with a simulated distribution of variances that resulted from random draws from the pool of all percent change in frequency values obtained from all birds. The distribution was constructed from 100 Monte Carlo simulations, which randomly selected \(n\) percent change in frequency values from the entire pool of values, where \(n\) equals the number of cells recorded in each bird. The median of the simulated variance distribution was compared with the variance of the values obtained from the bird, using a one-sample test (1-tailed). If the experimental variance was significantly less than the median variance of the simulated distribution, the values for that bird were considered “clustered.” This procedure was repeated for each bird.

**RESULTS**

Extracellular recordings of the spontaneous activity of 108 isolated RA neurons (median peak-to-peak spike height was 0.52 mV) were made in the brain slices. As previously reported in vivo (Adret and Margoliash 2002; Yu and Margoliash 1996) and in vitro (Mooney 1992), RA neurons exhibited spontaneous activity that was characterized by regular interspike intervals (Fig. 1B). The mean firing rate in control Ringer was 5.5 ± 2.8 (SD) Hz (range: 1–16 Hz, \(n\) = 88) and mean CV of firing rate was 0.15 ± 0.12 (SD) (\(n\) = 88). Figure 1C plots the mean firing rate and CV for each cell, revealing an indirect relation between the two; however, no distinct clusters emerged in this plot, suggesting that these recordings were made from a fairly homogeneous population of RA cells with respect to spontaneous activity.

**Effects of NE on spontaneous activity in RA**

The predominant effect of NE on RA neurons was to suppress spontaneous activity: 75% of cells significantly decreased their firing. Figure 2A shows a case in which 10 μM NE applied to the bath abolished the cell’s firing quickly and reversibly. Comparisons of pre- and postsuppression spike waveforms verified that the same cell was recorded on washout of NE (Fig. 2, B and C). In all, 47.9% (23/48) of cells tested exhibited complete suppression of their firing in NE. The effect of NE on all cells is plotted in Fig. 2B: in addition to complete suppression, NE decreased (but did not abolish) firing, increased firing, or did not change firing rates (Table 1). This range of effects was quantified by calculating the percent change in firing rate in NE relative to control (see METHODS). The resulting percentage is plotted for each cell in the summary graph in Fig. 8 (NE column). For the population of cells tested, NE caused a significant reduction in firing rate, from an average of 4.7 Hz in control conditions to an average of 2.0 Hz
in the presence of NE (Fig. 2E; paired t-test, \( P < 0.0001; n = 48 \)).

We investigated whether the NE-induced suppression resulted from a direct effect of NE on the cell recorded or from an indirect effect produced by other cells. Thus we examined the effect of NE under two conditions: when fast excitatory and inhibitory transmission was blocked with 1 mM kynurenic acid (KA) and 150 \( \mu M \) picrotoxin (PTX), or when synaptic transmission altogether was blocked with 100 \( \mu M \) CdCl\(_2\). Under both conditions, the suppressive effect of NE was maintained. Figure 3A shows an example in which NE abolished the spontaneous firing of an RA cell in the presence of 100 \( \mu M \) CdCl\(_2\). The scatter plot in Fig. 3B shows the effect of all

<table>
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<th>Condition</th>
<th>Decreased† (Abolished)‡</th>
<th>Increased</th>
<th>No Change</th>
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<tr>
<td>control, ( n = 48 )</td>
<td>38 (23)</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>KA+PTX, ( n = 52 )</td>
<td>35 (24)</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>CdCl(_2), ( n = 8 )</td>
<td>8 (6)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL, ( n = 108 )</td>
<td>81 (53)</td>
<td>16</td>
<td>11</td>
</tr>
</tbody>
</table>

*Condition refers to the Ringer solution prior to NE application; \( n \) refers to number of cells tested. †Changes in firing (decrease, increase, or no change) were determined for each cell by unpaired t-tests between pre-NE and NE trials. ‡Numbers in parentheses indicate the subset of cells that were silenced by NE application. NE, norepinephrine; RA, robust nucleus of the arcopallium.

