

# Nitric Oxide/Cyclic Guanosine Monophosphate Signaling in the Central Complex of the Grasshopper Brain Inhibits Singing Behavior

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## ABSTRACT

Grasshopper sound production, in the context of mate finding, courtship, and rivalry, is controlled by the central body complex in the protocerebrum. Stimulation of muscarinic acetylcholine receptors in the central complex has been demonstrated to stimulate specific singing in various grasshoppers including the species *Chorthippus biguttulus*. Sound production elicited by stimulation of muscarinic acetylcholine receptors in the central complex is inhibited by co-applications of various drugs activating the nitric oxide/cyclic guanosine monophosphate (cGMP) signaling pathway. The nitric oxide-donor sodium nitroprusside caused a reversible suppression of muscarine-stimulated sound production that could be blocked by <sup>1</sup>H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ), which prevents the formation of cGMP by specifically inhibiting soluble guanylyl cyclase. Furthermore, injections of both the membrane-permeable cGMP analog 8-Br-cGMP and the specific inhibitor of the cGMP-degrading phosphodiesterase Zaprinast reversibly inhibited singing. To identify putative sources of nitric oxide, brains of *Ch. biguttulus* were subjected to both nitric oxide synthase immunocytochemistry and NADPH-diaphorase staining. Among other areas known to express nitric oxide synthase, both procedures consistently labeled peripheral layers in the upper division of the central body complex, suggesting that neurons supplying this neuropil contain nitric oxide synthase and may generate nitric oxide upon activation. Exposure of dissected brains to nitric oxide and 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) induced cGMP-associated immunoreactivity in both the upper and lower division. Therefore, both the morphological and pharmacological data presented in this study strongly suggest a contribution of the nitric oxide/cGMP signaling pathway to the central control of grasshopper sound production. *J. Comp. Neurol.* 488:129–139, 2005. © 2005 Wiley-Liss, Inc.

**Indexing terms:** nitric oxide signaling; control of behavior; grasshopper brain; acoustic communication; second messenger pathways

Nitric oxide synthase (NOS) leading to the Ca<sup>2+</sup>/calmodulin-stimulated formation of nitric oxide (NO) has been detected in both invertebrates (e.g., cnidaria: Colasanti et al., 1995; molluscs: Moroz, 2000; crustaceans: Scholz et al., 2001; insects: Müller, 1997; Davies, 2000; Bicker, 2001) and vertebrates including mammals (for reviews, see Bredt and Snyder, 1992; Garthwaite and Boulton, 1995). It seems to represent an ancient signaling system conserved throughout large portions of the animal kingdom. Due to its production in the cytosol and its permeability through cell membranes, it may act both as an intracellular messenger and as an unconventional om-

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nidirectionally diffusible signaling molecule, independent of anatomically established synaptic connections.

NO serves various functions in the nervous system. It has been demonstrated to contribute to processes of cell migration (Gibson et al., 2001; Haase and Bicker, 2003), structuring of neuropils (Gibbs and Truman, 1998; Leamey et al., 2001), and the formation and maintenance of synapses (Wright et al., 1998). Nitric oxide's function as a neuronal transmitter involved in synaptic plasticity was initially demonstrated in the mammalian cerebellum (Garthwaite et al., 1988). Later, Elphick and co-workers (1993) detected NOS in the locust brain, and numerous subsequent studies on various insect species implicated NO signaling in the processing of visual (Bicker and Schmachtenberg, 1997; Schmachtenberg and Bicker, 1999; Jones and Elphick, 1999), olfactory (Müller and Hildebrandt, 2002; Wasserman and Itagati, 2003), and mechanosensory information (Ott et al., 2000), learning and formation of long-term memory (Müller, 1996, 2000; Müller and Hildebrandt, 2002), and modulation of synaptic release at the neuromuscular junction (Wildemann and Bicker, 1999).

