Inhibitory Network Interactions Shape the Auditory Processing of Natural Communication Signals in the Songbird Auditory Forebrain

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INTRODUCTION

Songbirds can modify their vocalizations based on auditory input, and have been used extensively as a model for vocal learning, which is necessary for the acquisition of spoken language (Doupe and Kuhl 1999; Konishi 1965b; Zeigler and Marler 2004). Like humans, songbirds rely on audition to generate auditory memories that are used as templates for the normal development of vocal behavior (Konishi 1965a; for reviews see Doupe and Kuhl 1999; Koppl et al. 2000; Nottebohm 1999; Zeigler and Marler 2004). In addition, song discrimination and the formation of auditory memories in adult animals is central for key behaviors, such as territorial defense, mate selection and individual recognition (Catchpole and Slater 1995; Kroodsma and Miller 1982; Nowicki and Searcy 2004). Thus a great deal of effort has been directed at understanding the anatomical and functional organization of brain areas involved in the auditory processing of birdsong. A particular focus has been the caudomedial nidopallium (NCM), an area considered analogous to the supragranular layers of the mammalian auditory cortex because it receives input from the primary telencephalic thalamo-recipent area field L and participates in intra-telencephalic circuitry that precedes the descending auditory projections from the arcopallium (Karten and Shimizu 1989; Mello et al. 1998; Yates et al. 1996; Wild et al. 1993). It is currently unknown whether NCM and other avian auditory areas have correspondence to portions of the primary versus higher-order auditory cortical areas of mammals. NCM displays strong electrophysiological responses to song stimulation, with greater selectivity for complex stimuli, compared with responses at earlier stations in the ascending auditory pathway, such as the thalamic-recipent field L2 (Chew et al. 1995, 1996; Muller and Leppelsack 1985; Sen et al. 2001; Terleph et al. 2006, 2007). NCM’s responses also show a preference for conspecific over heterospecific songs or artificial stimuli (Chew et al. 1996; Mello et al. 1992), suggesting that this area contributes to auditory discrimination of birdsong. Importantly, evidence from electrophysiological and gene expression studies suggest that NCM plays a role in the formation and/or storage of song auditory memories learned from tutors (Bolhuis et al. 2000; Phan et al. 2006; Terpstra et al. 2004). Furthermore, lesions to NCM can disrupt important aspects of the perceptual discrimination of birdsong (Gobes and Bolhuis 2007).

Here we explore the contribution of GABAergic inhibition to shaping auditory responses in NCM. GABAergic transmission, especially that mediated through GABAA receptors, plays a pivotal role in shaping receptive field (RF) properties in several sensory systems and experimental models. For example, GABAA receptor antagonism expands RFs in primary somatosensory (SI) and visual (V1) cortices (Dykes et al. 1984; Eysel et al. 1998; Ramoa et al. 1998; Tremere et al. 2001). In addition, direction and orientation selectivity in V1 neurons are controlled by GABAergic transmission (Sillito 1975a, 1977, 1979), indicating that inhibition contributes to the generation of complex response properties of sensory neurons. GABAA-mediated transmission also regulates the responses of neurons in auditory circuits. For instance, GABAA receptor

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blockade expands frequency tuning curves of central auditory neurons of mammals, including cortical ones (Chen and Jen 2000; Jen and Feng 1999; Suga et al. 1997; Yang et al. 1992), and plays a critical role in experience-dependent plasticity of auditory spatial maps in the avian (barn owl) brain (Zheng and Knudsen 1999, 2001).

Using a multi-disciplinary approach, we report here a marked role for GABA-mediated inhibition in the physiology of songbird NCM. First, using immunocytochemistry specific to the neurotransmitter GABA, we show that GABAergic cells comprise at least half of all neurons in NCM. Second, patch-clamp electrophysiological recordings in brain slices show that, under resting conditions, a high density of spontaneously active GABAergic synapses suppresses the excitatory synaptic inputs onto NCM neurons. Finally, coupling multi-electrode recordings in NCM of the awake songbird with pharmacological inputs onto NCM neurons, we show that GABA receptor blockade markedly alters the temporal organization of auditory responses without changes in frequency tuning. Taken together, our findings show that complex and unconventional interactions of inhibitory networks play a key role in the auditory processing of natural communication signals in the songbird auditory forebrain. We suggest that these interactions may be important for the processing of complex learned vocalizations as in the case of birdsong and human speech.

METHODS

Immunocytochemistry (ICC) and specificity controls

A total of 16 zebra finches (*Taeniopygia guttata*; *n* = 8 males and 8 females) were anesthetized and perfused transcardially with 20 ml of phosphate-buffered saline (PBS 0.1 M, pH 8 females) were anesthetized and perfused transcardially with 20 ml of an ice-cold mix of 1% paraformaldehyde and 2% glutaraldehyde in PBS. Brains were dissected out, cryoprotected overnight in 30% sucrose, and dried. The hemispheres were separated at the midsagittal plane, included in embedding medium (Tissue-Tek, Superfrost Plus glass slides).

We used a commercial rabbit anti-GABA antibody to detect GABAergic cells in our preparations, using a protocol previously described (Pinaud et al. 2006). Briefly, sections were hydrated for 30 min in PBS and sequentially incubated in a humid chamber in the following solutions: 1) blocking buffer (BB; 0.5% albumin and 0.3% Triton X-100 in 0.1 M PB) for 30 min at room temperature (RT); 2) primary antibody (1:200 dil in BB; Chemicon International, Temecula, CA), overnight at 4°C; 3) biotinylated goat anti-rabbit IgG antibody (1:200 dil in BB; Vector Laboratories, Burlingame, CA), for 2 h at RT; and 4) ABC reagent (1:100 dil in PBS; Vector Laboratories). Sections were developed by incubation in a filtered solution containing 0.03% dianinobenzidine, 0.15% nickel sulfate, and 0.001% hydrogen peroxide in PBS. All steps in the preceding text were separated by 3 × 10-min washes in PBS.