FIG. 2. Norepinephrine (NE) suppressed spontaneous activity of RA cells. A: raster plot shows that application of 10 \( \mu M \) NE abolished spontaneous activity of a single cell recorded in RA. Each tick mark indicates a spike that occurred during a 5-s sweep. Thick line on right indicates time of NE application, which lasted 1.42 min. Regular spontaneous activity recovered quickly during washout. B: left: recovery (post-NE, black) spike waveforms overlay baseline (pre-NE, red) waveforms. Right: area in red denotes mean waveform ± SD of baseline spikes. Black line plots mean waveform for recovery spikes, and error bars are SD. For both panels, the last 10 spikes before silencing are compared with the 1st 10 spikes recorded during recovery. C: scatter plot of spike height and width compares baseline spikes (red circles, \( n = 371 \)) to recovery spikes (black dots, \( n = 613 \)). D: scatter plot compares the firing rate in control Ringer (control) to that measured during the last minute of NE application. Each open circle represents a neuron. Diagonal line is where points would lie if there was no change in firing rate in presence of NE. Majority of cells showed a decrease in firing (i.e., lie below diagonal line). E: on average, there was a significant decrease in firing rate in NE for population of RA cells shown in D (*significant difference). Error bars represent SE.

FIG. 3. NE-induced suppression was likely a direct effect. A: NE abolished spontaneous activity of a neuron in RA recorded in presence of 100 \( \mu M \) CdCl\(_2\). Each point represents firing frequency of cell measured for each trial. B: scatter plot compares spike rate under baseline conditions to spike rate during NE application. Each point represents a cell; those recorded with fast excitatory and inhibitory transmission blocked (KA+PTX) are indicated with open circles, and those recorded with all synaptic transmission blocked (CdCl\(_2\)) are marked with solid circles. Diagonal line indicates where points would lie if firing rates were the same for baseline and NE conditions. C: on average, NE decreased firing rate from baseline frequencies, which were measured during either type of blockade of synaptic transmission. Error bars indicate SE, and asterisk marks significant decrease. D: effect of NE was similar at early and later time-points of NE application. Scatter plot compares degree of NE effect at end of NE application (later) and during the 2nd minute of NE application (early). Each point represents a cell. Diagonal indicates where points would lie if effect was the same for these 2 time-points. E: spike frequency is plotted against CV for each neuron that showed significant changes in firing rate in response to NE (solid circles). These data lie within range of values obtained from RA cells recorded under control conditions (open circles); control data are the same points shown in Fig. 1C. Each point represents a neuron.
applications of NE to cells under these two conditions. One-half of the cells tested under these conditions showed complete suppression of their firing rates in the presence of NE (30/60 cells), and for the population, there was a significant decrease in firing in the presence of NE (Fig. 3C; paired t-test, \( P < 0.0001; n = 60 \)). As for experiments conducted with control Ringer, neurons exhibited a range of responses, including significant decreases, increases, and no changes in their firing rates in response to NE (Fig. 3B; Table 1). This range of effects is also shown in Fig. 8 (NE in KA + PTX and NE in CdCl₂ columns), where the percent change in firing rate in NE for each cell is plotted. The average percent change in frequency was \(-63\%\) for NE, \(-50\%\) for NE in KA + PTX, and \(-96\%\) for NE in CdCl₂. The effect of NE did not differ across all three conditions (i.e., 1st 3 columns in Fig. 8; Kruskal-Wallis test, \( P < 0.1080 \)). For the subset of neurons with significant reductions in firing, the average percent change in frequency was \(-82\%\) (\( n = 23 \)) for NE in control Ringer, \(-85\%\) (\( n = 35 \)) for NE in KA + PTX, and \(-96\%\) for NE in CdCl₂ (\( n = 8 \)).

The measurements of spike frequency in the presence of NE were taken during the last minute of drug application, and thus reflect a stabilized change in spike frequency. The duration of drug application averaged 4.3 ± 1.8 (SD) min (\( n = 108 \)), but varied from cell to cell. To check for transient changes in spike frequency in NE application and to compare responses across cells at the same time point, we also measured the frequency of RA spiking during the second minute of NE application. These “early” spike rates were compared with the later spike rates previously measured for each cell in Fig. 3D. Whereas some cells showed slightly higher or lower spike rates at this earlier time-point than the later measurement, overall there were no consistent differences between these two time-points (\( P < 0.8893 \), paired t-test; \( n = 98 \)); some cells were not included in this analysis because NE application in these cases was not longer than 2 min. Similarly, the average percent change in frequency at this early point of NE application was \(-42\%\) relative to control (early NE column in Fig. 8).