The study described here adds another function of NO/cyclic GMP signaling in the insect nervous system, namely, as a major contributor to processes of selection and coordination of situation-specific behavior in the brain of acoustically communicating acridid grasshoppers. Many species generate distinguishable song types that serve as specific signals in the context of attracting partners for reproduction, courting, and agonistic behavior (for review, see Elsner, 1994). The sites in the central nervous system responsible for generation and selection of grasshopper song patterns are clearly separated, and each mechanism can be separately studied. Specific song patterns result from rhythmic movements of the sound-producing hind legs (stridulation) driven by central pattern generators in the third thoracic ganglion (Ronacher, 1989; Heinrich and Elsner, 1997). Each of several thoracic (sub)circuits responsible for the generation of a particular pattern is activated by tonic discharges of descending protocerebral command neurons (Hedwig, 1994; Hedwig and Heinrich, 1997). These command neurons seem to be selectively activated by presynaptic circuits residing in the central body complex of the protocerebrum (Heinrich et al., 2001a).

In immobilized but otherwise intact grasshoppers, species-specific sound production can be activated by focal injections into the central body neuropils of both nicotinic and muscarinic agonists (Heinrich et al., 1997). Studies with membrane-permeable drugs interfering with specific steps of intracellular signaling pathways identified both the adenylate cyclase- and the phospholipase C-initiated second-messenger pathways as mediators for the muscarinic acetylcholine receptor (AChR)-stimulated excitation (Wenzel et al., 2002). The same approach has now been extended to study the contribution of the NO/cGMP signaling pathway in the brain of grasshoppers to the control of stridulatory behaviors. In addition to these functional studies with various drugs interfering with NO-mediated signaling, we used established methods for identification of NOS-expressing and NO-responsive neurons. NOS-expressing neurons in sectioned grasshopper brains were labeled by immunocytochemistry with a universal anti-NOS antiserum and by NADPH-diaphorase histochemistry, which is widely used as a marker for NOS (see Ott and

Elphick, 2003 for a comparison of different protocols). We also attempted to identify cellular targets of NO by their capacity to stimulate soluble guanylyl cyclase, leading to the accumulation of cGMP, which can be detected by immunocytochemistry (De Vente et al., 1987; Truman et al., 1996). To potentiate NO-induced production of cGMP, we used the allosteric modulator of vertebrate sGC, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1; Ko et al., 1994), which has recently been demonstrated to influence insect sGC as well (Ott et al., 2004).

The functional and morphological studies presented here provide multiple lines of evidence showing that the NO/cGMP signaling pathway mediates inhibition in the central body complex, leading to the suppression of grasshopper sound production. It is demonstrated that 1) NO inhibits muscarine-stimulated stridulation through activation of sGC and accumulation of cGMP in neurons of the central body complex. These results are complemented by morphological studies demonstrating that 2) neurons intrinsic to the central body are capable of both generating NO as an intra- or transcellular messenger and responding to NO with accumulation of cGMP and therefore represent a potential endogenous source and target for NO-mediated inhibition of grasshopper sound production.

## MATERIALS AND METHODS

### Animals

Studies were performed with adult male grasshoppers of the species *Chorthippus biguttulus* (L. 1758). Most animals were caught in the vicinity of Göttingen, Germany and kept in separate groups of males and females in the laboratory for up to 2 weeks. Additional *Ch. biguttulus* were reared from eggs that had been collected during the previous summer and kept at 4°C for at least 4 months. After approximately 1 week at 26°C, the nymphs hatched and were fed wheat supplemental food for crickets (Nekton, Pforzheim, Germany). All experiments were conducted at room temperature (20–26°C).

### Drugs

Muscarine, 8-Br-cGMP, Zaprinast, IBMX, sodium nitroprusside (SNP), YC-1, and <sup>3</sup>H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ) were obtained from Sigma-Aldrich (Poole, UK). All drugs were dissolved in grasshopper saline made according to Clements and May (1974). YC-1 and Zaprinast were first dissolved as 100× stock solutions in dimethyl sulfoxide. SNP was either dissolved immediately before the experiment or, to serve as an inactive control, left in solution in an uncovered vial for at least 24 hours prior to its injection.