Omission of the primary antibody resulted in absence of cellular staining, demonstrating the specificity of our ICC detection system (secondary antibody plus ABC reagent). To determine the specificity of the anti-GABA antibody, we preabsorbed it with GABA conjugated to a carrier protein, analogous to the immunizing conjugate used to generate this antibody. We first generated a GABA-BSA conjugate as described previously (Walrond et al. 1993) with modifications. Specifically, we cross-linked GABA (5 mM; Sigma) with BSA (5 mg/ml) using glutaraldehyde (at 1% in 0.1M PB) for 1 h at RT, under stirring. This solution was then dialyzed against 30 volumes of cold 0.01 M PB (10 volume/day for 3 day, at 4°C). To preabsorb the anti-GABA antibody with the conjugate, we incubated the antibody at its working dilution with various concentrations of the postdialysis conjugate overnight at 4°C under agitation. The preabsorbed antibody was then used in the ICC procedure. We found that preabsorption with the GABA-BSA conjugate in the 10- to 50-μM range completely abolished GABA-like immunoreactivity in brain sections (Supplementary Fig. S1B). Further preabsorption controls using unbound BSA at the same concentrations as for the GABA-BSA conjugate did not alter GABA-like immunoreactivity in our preparations (Supplementary Fig. S1A). These procedures are in accordance with strict established guidelines for determining antibody specificity (Saper and Sawchenko 2003).

Cell measurements and counts

We used previously described methods to estimate maximum cell diameter and area and to quantify densities of neurons in NCM, using NeuroLucida software integrated with a Nikon E-600 microscope equipped with a motorized stage drive (LEP Mac5000) and coupled to a PC through a Lucivid system (Microbrightfield; Colchester, VT) (Pinaud et al. 2006). Briefly, to obtain cell diameter and area, we reconstructed the perimeters of 200 neurons, in three randomly placed NCM fields that encompassed the dorsal-to-ventral extent of this area, at three predefined stereotaxic levels (0.1, 0.5 and 0.9 mm from the midline, to provide a broad coverage of the medial-to-lateral extent of NCM). Given that no intra-animal differences were detected for cell sizes in dorsal versus ventral and medial versus lateral NCM comparisons (not shown), all data for soma diameter and area were combined for each animal. Cell diameter and area calculations were done using NeuroExplorer (Pinaud et al. 2006).

To generate estimates of local densities of GABAergic cells, a grid containing squares of 100 × 100 μm was superimposed on sections reacted for GABA ICC at the same planes detailed in the preceding text. A minimum of 15 such squares per stereotaxic level, per bird, evenly spaced through rostral and caudal NCM were used for counting labeled cells. To estimate neuronal cell density, we counted the number of neurons per unit area in Nissl-stained adjacent sections of the same animals using the same sampling as in the preceding text for GABA. The inclusion criteria for neurons were a large pale nucleus, usually with clear staining nucleolus, and prominent Nissl substance, whereas the exclusion criteria consisted of small cells with dark, homogeneously stained nuclei and scant cytoplasm. Because no regional differences were detected in NCM in relation to the overall neuronal and GABAergic cell populations or between sexes (not shown), results were averaged for each of the three planes examined and across birds (including *n* = 5 males and 5 females).

Whole cell patch-clamp electrophysiology

Twenty-three adult zebra finches (20 females and 3 males) were used in the in vitro experiments. All the quantitative data were obtained in females for consistency. Males were used only on a few occasions for qualitative comparisons. Birds were caught from local dealers and kept in aviaries at the animal facilities at the NSI or at the University of São Paulo in Ribeirão Preto. Birds were decapitated, and the brains were quickly dissected and placed in ice-cold artificial cerebrospinal fluid solution (ACSF) modified for slicing and consisting of (in mM): 87 NaCl, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgCl2, 25 glucose, 75 sucrose, 0.4 ascorbic acid, 2 sodium piruvate, and 3 myo-inositol, 354 mosM/kgH2O, pH 7.4 when bubbled with carbogen 95%O2-5%CO2. Parasagittal sections (200 μm thick; 4–5 sections starting at the midline) covering the medial-to-lateral extent of NCM and adjacent areas were obtained on a vibratome (Series 1000, Vibratome, St Louis, MO). Slices could be...
stored in this solution at RT for 4–6 h. These slices contained NCM as well as the adjacent auditory regions field L2 and the caudomedial mesopallium (CMM). Although these regions are thought to provide input to NCM, we note that it is currently unknown whether the projection fibers from these input sources are preserved in our slice preparations. For recordings, slices were transferred to a chamber mounted on a stage of an upright microscope (Olympus BX51WI) and continuously perfused with regular ACSF, which consisted of (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 25 glucose, 2 CaCl2, 1 MgCl2, 0.4 ascorbic acid, 2 sodium pyruvate, and 3 myo-inositol, 310 mosM/kgH2O, pH 7.4 when bubbled with 95%CO2-5%O2. Single neurons were visualized with DIC-IR optics, approached and patched with borosilicate glass pipettes (Sutter Instruments, Novato, CA) prepared using a P-97 horizontal puller (Sutter Instruments). Spontaneous postsynaptic currents (sPSCs) were recorded at ~70 mV in whole cell voltage-clamp with an EPC-10 patch-clamp amplifier (HEKA Elektronik, Germany) using the Pulse acquisition software. For recording, the pipettes were back-filled with filtered internal solution consisting of (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 4 ATP-Mg, and 20 phosphocreatine-Na, pH 7.3 with CsOH. A low-chloride internal solution, where CsCl was replaced by Cs-methanesulphonate (130 mM) and KCl (10 mM), was used in experiments intended to isolate GABAergic and glutamatergic transmission based on the sPSC reversal potential. In these experiments, cells were held at ~20 mV, a membrane potential between the reversal potential of GABAergic Cl- currents (~56 mV, after correction of a liquid junction potential of 11 mV) and of cationic glutamatergic currents (0 mV), resulting in outward Cl- currents and inward cationic currents. Reversal potentials and liquid junction potentials were calculated using the Patchers’s Power Tools of the IGOR Pro software (Wavemetrics, Lake Oswego, OR). For measuring membrane potential changes, we used a potassium gluconate-based internal solution (Wavemetrics, Lake Oswego, OR). Formeasuring membrane potential changes, we used a potassium gluconate-based internal solution (Wavemetrics, Lake Oswego, OR). For measuring membrane potential changes, we used a potassium gluconate-based internal solution (Wavemetrics, Lake Oswego, OR). Formeasuring membrane potential changes, we used a potassium gluconate-based internal solution (Wavemetrics, Lake Oswego, OR). Formeasuring membrane potential changes, we used a potassium gluconate-based internal solution (Wavemetrics, Lake Oswego, OR) calculated using the Patcher’s Power Tools of the IGOR Pro software for averaging across trials as displayed in Fig. 5.