For neurons that decreased but maintained spontaneous activity in the presence of NE, there was a decrease in spiking regularity. For these cells, there was a significant twofold increase in CV during NE application: the mean CV was 0.13 ± 0.18 (SD) before NE application (includes all Ringer types) and 0.30 ± 0.20 (SD) in NE (\( P < 0.0058 \), paired t-test; \( n = 28 \)). For those cells with significant changes (increases or decreases) in firing rate to NE, the relationship between frequency and CV was similar to that in control Ringer (Fig. 3E); thus spontaneous firing rate and regularity covaried and may be similarly regulated.

Next we examined whether those cells that did not decrease their firing rates in NE had a suppressive response to higher doses. When exposed to higher concentrations of NE (ranging from 20 to 100 \( \mu M \)), these cells maintained their spontaneous activity levels (Fig. 4A). The average spontaneous rate of these cells slightly increased in response to high doses of NE relative to that measured for 10 \( \mu M \) NE (Fig. 4B; paired t-test, \( P < 0.0235 \); \( n = 12 \)). There was no effect of the maximum dosage on firing CV (Fig. 4C; paired t-test, \( P < 0.3649 \); \( n = 12 \)). Thus a subpopulation of RA cells could not be induced to exhibit a suppressive response to NE.

The response of a cell to NE was not predictable from its baseline firing rate, its firing regularity (as measured by CV), or the individual bird from which it came. No correlation was found between the percent change in firing rate in NE and the baseline firing rate for each cell (\( r^2 = 0.027, P < 0.1016 \)). Similarly, there was no clear relation between the percent change in NE and CV for each cell (\( r^2 = 0.003, P < 0.5970 \)). Finally, neural responses to NE did not vary according to bird. Figure 4D plots the percent change in NE for each cell as a function of bird; in several birds, cells with a suppressive response coexisted with cells that did not (e.g., bird 2). A cluster analysis (see METHODS) did not find significant clustering of the percent change in NE values obtained from each bird.

Thus within the population of spontaneously active neurons in RA, there were distinct differences in responsiveness to NE.

**NE-induced suppression was mediated by \( \alpha_2 \)-adrenergic receptors**

Given the extensive evidence for \( \alpha_2 \)-adrenergic receptors in RA (Ball 1994; Casto and Ball 1996; Riters and Ball 2002; Riters et al. 2002), we investigated their role in the NE-induced suppression using yohimbine, a selective \( \alpha_2 \)-adrenergic receptor antagonist. Yohimbine readily reversed the suppressive
Yohimbine reversed the suppressive effect of NE. A: firing rate of a RA neuron is plotted against time during an experiment in which 1 mM KA +150 μM PTX, 10 μM NE, and 1 μM yohimbine were applied sequentially. Spontaneous activity returned in the presence of yohimbine + NE. B: firing rates of all neurons involved in the same experiment described in A are shown for baseline conditions in the presence of 10 μM NE and in the presence of 10 μM NE + 1 or 5 μM yohimbine (yoh). Lines connect responses recorded from the same neuron. Brackets and asterisks indicate those comparisons that yielded statistically significant differences. C: scatter plot compares baseline firing rate to that obtained in the presence of yohimbine alone. Each point represents a neuron, and diagonal line indicates where points would lie if these values were equal. Cells tested with 1 or 5 μM yohimbine are indicated by circles or squares, respectively.

This lack of change in yohimbine alone was also reflected in the percent change in frequency in NE (yoh column in Fig. 8), which averaged 12%. Moreover, significant effects were not apparent when the two concentrations of yohimbine were considered separately (paired t-test between control and yohimbine firing rates; for 1 μM P < 0.9162, n = 8 and for 5 μM P < 0.3396, n = 6). Thus we found no evidence for endogenous NE-induced suppression of firing rates.