### Pharmacological experiments

**Preparation and recording.** The grasshopper's pronotum was fixed with wax to a holder, and the head was likewise attached to the pronotum. The cuticle of the head was incised posterior to the compound eyes, and the frontal part of the head was tipped forward to expose the dorsal surface of the brain. To record the stridulatory movements of the hind legs, small pieces of reflecting foil (Scotchlite 3M, type 7610) were glued to the distal parts of the femura. Up and down movements of the hind legs were detected by two optoelectronic cameras (Helversen and Elsner, 1977) that converted the position of the leg into a

proportional voltage signal. Recordings of hind leg movements and the pressure pulse were digitized and stored on a personal computer.

**Injection of drugs.** Drugs were applied by pressure injection (pressure pump PV 820, WPI Instruments, Waltham, MA) through a capillary with two or three chambers that were pulled to a single tip 10–15  $\mu\text{m}$  in diameter to enable individual injections of small (1–10 nl) volumes of different drugs to be made into the same site within the grasshopper brain. The location of the central body complex can be estimated from the pattern of tracheae on the dorsal surface of the protocerebrum. Injection capillaries were inserted in a stepwise manner until a position was reached where periodic injections ( $\Delta t = 5$  minutes) of a fixed volume of muscarine ( $10^{-4}$  M) stimulated stridulation of similar duration. Earlier studies, in which the sites of muscarine stimulations were labeled by co-injection of nondiffusible dextrans or latex beads, had demonstrated that only injections into the upper or lower division of the central body or into a small neuropil situated posterior and dorsal to the central body could stimulate singing behavior (Heinrich et al. 1997, 2001a). A comparison of songs stimulated at different sites within the central body upper or lower division revealed no differences.

**Evaluation and statistical analysis of experimental results.** The data were examined with the analysis program NEUROLAB (Hedwig and Knepper, 1992) and processed with the following tools: generation of histograms and graphs with Excel (Microsoft), statistical evaluation with PRISM (Graphpad, San Diego, CA), and assembling of figures with Photoshop (version 5.5, Adobe Systems, Mountain View, CA). Prior to testing for potential inhibitory effects on muscarine-stimulated stridulation of a second drug, the average duration of muscarine-stimulated songs was calculated from three consecutive trials. Identical muscarine injections were applied at regular intervals of 5 minutes throughout the entire experimental series to maintain a similar level of overall excitation. The test substance was injected in between two regular stimulations with muscarine. Analyses were performed by using relative values calculated by setting the longest duration of all songs stimulated at a particular injection site as 100%. Potential changes in the duration of muscarine-induced stridulation following the injection of another drug were evaluated by a nonparametric Kruskal-Wallis test followed by Dunn's multiple comparison test. To provide an estimate of the variance of the stimulated song durations, SDs of the means were calculated and included in the histograms.

### Morphological studies

**Preparation.** Brains of male *Ch. biguttulus* were extracted and fixed for 16–20 hours in 4% paraformaldehyde at 4°C. The tissue was dehydrated through an ethanol series and embedded in wax with a low melting temperature (1 g 1-hexadecanol plus 99 g poly[ethylenglycol 400] distearate). Transverse sections (referring to the neural axis) of 12  $\mu\text{m}$  thickness were cut with a microtome (Reichert-Jung BioCut 110) and transferred to microscope slides. Adhesion of the sections was increased by placing the slides into formaldehyde vapor for 30 minutes. After extraction of the wax and rehydration, the sections were processed for NADPH-diaphorase activity or immunocytochemistry.