Analysis of auditory responses

Multiunit responses (typically 5–10 units) were recorded at each site to enable valid comparisons between conditions (before vs. during drug administration) because these responses are stable over a period of 1–2 h. Single units cannot always be held for the necessary time period in the awake preparation. These multiunit neural responses to song stimuli were quantified as the difference between the root-mean-square (RMS) value obtained for each electrode during a response window (from stimulus onset to stimulus offset plus 100 ms) and the RMS during the control period of each trial (a 500-ms window occurring prior to stimulus onset). To compute the RMS, each digitized value is squared, the mean of these squares over the response interval is computed, and the square root of that mean is taken. This provides a method for rectifying the multiunit activity and computing its average power.

In an additional analysis, the RMS was calculated over 1-ms windows to provide a measure of the local burstiness or temporal variance of the responses over short time intervals. To measure this temporal variance, the SD of these values (the RMS of each 1 ms window) was calculated over time during the response period for...
Histological confirmation of recording sites

To confirm recording locations, electrolytic lesions (10 µA of current for 10 s, 3 lesions per hemisphere) were made at several recording sites in each brain. Animals were killed by sodium pentobarbital (Nembutal) overdose and perfused transcardially with saline followed by a 4% paraformaldehyde solution. Fixed brains were removed and cut on a vibratome parasagitally (50-µm sections), and processed for Cresyl-violet staining following standard protocols (Pinaud et al. 2006).

All animal handling and experimentation procedures described in the preceding text were approved by the IACUC committees of OHSU, Rutgers University, and University of São Paulo, and are in accordance with National Institutes of Health guidelines.

RESULTS

GABAergic neurons are highly prevalent in NCM

We have previously cloned the zebra finch homologue of the 65-kDa glutamic acid decarboxylase gene (zGAD65), a specific GABAergic cell marker, and shown by in situ hybridization that zGAD65-positive cells account for ~40% of the overall neuronal population in NCM (Pinaud et al. 2004). However, the representation of GABAergic neurons may have been underestimated, given that cells expressing zGAD67 (the other synthetic enzyme for GABA) were not detected with our probe. We have now conducted an immunocytochemical (ICC) analysis with a specific anti-GABA antibody that labels GABAergic cells irrespective of their synthetic enzyme. Our procedure reliably identified known populations of GABAergic neurons in several brain areas (Supplementary Fig. S2). In NCM, we found a particularly high density of evenly distributed GABAergic neurons (Fig. 1, A and B) with relatively few clusters of two to four neurons (Fig. 1B, *) that occurred in a wide range of sizes. These cells had an overall normal distribution of sizes (mean diameter = 16.1 µm and median diameter = 15.5 µm). Interestingly, the mode of the distribution was 9.8 µm, indicating a higher frequency of small-sized cells (as can be seen in Fig. 1C, *). We also observed the occurrence

![Fig. 1](http://jn.physiology.org/)
of larger cells (>20 μm diam), suggesting the possible presence of a second population of cells. We note, however, that these were much less frequent than the smaller ones, so that the general cell size distribution was not bimodal and had no obviously abnormal right tail. Nonetheless, our qualitative observations are consistent with our previous findings using zGAD65-specific riboprobes (Pinaud et al. 2004).

Quantitative analysis revealed that GABAergic neurons account for 51.9 ± 1.4% (mean ± SE) of the overall neuronal population. A reconstruction of 200 GABA-positive neurons (n = 4 birds) revealed an average cell diameter of 9.2 ± 0.6 μm (range: 3.3–20.8 μm), which was significantly smaller than the average diameter of the general neuronal population in NCM (11.8 ± 0.3 μm; P = 0.0016; Student’s t-test; Fig. 1D). The area of GABAergic cell somata averaged 34.6 ± 0.9 μm² and was also significantly different from the average soma area (38.1 ± 1.0 μm²; P = 0.00046; Student’s t-test; Fig. 1D). GABAergic cell density in NCM proved to be substantially higher than other auditory stations such as the brain stem nucleus angularis (19.3 ± 1.5% of the overall neuronal population), the HVC shelf (29.7 ± 0.6%), field L2 (44.9 ± 2.0) and structures of the song-control system, such as the lateral magnocellular nucleus of the anterior nidopallium (LMAN; 29.2 ± 3.0%). CMM exhibited a similarly high density of GABAergic neurons (51.8 ± 1.4 of the overall neuronal population) as compared with NCM.

The data described in the preceding text shows that the GABAergic neuronal population in NCM is primarily composed of small neurons, which are typically encountered in local neuronal circuits. More importantly, GABAergic neurons are the prevalent cell type in NCM, suggesting that inhibitory transmission may play a key role in the physiology of this auditory area.