Another possible explanation is that the suprarecovery observed with NE + yohimbine treatment reflected suppression-induced plasticity in spontaneous firing rate (Nelson et al. 2003). To examine this, we compared each cell’s firing rate before NE application to its recovered firing rate upon washout of NE (as defined as the rate at 5 min after the return of spiking); both the pre-NE and recovery time-points are shown in Fig. 6A. Only cells with NE-induced suppression were included in this analysis. Figure 6A shows an example of a neuron that had a slightly (and significantly) elevated firing rate after recovery from NE-induced suppression. Its pre-NE firing rate was 5.4 Hz, whereas upon recovery its rate was 6.8 Hz; this amounted to an increase of 25.9% relative to baseline. Many cells had similar elevations during recovery, as shown in Fig. 6B, which plots pre-NE baseline rates against recovery rates. Significant increases were observed for 48.8% of cells (2041), and for the population, there was a significant increase in firing rate upon recovery from NE effects (Fig. 6C; P < 0.0242, paired t-test; n = 41). This constituted a 39.8% average increase relative to baseline (post-NE column in Fig. 8). Whether a cell showed an elevated firing rate during recovery was not apparent from the amount of time NE was applied (percent change in frequency during recovery vs. minutes NE applied; \( r^2 = 0.046, P < 0.1791 \)) nor from the degree of NE-induced suppression (percent change in frequency during recovery vs. percent change in frequency in NE; \( r^2 = 0.0596, P < 0.1242 \)). There was an inverse relationship between the baseline firing rate and the percent change in firing during recovery from NE; cells with lower firing rates were more susceptible to suprarecovery than cells with higher firing rates. This trend was weak, however (\( r^2 = 0.1356, P < 0.046, P < 0.0006; n = 11 \)). This suprarecovery was also apparent in the percent change in frequency in NE + yohimbine for all cells (NE+yoh column in Fig. 8); the average was 140%. This suggested that, in addition to antagonizing the exogenously applied NE, yohimbine may have blocked the effects of endogenously released NE.

To test this, we monitored the effect of yohimbine alone on the firing rates of RA cells that were previously determined to suppress their firing in NE. After NE washout, application of yohimbine alone did not increase firing rates relative to baseline (pre-yohimbine) levels (\( n = 14 \)). Figure 5C compares the baseline firing frequency for each cell to the frequency in the presence of yohimbine alone; most points lie along the diagonal line, which indicates similar firing rates under both conditions. Overall, there was no significant difference between baseline firing rates and those measured in the presence of 1 or 5 μM yohimbine alone (P < 0.6044, paired t-test; \( n = 14 \)). This lack of change in yohimbine alone was also reflected in the percent change in frequency (yoh alone column in Fig. 8), which averaged 12%. Moreover, significant effects were not apparent when the two concentrations of yohimbine were considered separately (paired t-test between control and yohimbine firing rates; for 1 μM P < 0.9162, n = 8 and for 5 μM P < 0.3396, n = 6). Thus we found no evidence for endogenous NE-induced suppression of firing rates.

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0.0179). Regardless, washout from the suppressive effects of NE was associated with increases in firing rates.

Because the NE-induced suppression was blocked by yohimbine, we tried to mimic the effect of NE with a selective $\alpha$-adrenergic receptor agonist, clonidine. Clonidine reliably decreased the spontaneous firing rates of RA cells, as shown by the cell in Fig. 7A. All cells showed a decrease in the presence of 10 or 50 $\mu$M clonidine (Fig. 7B); for 18/19 cells (94.7%), this decrease was significant. Across the population, clonidine caused a significant decrease in firing rate relative to baseline levels ($P < 0.0006$, paired t-test; $n = 19$), and the average percent change in frequency was $-47.2\%$ (clonidine column in Fig. 8). For the subset of cells with a significant reduction in the presence of clonidine, the average percent change in frequency was $-48.3\%$. Thus, consistent with a role for $\alpha$-adrenergic receptors, clonidine mimicked the suppressive effect of NE. Unlike NE, however, clonidine rarely abolished spontaneous activity of RA cells (Figs. 7B and 8). Clonidine eliminated firing in 5.3% (1/19) of cells tested, whereas NE eliminated firing in 49.1% (53/108) of cells tested; this difference in incidence was significant ($P < 0.0003$, Fisher’s exact test). Finally, clonidine did not significantly alter spiking regularity, as measured by the CV of firing frequency ($P < 0.1054$, Wilcoxon; $n = 19$).