**NADPH-diaphorase staining.** Prior to the staining procedure (originally described by Schürmann et al., 1997), brain sections were stored for at least 1.5 hours at room temperature in phosphate-buffered saline (PBS; 0.1 M) containing 0.25% Triton X-100. Tissue sections were incubated for 30 minutes at 37°C in the dark with 0.6 mM nitroblue tetrazolium salt (Sigma) and 0.25 mM  $\beta$ -NADPH (Sigma) in PBS (pH adjusted to 8). Control sections were incubated with nitroblue tetrazolium salt only. The staining reaction was terminated by three washing periods in PBS. For microscopic analysis the sections were dehydrated and enclosed in Roti-Histokitt (Roth, Karlsruhe, Germany).

In addition, whole mount preparations of grasshopper brains were processed for NADPHd activity following the protocol of Ott and Elphick (2002, 2003). Briefly, dissected brains were fixed for 2 hours in a 9:1 solution of methanol and formalin. After washing in 0.1 M Tris buffer (pH 7.2), the brains were initially incubated for 1 hour on ice in acetate buffer (0.1 M; pH 4) and subsequently left overnight in sodium acetate buffer (0.1 M; pH 5) containing 0.2% Triton-X-100. For staining, the reagents  $\beta$ -NADPH (0.2 mM) and nitroblue tetrazolium salt (0.2 mM) dissolved in Tris buffer with 0.2% Triton X-100 were first allowed to penetrate the tissue for 3 hours on ice, before the staining reaction was carried out at room temperature (30–60 minutes) and eventually terminated by rinsing in distilled water. Prior to microscopic examination, the brains were cleared by incubation for 15–30 minutes in methanol/glacial acetic acid (3:1), three times for 15–30 minutes in methanol and 30–60 minutes in cedar oil.

**Anti-NOS immunocytochemistry.** Prior to staining, the brain sections were incubated for 1 hour in blocking buffer (PBS 0.1 M containing 0.5% Triton X-100, 0.25% bovine serum albumin, and 1% normal goat serum). Brain sections were incubated for 2 days at 10°C with the primary anti-rabbit uNOS antiserum (Affinity BioReagent, Golden, CO) at a dilution of 1:100 in blocking buffer. After washing three times with PBS a Cy3-coupled secondary antibody (Jackson ImmunoResearch, West Grove, PA) was applied for 1 hour (1:100 in blocking buffer). The staining procedure was terminated by washing the brain sections three times in PBS. Sections were dehydrated and mounted with Fluoromount (BDH Laboratory Supplies, Poole, UK) before immunofluorescence was analyzed with a microscope (Leica DMRB or Leica TCS SP2).

**Anti-cGMP immunocytochemistry.** Prior to fixation and sectioning (see above), brains of male *Ch. biguttulus* were extracted and transferred to saline or saline containing SNP ( $10^{-3}$  M) plus Zaprinast ( $10^{-3}$  M) for 20 minutes at room temperature. Alternatively, brains were preincubated on ice for 45 minutes with YC-1 ( $5 \times 10^{-4}$  M) followed by incubation at 33°C with YC-1, SNP, and Zaprinast (same time and concentrations as above). After fixation and sectioning, the brain tissues were incubated overnight at room temperature with the primary rabbit anti-cGMP antiserum (courtesy of Dr. Jan de Vente; De Vente et al., 1987) at a dilution of 1:4,000 in PBS. After three washes in PBS, a Cy3-coupled secondary antibody (Jackson ImmunoResearch, diluted 1:60 in PBS) was applied for 2 hours.