**Spontaneous GABA release suppresses the activity of excitatory circuitry in NCM slices**

To characterize the potential roles for GABAergic and glutamatergic inputs to NCM neurons at rest, we conducted whole cell patch-clamp recordings in slices containing the NCM and the adjacent projection areas field L and CMM. We found that all recorded neurons in NCM receive strong synaptic input, as revealed by a high frequency of spontaneous postsynaptic currents (sPSCs; Fig. 2A–C). These sPSCs exhibited a frequency of 3.1 ± 0.3 (SE) Hz (n = 31 neurons from 21 birds) and amplitude and half-width of −48 ± 3.2 pA and 9.9 ± 0.7 ms, respectively.

To assess the fraction of fast neurotransmission contributed by excitatory or inhibitory mechanisms, we used BIC, a competitive GABA_A receptor antagonist, and DNQX, an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptor antagonist. Application of BIC to the recording bath significantly decreased the frequency of sPSCs from 2.7 ± 0.3 to 0.4 ± 0.1 Hz (n = 23; P < 0.0001, paired t-test; Fig. 2A–C), resulting in an average 87 ± 3% suppression. The remaining, BIC-resistant, events had significantly smaller amplitude (P = 0.0005; paired t-test; Fig. 2A–C) and were completely blocked by co-application of DNQX to the recording bath (Supplementary Fig. S3), indicating that these were excitatory. Thus the vast majority of spontaneous synaptic currents recorded in the brain slices containing NCM are GABAergic.

It is noteworthy that a distinct form of synaptic activity was elicited by BIC in a significant fraction of cells (9 of 28–32%). This consisted of regular bursts of synaptic currents (Fig. 2D, top trace). Compared with spontaneous inhibitory postsynaptic currents (sIPSCs) or mean spontaneous excitatory postsynaptic currents (sEPSCs), these bursts were notably larger (mean amplitude of 232.5 ± 37.6 pA) but occurred at a much lower frequency (0.08 ± 0.01 Hz; n = 9) and in a seemingly rhythmic pattern (Fig. 2D). These events are suprathreshold as they were able to elicit a train of action potentials, as seen in current-clamp mode (Fig. 2E). Application of DNQX (10 μM) to the recording bath eliminated these events, confirming that they originate from glutamatergic synapses normally inhibited by the spontaneous GABAergic tone. Although we cannot determine if the recorded neurons are glutamatergic or GABAergic, it is reasonable to conclude that under resting conditions, GABAergic sIPSCs suppress components of the excitatory network that drives NCM neurons in our slice preparation and that only becomes active when the basal GABAergic tone is inhibited.

We next applied DNQX (without BIC) to the bath, to investigate the contribution of AMPA/kainate receptors to the sPSCs. DNQX failed to trigger significant changes in sPSCs frequency (control: 4.0 ± 0.7 Hz; DNQX: 3.4 ± 0.6 Hz; n = 9; P = 0.41; paired t-test) or amplitude (control: 51.5 ± 6 pA; DNQX: 41.5 ± 3.6 pA; n = 9; P = 0.08; paired t-test; Fig. 3A–C). In fact, in only three of nine cells DNQX had a detectable effect on sPSCs frequency. Subsequent application of BIC inhibited all the remaining events (not shown). By comparing the GABAergic sIPSCs (DNQX-resistant events) with the glutamatergic sEPSCs (BIC-resistant events), it is clear that spontaneous GABAergic activity is substantially stronger and more frequent than spontaneous glutamatergic activity (mean peak conductances of 592 vs. 270 pS, and 3.4 vs. 0.4 Hz for sIPSCs and sEPSCs, respectively).

In addition, GABAergic sIPSCs were driven by action potentials because they were strongly inhibited by application of TTX (frequency from 3.6 ± 0.7 to 0.4 ± 0.07 Hz; amplitudes from 44.5 ± 6.7 to 24.9 ± 1.8 pA, both P < 0.05, paired t-test; n = 9). These findings were different from those obtained for glutamatergic sEPSCs, which were basically composed by miniature currents given they were not significantly affected by TTX application (frequency from 0.74 ± 0.1 to 0.49 ± 0.2 Hz; amplitudes from 35.1 ± 3.7 to 30.2 ± 3.1 pA, both P > 0.05, paired t-test; n = 6).

The preceding findings suggest that GABAergic neurons are spontaneously active, independent of excitatory drive. To confirm this hypothesis, we performed experiments using a low-chloride internal solution (see methods) and held cells at −20 mV. Under these conditions, chloride-mediated synaptic currents (GABAergic) are outward, whereas cationic synaptic currents (glutamatergic) are inward. As predicted, we observed a high frequency of outward currents (2.6 ± 0.3 Hz; n = 9) and only minor inward currents (0.6 ± 0.07 Hz; n = 6; quantified at −50 mV to increase signal-to-noise ratio; Fig. 3D). Application of DNQX inhibited all inward currents but did not affect either the frequency (control: 2.4 ± 0.8 Hz; DNQX: 3.1 ± 1.1 Hz; n = 6; P = 0.13, paired t-test) or the amplitude (control 20.0 ± 2.7 pA; DNQX: 20.2 ± 2.3
pA; n = 6; P = 1.0, paired t-test) of outward currents (Fig. 3E). These results confirm that spontaneous GABAAergic synaptic activity in NCM is not dependent on glutamatergic interneuron activity and likely reflects spontaneous activity from GABAAergic neurons.

**GABA<sub>A</sub> receptors regulate temporal dynamics of song-evoked responses**

The experiments in the preceding text suggest a prominent role for GABA in NCM synaptic physiology and possibly function. To directly investigate the contribution of GABAAergic transmission to birdsong auditory processing in NCM, we combined multi-electrode extracellular recordings in awake restrained birds with local pharmacological interventions during playback of a randomized series of conspecific and heterospecific songs and tone stimuli (see Methods).