Given the range of responses to NE, we directly compared the response of a cell to NE with its response to clonidine (Fig. 7C). In these experiments, NE application preceded clonidine application, because effects of clonidine were not readily reversible. After NE was applied, a period of washout allowed spiking to resume its predrug frequency and regularity (on average 8 min after NE application); clonidine application followed. We found no significant difference in the average percent change in frequency elicited by NE and by clonidine ($P < 0.1280$, paired t-test; $n = 14$); however, clonidine and NE did not have identical effects in the same cell. For example, cells that lost all spontaneous activity in the presence of NE maintained firing in the presence of clonidine, albeit at a decreased level relative to control. Moreover, three cells that slightly increased their firing in response to NE had a suppressive response revealed in the presence of clonidine. This raised the possibility of roles for other adrenergic receptor types in regulating spontaneous activity in RA. Overall, clonidine mimicked the suppressive effect of NE, but not to the same degree.

**NE-induced suppression involved a conductance increase**

To determine whether the NE-induced suppression involved a change in conductance, we made perforated patch recordings from spontaneously active RA cells. Perforated patch recordings maintained the spontaneous activity of RA neurons more reliably than whole cell recordings, which allowed us to assay the effect of NE on spontaneous activity as well as on conductance. The top of Fig. 9A shows the average voltage responses of one cell to a $-20$-pA current pulse under control conditions and in the presence of 10 $\mu$M NE. The bottom compares the neuron’s responses to multiple hyperpolarizing current pulses in control conditions and in NE in an I-V plot. NE induced a decrease in input resistance for this cell, as indicated by the decrease in slope of the I-V relationship in NE. Similarly, increases in conductance were measured for all cells that significantly decreased their firing in the presence of NE; the average increase in conductance was $1.59 \pm 1.08$ (SD) nS ($n = 10$). This was equivalent to a 81.4% increase relative to control conductance; the individual values for all 10 cells are shown on the ordinate of Fig. 9B. The degree of conductance change tended to be larger for cells with higher baseline firing rates (Fig. 9B); however, this was not statistically significant ($r = 0.2827$, $P < 0.1138$). Furthermore, there was no significant correlation between the percent change in conductance and percent change in firing rates induced by NE.
NE also decreased excitability for three cells that were tested with depolarizing current pulses. This is shown by the voltage responses in the top of Fig. 9C, where a +40 pA current pulse evoked spiking under control conditions, but not in the presence of NE. Spiking was nearly eliminated in the presence of NE for all cells tested (Fig. 9C). For each cell, the decreases in excitability were significant: when matched for current pulse amplitude and membrane potential, the number of spikes elicited under control conditions was significantly less than the number evoked in the presence of NE (paired t-test, $P < 0.01$). Thus NE also decreased excitability in RA cells.

The NE-induced conductance had a reversal potential consistent with a $K^+$ conductance. The reversal potential for the NE-induced conductance in the cell shown in Fig. 9A was $-81 \text{ mV}$; the average across all cells was $-82.8 \pm 21.01 \text{ (SD) mV}$ ($n = 10$). Also consistent with a $K^+$ conductance, NE induced a slight hyperpolarization in some cells, as indicated by the positive holding current required in NE to match $V_m$ to control levels (mean $= 22.5 \pm 12.6 \text{ (SD) pA; } n = 4 \text { cells that hyperpolarized}$. Some cells had less negative reversal potentials that might also reveal a role for $Cl^-$ conductances (range $-65.4 \text{ to } -135.5 \text{ mV}$). Five cells were filled with biocytin after rupturing the patch at the end of the experiment; each cell had thick spinous dendrites, consistent with RA projection neuron morphology (Spiro et al. 1999).

Finally, we examined whether hyperpolarization of RA cells could induce long-lasting increases in firing rate (Nelson et al. 2003). Four cells were hyperpolarized with negative holding current to a level that eliminated spontaneous activity for $\sim 2 \text{ min}$. On release of hyperpolarization, there was an average increase of 4.1% in firing rates relative to control, but this did not reflect a significant difference between pre- and posthyperpolarization firing rates (paired t-test, $P < 0.2385; n = 4$). Thus at least short periods of hyperpolarization did not enhance firing rates.

**Spikes could be synaptically evoked from RA neurons silenced by NE**

NE could have rapid effects on spontaneous activity in RA. Figure 10A shows the effect of a 25-ms puff of 10 mM NE from a pipette positioned near a neuron recorded extracellularly. The cell lost its spontaneous activity within 2 s of the puff and resumed firing in <10 s. This effect was blocked by yohimbine. NE was similarly fast-acting and reversible for three other cells, indicating that it is capable of operating on a timescale of seconds.