## RESULTS

The species-specific sound production (stridulation) of the acoustically communicating grasshopper *Ch. biguttulus* can be stimulated by pressure injection of cholinergic agonists into the upper and lower division of the central body complex in the brain (Heinrich et al., 1997; 2001a). Muscarine, activating muscarinic ACh receptors connected to the adenylate cyclase/cyclic AMP/protein kinase A signaling pathway, has been demonstrated to stimulate stridulation reliably (Wenzel et al., 2002). In a particular experiment, repeated injections of muscarine to the same stimulation site in the central body at regular intervals of 5 minutes led to an initial increase in the duration of the stimulated stridulation due to an accumulation of second messengers. After two to four such stimulations, each muscarine pulse releases stridulatory activity of uniform intensity and duration (Heinrich et al., 2001b; Wenzel et al., 2002). Repeated stimulation of stridulation with muscarine can therefore be used to test for putative inhibitory effects of other neuroactive drugs on the control circuits for sound production in the grasshopper brain.

### SNP inhibits muscarine-stimulated stridulation

To explore putative inhibitory effects of NO on the performance of stridulation, double-barreled glass capillaries pulled to a single tip were filled with muscarine ( $10^{-4}$  M) and the NO donor SNP ( $10^{-3}$  M), respectively. The capillary was placed in the central protocerebrum (area indicated in Fig. 1A) of an adult male *Ch. biguttulus* at a site where periodic ( $\Delta t = 5$  minutes) injections of a fixed volume of muscarine elicited species-specific stridulatory activity of similar duration (Fig. 1B,C). An injection of SNP to the same site within the protocerebrum interposed between two muscarine pulses resulted in a gradually developing, complete and fully reversible inhibition of muscarine-stimulated stridulation. Muscarine-induced stridulation was maximally, and in this experiment completely, suppressed at 17 and 22 minutes after the application of SNP. Inhibitory effects persisted until 27 minutes after the injection. Subsequent stimulations with muscarine elicited stridulation of similar duration as before SNP treatment. After full recovery of the muscarine response, SNP-mediated inhibition could be repeated with the same outcome.

The experiment shown in Figure 1 is a typical example with regard to the nature and time course of SNP effects (compare also with cumulative results in Fig. 2A). However, single injections of SNP could also lead to a partial instead of a complete suppression of muscarine-stimulated stridulation. The amount and duration of inhibition increased when two or three pulses of SNP were applied, indicating a dosage effect of SNP on the reduction of overall excitation in the neural circuits controlling stridulation. In all experiments described below, only one pulse of SNP (or any other drug interfering with muscarine-induced stimulation) was applied. This pulse was delivered to the identical site in the protocerebrum where muscarine elicited the stridulation and consisted of a similar volume (approximately 1–3 nl) as the muscarine pulses.

### NO-induced production of cGMP in the cephalic control circuits mediates inhibition of stridulation

The experiments summarized in this section provide compelling evidence that NO mediates inhibition of muscarine-induced stridulation through activation of soluble guanylyl cyclase (sGC) and the accumulation cGMP.

**NO mediates inhibition of muscarine-stimulated stridulation.** Upon dissolving into aqueous solution, SNP spontaneously liberates NO. To test whether the liberated NO or the remaining portion of the SNP molecule accounts for the inhibitory effects on muscarine-stimulated stridulation, we compared freshly made solutions of SNP with equally concentrated solutions made on the day before that had been stored overnight in a small tube with an open lid at room temperature. Freshly made solutions of SNP ( $10^{-3}$  M), injected to the same sites in the protocerebrum where muscarine reliably elicited stridulation, caused a reversible inhibition of muscarine-stimulated behavior (Fig. 2A). Although complete suppression of stridulation was only seen in a few experiments (see Fig. 1), a reduction in the duration of muscarine-stimulated stridulation was observed in each of the experiments performed. In contrast, depleted solutions of SNP, from which NO should have been evaporated, were not found to inhibit muscarine-stimulated stridulation in any experiment (Fig. 2B). This suggests that after dissociation of the SNP molecule in solution, the NO component mediates the inhibitory effects on the performance of stridulation.