Before injections, typical song-evoked responses were detectable at recording sites in both hemispheres (Supplementary Fig. S4). These responses typically consisted of bursts of multiunit action potentials in response to song syllables with a sustained (tonic) activity that bridged the intervals between syllables and outlasted the stimulus (Figs. 4A and 5B, control electrodes). Note that NCM has a high level of irregular spontaneous activity that can be seen preceding the stimulus in the panels of Fig. 4. Application of BIC dramatically changed the pattern of NCM responses to song playback. First, song-evoked responses were converted from tonic, to large phasic bursts of activity that exhibited
ON-OFF-like features (Figs. 4B and 5B, BIC electrodes). This BIC-induced phasic behavior during song presentation was especially robust in response to the first syllable. Second, the sustained firing between syllables was largely abolished by BIC application (Figs. 4B and 5B, BIC electrodes). As described in METHODS, drug doses were maintained at a level that affected spontaneous activity somewhat, producing increased burstiness without changing the average level of activity.

To gain insight into this reorganization of song-evoked responses, we calculated a ratio between the response amplitude after and before BIC application (Fig. 6A; a value of 1 indicates no effect). We found no difference in this ratio when comparing BIC and control sites (Student’s t-test, df = 22, $t = 0.453$, $P = 0.655$, 2-tailed), indicating that BIC did not change the overall size of the response. However, a significant difference was obtained between experimental and control sites when comparing the temporal variance (Student’s t-test, df = 22, $t = 3.018$, $P = 0.006$, 2-tailed), reflecting the increase in phasic bursts separated by periods of lowered activity (Fig. 6A). Interestingly, despite this clear change in the temporal pattern, the total response magnitude was unchanged, indicating that BIC altered network response properties at the recorded NCM sites without affecting the overall size of the recruited cell population (e.g., by lowering action potential threshold).

We next conducted the same in vivo experiment as in the preceding text except that picrotoxin (PTX), which blocks both

FIG. 3. Frequency and amplitude of sIPSCs are Independent of glutamatergic input. A: spontaneous synaptic currents recorded in an NCM neuron before (left) and after (right) application of 20 μM of dinitroquinoxaline-2,3(1H,4H)-dione (DNQX). B: cumulative frequency histograms of the inter-event intervals (left) and sPSC amplitudes (right) from the same cell shown in A. The number of analyzed events is shown in parenthesis. C: effects of DNQX on the frequency and amplitude of the synaptic events. D: recordings made at –20 mV using a low-chloride internal solution. The outward currents are GABAergic and blocked by BIC, while the inward currents are glutamatergic and blocked by DNQX. E: summary of the effects of DNQX on the outward sIPSCs frequency (left) and amplitude (right).
GABA_A and GABA_C receptors, was used instead of BIC. As observed with BIC, local PTX administration converted the sustained responses found in the preinjection condition to highly phasic ON-OFF-like responses (Fig. 4C). In addition, quantitative analysis showed no significant differences between the BIC and PTX effects (Supplementary Fig. S5). These data suggested that there were no additional quantitative effects of GABA_C blockade on the measured responses. Thus, local BIC and PTX application alters the temporal organization of song-induced spikes in NCM. A: raw traces depicting multiunit responses to song playback from representative electrodes located in control and experimental hemispheres prior to drug application. Note that responses are sustained throughout the duration of the stimulus in both hemispheres. NCM typically has a high level of irregular spontaneous activity that can be seen preceding the stimulus. Song syllables can vary widely in amplitude, as seen on the stimulus envelope traces. B: application of BIC in the experimental hemisphere drastically changes the response profile of song-activated units. Note that the response becomes highly phasic and synchronized, particularly in relation to the 1st syllable, and that sustained activity between syllables is largely abolished. C: PTX treatment induces changes similar to those observed with BIC.
we combined results from BIC and PTX experiments for the analyses that follow.

To further examine the drug-induced shift from a tonic to a phasic response pattern (see schematic model in Fig. 6B), we stimulated birds with pure tones, which are acoustically simpler than songs, and monitored the response profiles in NCM before and after either BIC or PTX injections. Under control conditions, responses to tone stimuli included both a phasic component at stimulus onset, and tonic activity persisting throughout the stimulus at a lower level (Fig. 6C; blue trace). Interestingly, under drug treatment, phasic onset responses significantly increased in size while the tonic component was markedly reduced (Fig. 6C; red trace). We quantified this relationship by computing a phasic/tonic response ratio for the best frequency at each site. When these ratios were compared between the predrug and drug conditions and between the control and experimental hemispheres, using a repeated-measures ANOVA, there was a significant effect of drug treatment on the phasic/tonic response ratio \( \frac{F(1,19)}{11005} 9.06, P < 10^{-3} \) and a significant interaction between drug treatment and hemisphere \( \frac{F(1,19)}{11005} 16.81, P < 10^{-3} \); post hoc testing with Bonferroni correction showed a significant increase in the phasic/tonic ratio only the drug hemisphere, \( P < 10^{-6} \). These results obtained with tone stimuli clearly confirm that BIC shifts neuronal responses from the tonic/sustained period to the phasic response window (schematized in Fig. 6B), as suggested by the increased phasic, and lower tonic, responses to full songs under BIC (Fig. 5B, arrows in bottom BIC graphs).