Despite elimination of spontaneous activity by NE, RA neurons retained the capacity to fire action potentials. Stimulation of HVC, a source of afferent input to RA, in the same slice could induce NE-silenced cells to fire (see ticks enclosed by box in Fig. 10A). Figure 10B shows these evoked spikes in more detail. When three trains of 100-Hz stimulus puffs were delivered to HVC, the RA neuron followed with nearly one spike per stimulus pulse. Thus NE did not block the ability of

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**FIG. 9.** NE induced a conductance increase in RA neurons. **A:** top: voltage responses of an RA neuron to $-20 \text{ pA}$ current pulses are shown for control conditions and in the presence of NE. Each voltage response is an average of 3 traces. Membrane potential was held at $-80 \text{ mV}$. Scale bars are 500 ms and 4 mV. Bottom: $I-V$ plot for an individual RA neuron shows its voltage response to current injection in control (solid circles) and NE (open circles) conditions. Lines show best linear fits through points. **B:** percent increase in conductance in response to current injection in control (solid circles) and NE (open circles) conditions. Lines show best linear fits through points. **C:** percent increase in conductance in response to current injection in control (solid circles) and NE (open circles) conditions. Lines show best linear fits through points. **D:** percent increase in conductance in response to current injection in control (solid circles) and NE (open circles) conditions. Lines show best linear fits through points.
HVC inputs to drive action potential firing in RA neurons. This was true in three other cells tested.

These results raised the possibility that NE could function to raise the signal-to-noise ratio of evoked RA activity through elimination of spontaneous activity. For example, in cell 1 of Fig. 10C, the ratio of evoked spikes (those occurring during HVC stimulus trains; see METHODS) to the total number of spikes (counted between the first and last stimulus pulse) under control conditions was 0.55, whereas the ratio in the presence of NE was 0.93. This change represents a 60.5% increase in the signal-to-noise ratio. In four cells tested, NE induced a mean increase in signal-to-noise ratio of 62.1 ± 47.4 (SD)%. One of these cells, however, showed a negligible increase in signal-to-noise of 1.5% in NE (cell 2 in Fig. 10D). The lack of a strong increase in this case was caused by stimulation-induced silencing of spontaneous activity under control conditions. This suggests that when RA neurons are driven strongly, intrinsic (e.g., afterhyperpolarizations) or polysynaptic mechanisms may also suppress spontaneous firing.

The increase in signal-to-noise ratio was accompanied by a decrease in signal: the number of evoked spikes in NE was significantly less than the number evoked under control conditions in three of four cells ($P < 0.05$, unpaired t-test). This probably reflects the increased conductance and decreased excitability that RA cells experienced in the presence of NE.

**DISCUSSION**

We found that NE modulated the neural activity of vocal premotor cells in vitro. NE suppressed spontaneous activity in RA, either decreasing the firing rate or abolishing firing altogether. This suppression likely resulted from a direct effect of NE on the cell recorded, and was mediated by $\alpha_2$-adrenergic receptors. The NE-induced suppression involved an increase in conductance, which did not prevent RA neurons from firing when activated by HVC afferents. This could result in increased signal-to-noise ratio for evoked spiking in RA.

**Mechanisms of NE action**

The predominant effect of NE was to suppress spontaneous activity in RA. This was likely mediated by $\alpha_2$-adrenergic receptors because the suppression was blocked by the antagonist yohimbine and partially mimicked by the agonist clonidine. This pharmacology agrees with previous studies that have localized $\alpha_2$-adrenergic receptors to RA (Riters and Ball 2002). Given the moderate noradrenergic projections to RA (Appelants et al. 2002; Mello et al. 1998), NE is the likely ligand for these receptors. However, a role for dopamine cannot be ruled out because it can also activate adrenergic receptors (Curnell et al. 2002; Malenka and Nicoll 1986) and is prevalent in RA as well (Harding et al. 1998; Sakaguchi and Saito 1989).