**NO activates a soluble guanylyl cyclase.** The membrane-permeable drug ODQ has been shown in a number of vertebrate and invertebrate preparations to inhibit sGC, a potential target of NO (Garthwaite et al., 1995; Ball and Truman, 1998; Feelisch et al., 1999; Zhao et al., 2000). Using an injection capillary with three chambers that had been pulled to a single tip, muscarine ( $10^{-4}$  M), SNP ( $10^{-3}$  M), and ODQ ( $10^{-3}$  M) were consecutively injected to the same site within the central protocerebrum. At the same injection sites where muscarine reliably elicited stridulation, SNP reversibly reduced the duration of muscarine-stimulated stridulation (Fig. 2C; filled circles), whereas the same volume of SNP in combination with an injection of ODQ had no inhibitory effect on the behavior stimulated with muscarine (Fig. 2C; open circles). The inhibitory effect of SNP therefore seems to be based on a NO-mediated activation of sGC.

**Inhibition is mediated by accumulation of cGMP.** To substantiate our results further, we performed two experiments in which we directly elevated cytosolic concentrations of cGMP, the intracellular messenger produced by activated sGC and other guanylyl cyclases. Injections of 8-Br-cGMP, a membrane-permeable analog of endogenously generated cGMP, caused a reversible inhibition of muscarine-stimulated stridulation (Fig. 2D). When compared with the inhibition induced by SNP (compare Fig. 2A), 8-Br-cGMP-mediated inhibition appeared after a shorter latency and persisted for shorter periods. A weaker but still significant reduction of stridulation resulted from injection of Zaprinast, an inhibitor of cGMP-hydrolyzing phosphodiesterase (Fig. 2E). Inactivation of the phosphodiesterase apparently leads to an accumulation of endogenously generated cGMP sufficient to mediate the inhibitory effect on stridulation.