**Correlation of phasic and tonic components is preserved during drug treatment**

We have shown that blockade of inhibition, which would in theory enhance the excitability of NCM circuitry, instead triggers a marked suppression of the sustained component of song-evoked responses. One potential explanation for the drug-induced suppression of tonic responses is that when neuronal activity is recruited to the phasic period, after pharmacological treatment, the patterns of activity of the responding neurons becomes better correlated. One possibility is that the refractory periods of the responding neurons become synchronized; however, the time scale of the phasic-tonic relationship is tens of milliseconds, much longer than a biophysical refractory period. Nonetheless it is probable that the activity we record is strongly...
influenced by an oscillatory network. Thus it is possible that GABAergic antagonists effectively change the oscillation period of this network, leading to reduced excitability during the tonic interval. We could not directly test this hypothesis, but we investigated several relevant parameters in the data we collected. First, we observed that neurons recorded in NCM slices could fire at rates >20 Hz under BIC treatment (Fig. 2E shows a representative neuron) (see also Supplementary Fig. S6), corresponding to an interspike interval of 50 ms, which is a typical duration for the phasic burst. Although this finding suggests that NCM neurons could participate in both phasic and tonic periods of the response, this observation might not be true of all NCM neurons. Second, in vivo evoked auditory responses in NCM were unaffected by spontaneous bursts that, by chance, immediately preceded stimulus onset (Supplementary Fig. S7). Although this observation is highly supportive, it is not conclusive because we recorded a multiunit population, which prevents us from ruling out the possibility that different subpopulations participated in the spontaneous and stimulus-elicted activity. Finally, to answer these concerns, we undertook an analysis of the correlation and regression between the phasic and tonic activity for each response to tone stimuli at the best frequency for each site. In the predrug condition, these correlations were significant across recording sites in each bird ($r^2$ range: 0.204–0.551, $P < 0.05$), and the regressions had positive slopes. When the slopes before and after BIC or PTX treatment, and between control and drug hemispheres, were compared using a repeated-measures ANOVA, we found a significant change in regression slope with drug treatment ($F(1,12) = 6.34, P = 0.027$) but no significant interaction between drug treatment and hemisphere. In post hoc tests with Bonferroni correction, we found no significant difference in slopes in either the control or experimental hemisphere before and after drug application (control hemisphere, $P = 0.352$, experimental hemisphere, $P = 0.232$). Thus the overall decrease reflects the increase in phasic responses and...
decrease in tonic responses that was documented earlier for group data. However, the regression slopes remained positive in all birds tested and correlations were significant in the treated (\( r^2 \) range = 0.218–0.363, \( P < 0.05 \)) and untreated (\( r^2 \) range = 0.261–0.383, \( P < 0.05 \)) hemisphere in six of seven birds tested. If synchronized excitability (or depression) that depended on an initial high frequency were the explanation for the suppression of tonic/sustained responses following GABAergic blockade, then trials with higher phasic activity (representing some combination of more units recruited, greater synchronization and higher firing rates) should have exhibited lower tonic responses. Interestingly, the opposite is in fact observed: trials with higher phasic responses also clearly have higher tonic responses, as determined by the positive correlations. While this does not rule out a subtle effect of changes in the periodicity or strength of an oscillatory network, it does suggest that the lower tonic response does not inversely reflect synchronization related to the higher phasic response.

**GABAergic transmission regulates the temporal pattern of song-evoked responses**

Whereas all song syllables normally elicited neuronal responses in NCM, the responses to a number of song syllables were either reduced or absent under BIC or PTX (Fig. 5B; examples in shaded boxes), indicating that GABAergic transmission blockade is changing the temporal profile of auditory processing in NCM. To demonstrate this effect, we compared the correlation between the stimulus envelope and the averaged response for the control versus experimental conditions. NCM responses do not closely parallel the amplitude waveform of the stimulus, even in the control condition, but this analysis demonstrates the type of changes in correlation associated with temporal reorganization of responses. A repeated-measures ANOVA that included drug type (BIC and PTX) and hemisphere as factors showed that GABA-receptor antagonists significantly reduced the correlation in the experimental hemisphere compared with the control hemisphere \([F(1,7) = 53.4, P = 0.0016]\). There was no interaction with drug type, confirming similar effects for both BIC and PTX \([F(1,7) = 1.8, P = 0.222]\). These comparisons are presented as a SECI (see Methods), which shows no change for the control hemisphere and comparable changes in the experimental hemisphere for both BIC and PTX (Fig. 6D). These findings indicate that the BIC- and PTX-induced alterations in firing behavior resulted in a decreased temporal correlation of NCM responses to song elements. Thus we infer that under normal conditions, local GABAergic transmission contributes to the ability of NCM activity to follow the temporal envelope of the stimulus and thus could contribute to the fidelity of auditory processing in NCM.

**GABA\(_A\) antagonism does not influence frequency tuning in NCM**

The changes in the temporal profile of the responses described in the preceding text might reflect decreased discrimination capabilities of NCM induced by expanded frequency tuning. In fact, this effect has been previously shown in central auditory neurons where local BIC treatment triggers significant expansion of frequency tuning curves in both single cell and population responses (Chen and Jen 2000; Suga et al. 1997; Yang et al. 1992). To explore this possibility, we determined the frequency tuning curves for NCM sites in both control and experimental hemispheres before and after BIC application. We observed, however, no effect of BIC on frequency tuning curves in NCM (Fig. 7), and an ANOVA showed no significant

**FIG. 7.** GABAergic transmission does not shape frequency tuning in NCM. Tuning functions based on normalized phasic (0–50 ms) responses to tone stimuli (250–5,000 Hz) before (blue) and after (red) BIC application are shown for 4 recording sites (2 left hemisphere—control; 2 right hemisphere—BIC). Bottom traces in each graph show background levels of activity. Different sites show different best frequencies and tuning widths, but BIC does not cause any changes in the overall shape of the tuning functions.
Such changes suggest that during normal auditory processing, GABAergic transmission is necessary for normal temporal organization of song-evoked auditory responses in awake birds. Thus our study establishes that GABA-mediated transmission plays an important role in auditory processing of natural communication signals in the songbird NCM. More specifically, we observed that NCM responses to birdsong, recorded in awake restrained birds, shift from a primarily sustained (tonic) firing mode to a highly synchronized, phasic-like response pattern, on local blockade of GABA_\text{A} receptors. This marked alteration in the temporal organization of NCM responses occurs without changes in the overall response amplitude at the recorded sites. Blockade of GABAergic transmission also suppresses the evoked responses to some song syllables. This action appears to be mediated primarily through GABA_\text{A} receptors because spontaneous inhibitory transmission in NCM slices could be completely abolished by BIC, and no further effect was observed when both GABA_\text{C} and GABA_\text{A} receptors were blocked in our in vivo auditory experiments.