The inability of clonidine to consistently abolish firing, even in RA cells that were silenced by NE, is puzzling. One possibility is that a complete loss of spontaneous activity requires coordinated activation of multiple adrenergic receptor subtypes; however, this seems unlikely because yohimbine completely reversed such a loss of activity in NE. Alternatively, the disparate actions of yohimbine and clonidine may reflect small differences in their affinities for $\alpha_2$-adrenergic receptor subtypes: in mammals, yohimbine has highest affinity for the $\alpha_2$C subtype, whereas clonidine has highest affinity for the $\alpha_2$A subtype (MacDonald et al. 1997). This difference could be more pronounced in avian adrenergic receptors. Nevertheless, studies in other species have found a similar inability of clonidine to mimic fully $\alpha_2$-adrenergic receptor–mediated effects (Blanton and Kriegerstein 1992; Liu and Alreja 1998; Williams and Reiner 1993), which may indicate the existence of a novel $\alpha_2$-adrenergic receptor subtype. A third possibility is that the action of clonidine on imidazoline receptors could interfere with the suppressive effect of $\alpha_2$-adrenergic receptor activation, although previous studies have found only a synergistic effect between these two receptor types (Georges and Aston-Jones 2003; Georges et al. 2005).

There was some heterogeneity in RA cellular responses to NE. While the majority of neurons decreased their firing in response to NE, other neurons showed no change or even slight increases in their firing rates. Similar to this latter type, RA neurons recorded in vivo in anesthetized birds did not suppress their firing in response to NE infusion (Dave et al. 1998); however, it is difficult to compare these results with ours given the difficulty in knowing NE concentration near cells recorded in vivo. The heterogeneous responses to NE in our study could

**FIG. 10.** NE-induced suppression of spontaneous activity did not prevent RA cells from spiking in response to afferent input. A: raster plot shows how spontaneous activity of a single RA neuron was briefly interrupted by local application of NE. Dotted vertical line indicates the time of a 25-ms puff of 10 mM NE. Effect of the NE puff was blocked by 1 $\mu$M yohimbine (indicated by thick vertical line on right of raster plot). During trials 5–7, HVC was stimulated with 3 pulse trains (indicated by arrowheads), which evoked spiking from the RA cell. Boxed area is enlarged in B. B: enlarged view of boxed area in raster plot in A shows how stimulation of HVC can evoke spikes in a RA neuron whose spontaneous activity has been silenced by local application of NE. Each stimulus consisted of 3 trains, made up of 3 pulses occurring at 100 Hz each. One spike was evoked for most stimulus pulses. C: bath-applied NE caused an increase in ratio of evoked to spontaneous spikes for cell 1. Raster plot shows that, under control conditions, evoked RA spikes (arrowheads indicate stimulus trains delivered to HVC) occurred with a continuing background of spontaneous activity, whereas in NE, spikes were evoked during a silenced background. This resulted in an increase in signal-to-noise ratio. D: raster plot for a different cell shows how, under control conditions, HVC-driven activity in RA also exhibited brief suppressions of spontaneous activity. Thus the ratio of evoked to spontaneous spikes for this cell was comparable between control and NE conditions.
be caused by RA neurons that had different complements of adrenergic receptor types. For example, those cells lacking a suppressive response to NE may have lacked the \( \alpha_2 \)-adrenergic receptor or had in addition other receptor types that counteracted the effect mediated by \( \alpha_2 \)-adrenergic receptors. The latter possibility was raised by three cells that did not exhibit NE-induced suppression, yet decreased their firing when tested with clonidine (Fig. 7C). Although not tested for in this study, there is some evidence for \( \beta \)-adrenergic receptors in RA (Revilla et al. 1999). The suprarecovery observed under NE + yohimbine conditions could also be interpreted as reflecting the actions of other receptor types: these would increase firing rate, and the effect would be more evident in the absence of \( \alpha_2 \)-mediated suppression.

Another potential source of response heterogeneity is androgen level: \( \alpha_2 \)-adrenergic receptor binding is seasonally regulated in starlings, and is sensitive to androgen levels (Riters et al. 2002). Although zebra finches are not seasonal birds, differences in their androgen levels could change the density of \( \alpha_2 \)-adrenergic receptors in RA between individual birds, resulting in heterogeneity of responses to NE either within or between birds. Although the effect of NE on RA cells did not seem to vary according to bird in our data set; this remains a possibility given the restricted sampling of neurons in each bird.

The NE-induced suppression of spontaneous activity in RA was mediated by a conductance increase. The average reversal potential for the NE-induced conductance was consistent with \( \beta \)-adrenergic receptors in RA (Revilla et al. 1999). The suprarecovery observed under NE + yohimbine conditions could also be interpreted as reflecting the actions of other receptor types: these would increase firing rate, and the effect would be more evident in the absence of \( \alpha_2 \)-mediated suppression.

Consequences of NE action in RA

The RA neurons modulated by NE in vitro were similar to those with auditory and premotor activity in vivo in that they displayed regular spontaneous activity. Although the firing rates of RA neurons in our slice preparation had lower frequencies than reported in vivo (Adret and Margoliash 2002; Dave et al. 1998; Leonardo and Fee 2005; Vicario and Raksin 2000; Vicario and Yohay 1992; Yu and Margoliash 1996), the activity in vitro was still marked by regularity. The lower firing rates in this study likely reflect differences between in vitro and in vivo preparations, including synaptic drive, temperature, and oxygenation. Regarding the cell types modulated by NE in this study, spontaneous activity is a property shared by both projection neurons and interneurons (Spiro et al. 1999). Although we cannot rule out the possibility that some of the neurons in this study were interneurons, we think the majority were projection neurons given the regularity of their spontaneous activity and the morphology of the subset of filled cells (Mooney 1992; Spiro et al. 1999). Thus NE-induced suppression could profoundly influence the auditory and premotor output of the nucleus.

NE-mediated increases in signal-to-noise ratio of sensory responses have been found in the auditory (Foote et al. 1975), visual (Kasamatsu and Heggelund 1982), and somatosensory (Waterhouse and Woodward 1980) systems. These resulted from decreases in spontaneous activity and/or increases in evoked activity (Madison and Nicoll 1982). In the song system, a similar role has been found for NE in modulating the salience of auditory signals. NE application to the forebrain nucleus interface of the nidopallium (NIF) increases or decreases auditory responses in a dose-dependent manner (Cardin and Schmidt 2004). Similarly, NE could increase the salience of signals within RA, either through decreasing spontaneous activity, increasing evoked activity, or both. The increases in signal-to-noise ratio that we observed for RA cells resulted from the loss of spontaneous activity alone. Although NE also decreased evoked activity and excitability, this reduction in signal was outweighed by the decrease in noise, resulting in an increased signal-to-noise ratio overall. One cell showed a loss of spontaneous activity between bouts of HVC stimulation under control conditions (in the absence of NE), suggesting the activation of an afterhyperpolarization (Spiro et al. 1999) or inhibitory circuitry when RA neurons are strongly driven by HVC. Thus a combination of NE, intrinsic, and synaptic properties could contribute to increasing the gain of auditory or premotor signals relayed through RA.

Upon release from the effect of NE (either during washout of NE or during yohimbine + NE application), enhanced firing rates relative to pre-NE levels were observed. This raised the possibility that a prolonged loss of spontaneous activity could cause a compensatory increase in firing rate, as in the vestibular nucleus, where prolonged hyperpolarization leads to firing rate plasticity (Nelson et al. 2003). Although we found no lasting effect of short hyperpolarizations on the firing rates of a subset of RA neurons, future experiments may reveal a similar type of plasticity.

Implications for behavior

LC activation is related to the state of arousal of an animal (Berridge and Waterhouse 2003) and has been associated with awake states (Aston-Jones and Bloom 1981), processing of salient sensory cues (Aston-Jones et al. 1994), and execution of motor responses after simple decisions (Aston-Jones and Cohen 2005; Clayton et al. 2004). In songbirds, NE gates state-dependent auditory responses in the song system (Cardin and Schmidt 2004; Dave et al. 1998). Because RA is a sensorimotor nucleus, NE release in RA could modulate both auditory and premotor activity during singing. Consistent with this, wide-scale NE depletion decreases singing frequency (Barclay et al. 1996) and modulates singing-related gene expression (Castelino and Ball 2005).

The NE-induced suppression we found in vitro is reminiscent of the diminished spontaneous activity that occurs in RA in vivo during the transition from tonic to burst firing. During singing, RA cells reduce their tonic, spontaneous firing and produce premotor bursts that are remarkably precise (Chi and Margoliash 2001; Dave et al. 1998; Leonardo and Fee 2005; Yu and Margoliash 1996). Similar changes also occur during auditory responses in anesthetized birds, but they are less pronounced (Dave et al. 1998; Shea and Margoliash 2003). NE could contribute to this transition to burst firing, thus promot-
NE modulates RA spontaneous activity in vitro


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