GABAergic transmission is known to play an important role in shaping the response properties of neurons from several sensory modalities. Consistent with its predominantly inhibitory action, GABA seems to restrain, both spatially and temporally, the excitatory activity that occurs during sensory processing in the majority of systems studied. Typically, antagonists of GABA-mediated transmission decrease the threshold for action potential generation, thereby increasing the excitability of neuronal networks and their firing frequency. In the auditory system, the focus of our interest, GABA_\text{A} receptor antagonism markedly expands frequency-tuning curves at the level of the mesencephalon, thalamus, and/or cortex in various vertebrate species and shifts evoked responses from predominantly phasic to tonic (sustained) patterns (Chen and Jen 2000; Jen and Feng 1999; Lu et al. 1998; Pollak and Park 1993). GABAergic transmission has also been shown to play a role in more complex aspects of auditory processing as in the case of experience-dependent plasticity of auditory spatial maps in the barn owl (Zheng and Knudsen 1999, 2001). Importantly, GABAergic regulation of the integration dynamics in the time domain influences the statistical probability and/or timing precision of action potential generation, a mechanism postulated to encode perception. In the big brown bat (\textit{Eptesicus fuscus}), for example, auditory cortical neurons that typically have brief response patterns, hence “phasic” responders, can be converted to “tonic” (sustained) responders by blocking GABA_\text{A} receptors (Chen and Jen 2000; Jen and Feng 1999). Such changes suggest that during normal auditory processing, GABA suppresses sustained excitatory drive in response to stimulation. Comparable findings have also been reported in the primary cortical visual area V1, where blockade of inhibition converts phase-locked response patterns to tonic responses through enhanced overall neuronal excitability (Sillito 1975b, 1979).

Our present findings for the songbird NCM depart substantially from these previously described GABAergic mechanisms. For instance, one might have predicted that local BIC application would result in an expansion of tuning curves as occurs in other systems, leading to decreased frequency discrimination ability in NCM. However, frequency tuning curves in NCM were not affected by BIC, indicating that frequency discrimination in NCM is insensitive to GABA_\text{A}-mediated inhibition. If GABAergic transmission regulates frequency tuning in the songbird auditory system, it does so outside NCM, possibly in preceding stations in the auditory pathway such as the primary telencephalic thalamo-reciprocal field L2. In fact, field L2a neurons in canary NCM exhibit strong inhibition at frequencies that flank the neuron’s preferred frequency (Terleph et al. 2006). Such inhibitory sidebands are not found in NCM, supporting the notion that inhibition does not play a role in frequency encoding in this auditory area (Terleph et al. 2006).

Another remarkable difference from the previous literature was the tonic-to-phasic shift observed under BIC and PTX treatment. Although this finding attests to the importance of GABA_\text{A}-mediated inhibition in NCM, the mechanism is presently unclear. A possible explanation is that, under normal GABAergic transmission (control condition), the refractory periods of song-responsive cells in our multiunit recordings are asynchronous, so cells would be able to encode the auditory stimulus by responding at different times, and/or with different latencies to specific acoustic features. Under BIC, however, song-responsive cells would show a strong phasic activation and then synchronize their refractory periods, resulting in a lack of responsiveness over a specific time window.

According to this hypothesis (synchronization of refractory periods), we would expect firing activity to be completely suppressed during the period that follows a phasic response. However, our observations make this possibility highly unlikely based on both phenomenological and analytical grounds. First, NCM neurons in slices fire, under BIC, at rates >20 Hz, so such neurons would not be expected to become refractory over the time periods of the phasic and tonic responses we observed. Second, evoked auditory responses in the NCM of awake birds were still reliably elicited following spontaneous bursts of activity that immediately occurred prior to stimulus onset. These data strongly suggest that the recorded NCM sites recover from inactivation rapidly and can respond to successive inputs well within the range of intersyllable intervals in a typical song. Finally, we showed that phasic and tonic responses are positively correlated in control and BIC conditions, which is inconsistent with the hypothesis that higher phasic responses reflect greater synchronous excitation that leads to synchronized refractoriness.

An interesting alternative hypothesis is that GABA_\text{A}-mediated inhibition normally suppresses a secondary inhibitory network in NCM. Such a network would be “released,” or disinhibited, from GABA_\text{A}-receptor influence on the local application of BIC and actively prevent NCM neurons from responding in a
sustained/tonic fashion to song. Such hypothesis could also explain the lack of neuronal responses to some syllables, reflected in decreased fidelity of auditory responses. Based on our data, though, this secondary network would have to rely on a BIC-insensitive inhibitory mechanism, which could be implemented in various ways. For example, inhibitory neurons that relied on GABA_B-mediated transmission could be under the control of the BIC-sensitive inhibitory network and thus actively suppress sustained song-evoked firing under BIC. However, the BIC-induced tonic-to-phasic reversal of NCM’s firing pattern occurred in a time scale substantially faster than that typically carried out by metabotropic GABA_B receptors (Bowery et al. 2002; Dutar and Nicoll 1988a,b; Tamas et al. 2003). The possibility that GABA_B receptors were tonically disinhibited by BIC or PTX treatment over the whole course of the experiment cannot be ruled out with our data. Importantly, however, BIC abolished all measurable sIPSCs in our slice recordings, which indicates no GABA_B influence was present, although this might not generalize to the in vivo condition. Thus the involvement of GABA_B receptors seems at present unlikely but cannot be ruled out.

Another possible scenario is that GABA might act as an excitatory transmitter at a subset of NCM synapses as described for the mammalian brain (Cohen et al. 2002; Gulledge and Stuart 2003; reviewed in Stein and Nicoll 2003); the blockade of such synapses could decrease some components of song-evoked responses. Yet another possibility is that NCM contains distinct populations of GABA_A-mediated inhibitory synapses with different sensitivities to BIC. Under this hypothesis, our BIC application (which was not maximal, intentionally, so as to avoid seizure-inducing concentrations) would have blocked the synapses with highest affinity for BIC, whereas less BIC-sensitive synapses would still be able to exert an inhibitory effect on NCM responses. Preliminary data indicate that GABAergic cells in NCM can themselves express the GABA_A receptor (not shown), providing a possible substrate for a GABA_A-dependent double-inhibitory circuit if different classes of GABA_A synapses differ in BIC sensitivity.

Finally, one cannot exclude the possibility that the BIC-insensitive inhibition in NCM might be mediated by an inhibitory neurotransmitter different from GABA. For example, metabotropic glutamate receptors, which can trigger inhibitory

![Diagram](http://jn.physiology.org/figure/100/4/453/fig8)

**FIG. 8.** Anatomical-functional NCM model. This simplified model suggests that under resting conditions, spontaneously active GABAergic synapses suppress NCM’s excitatory network and that on auditory stimulation, spontaneously active GABAergic synapses are suppressed by a secondary inhibitory network that is inactive at rest and that is activated by auditory stimuli. The inhibition of spontaneously active GABAergic synapses could arise from locations remote to NCM, such as field L (A and B), as well as from GABAergic neurons intrinsic to NCM (C and D). GABAergic neurons are illustrated in red while excitatory cells are black; bright colors indicate activated neurons while faded colors illustrate resting cells.
effects under physiological conditions (Fiorillo and Williams 1998) and are highly expressed in NCM (Wada et al. 2004), could be involved. Alternatively, glycinegic transmission, which plays an important role in the physiology of auditory brain stem areas of other vertebrates (Caspary et al. 2005; Kotak et al. 1998; Suneja et al. 1998) could participate in a putative double-inhibitory (disinhibitory) circuit. Indeed although our preliminary attempts to verify glycinegic transmission in NCM slices have been unsuccessful, recent in situ hybridization data show that glycine receptor components are expressed in NCM, and in vivo recordings in awake zebra finches show that local application of strychnine, a glycinegic receptor antagonist, can modulate some aspects of the NCM response to birdsong (Mello et al. 2007). Further studies are needed to establish the precise nature of the novel and complex inhibitory interactions we have uncovered here. In addition, subsequent investigations will be necessary to more precisely characterize the role of inhibitory transmission on the detection and analysis of the spectral and temporal features of song.

Our patch-clamp data show that spontaneously released GABA actively inhibits the excitatory network that drives NCM neurons in our slice preparations on BIC application. It seems likely that the elements of this excitatory network may be intrinsic to NCM because about half of NCM neurons are likely excitatory (Pinaud and Mello 2007; Pinaud et al. 2004). Alternatively, the excitatory drive we observed could come from the adjacent auditory field L and/or CMM, which are present in our slices and are thought to provide input to NCM (Vates et al. 1996), although we currently do not know whether the fibers from these input pathways are present in the slices. We do not know the extent to which the events observed in the slices also occur in vivo. It is tempting to speculate, however, that during auditory stimulation, the GABAergic cells that suppress the excitatory drive onto NCM neurons may be inhibited so that the excitatory network can become functional and process and transmit auditory information to other brain areas (Fig. 8). Should this configuration be confirmed, it will be important to determine whether the inhibition directed at spontaneously active GABAergic cells arises from sources extrinsic or intrinsic to NCM (Fig. 8; top vs. bottom schematics).

Unconventional interactions of inhibitory networks similar to those reported here may also be present, but still undetected, in cortical areas of other sensory systems or species. NCM is a telencephalic pallial area comparable, based on connectivity, to supragranular layers of the mammalian auditory cortex because it receives projections from the primary thalamo-recipient region field L and participates in intra-telencephalic circuitry that precedes the descending auditory projections originating in the arcopallium (Karten and Shimizu 1989; Mello et al. 1998). To our knowledge, a high-resolution analysis of the role of GABAergic inhibition in the encoding of complex sounds has not yet been performed at a comparable brain level in mammals although we should point out that we do not know the extent to which NCM and other avian auditory regions may compare with parts of the primary and higher-order auditory cortical fields in the mammalian brain. It is also possible, however, that the mechanisms we uncovered here are specific to NCM and reflect specializations related to the processing and encoding of learned vocalizations and/or behaviorally-relevant vocal communication signals. Songbirds are among the very few animals that evolved vocal learning (besides humans, cetaceans, bats, and 2 other avian orders), whereas most species, including non-human primates and rodents, lack this trait (reviewed in Jarvis 2004). Interestingly, in some auditory stations (e.g., the mammalian medial geniculate nucleus), an increase in GABAergic cell number has been shown to parallel phylogenetic increases in the computational requirements associated with a growing complexity of auditory-related behaviors (Winer and Larue 1996). In NCM, about half of the neuronal population is GABAergic, and a large proportion of these neurons participate in the auditory response to song (Pinaud et al. 2004); in contrast, only 25–30% of the neuronal population in the presumed mammalian NCM counterpart is inhibitory (Jones 1993). This high density of GABAergic elements in NCM and the apparently unique and complex interaction of inhibitory circuits we described here could reflect the high demands of encoding and/or memorization of learned natural communication signals. Should this hypothesis prove correct, our results could shed an important light on the function and evolution of auditory processing areas in other vocal learners as well as the neural basis of speech acquisition and language processing in humans.

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REFERENCES