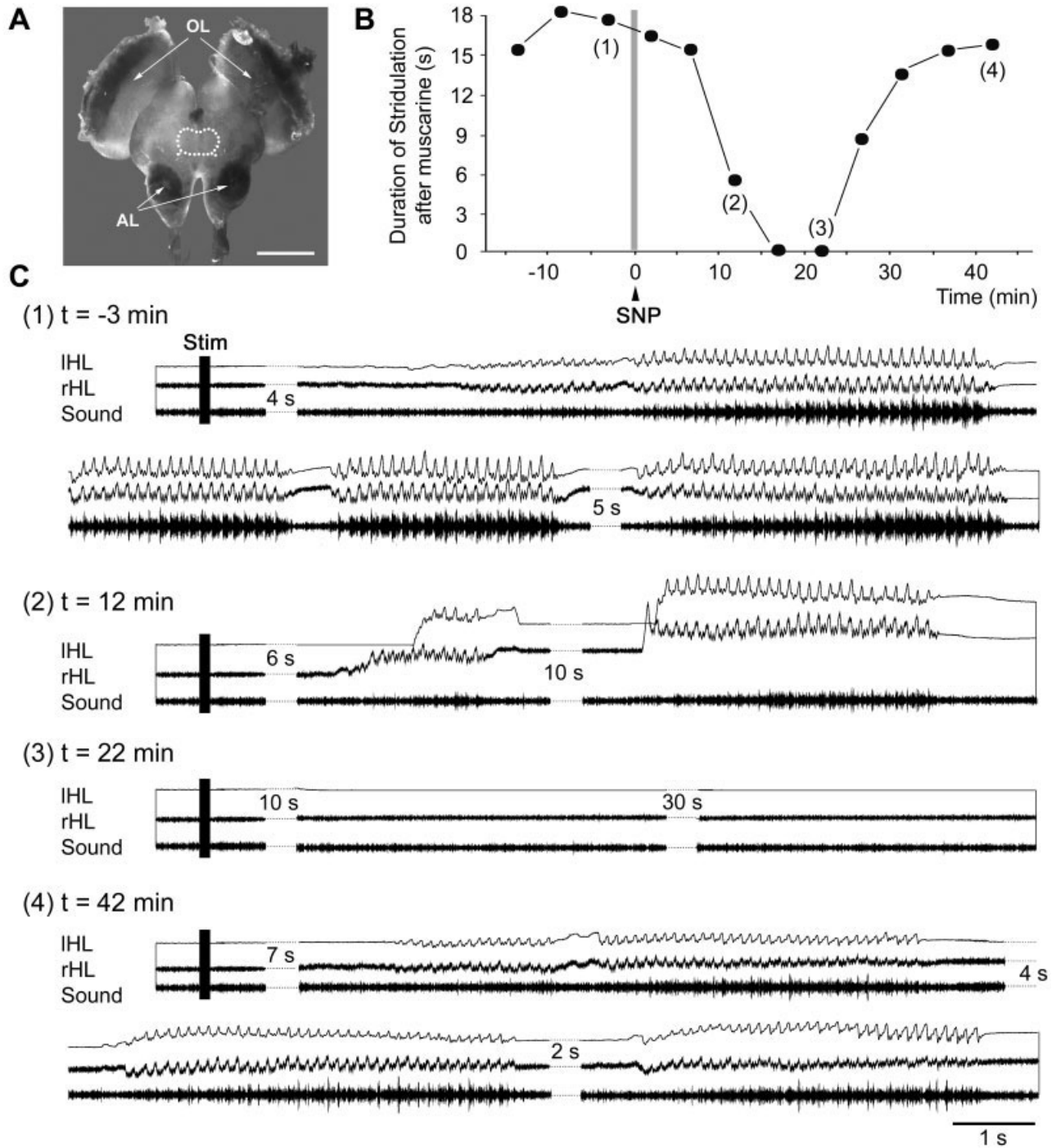


Fig. 1. Sodium nitroprusside (SNP) reversibly inhibits muscarine-stimulated stridulation in male *Ch. biguttulus*. **A**: Ventral view of a dissected brain. White dots indicate the area where the injection capillary was inserted to apply drugs to the central body complex neuropil. After the capillary was placed at a site where repeated injections of muscarine reliably elicited stridulation of similar duration, both the injection site and the volumes injected with each stimulation pulse remained unchanged during the entire experiment. **B**: Repeated injections of identical small volumes (1–3 nl) of muscarine ( $10^{-4}$  M) to the same site within the protocerebrum stimulated

song production of similar duration. Following one injection of SNP ( $10^{-3}$  M), muscarine-stimulated stridulation was at first reduced, then entirely suppressed, and finally gradually recovered to durations obtained before application of SNP. **C**: Original recordings of muscarine-stimulated stridulatory movements of the left (IHL) and right (rHL) hind leg and the resulting sound patterns. Recordings 1–4 show the entire stridulatory activity resulting from the stimulations indicated in B. Data presented in B and C show the results of one typical experiment. OL, optic lobes; AL, antennal lobes Stim, time of muscarine stimulus. Scale bar = 500  $\mu$ m in A.

### NADPHd activity and NOS immunoreactivity in the central body complex

NOS-dependent NADPH-diaphorase activity is supposed to be resistant to formaldehyde fixation of vertebrate (Matsumoto et al., 1993) and some, although not all, invertebrate tissues (Ott and Elphick, 2002, 2003); it is widely used as a histochemical marker for the presence of NOS (Dawson et al., 1991; Müller, 1994; Müller and Bicker, 1994). To identify putative endogenous sources for NO in brain areas contributing to the control of grasshopper stridulation, the NADPH-diaphorase staining procedure was applied to whole mount preparations (following

the protocol of Ott and Elphick, 2002, 2003) and sections of *Ch. biguttulus* brains. Our stainings of *Ch. biguttulus* brains confirmed the results of earlier studies on other insect nervous systems. In particular, distinct NADPH-diaphorase staining was present in the antennal (Fig. 3A) and optic lobes (Fig. 3B), two areas involved in the processing of sensory information that are known to express NOS from various earlier studies on locusts (e.g., Elphick et al., 1996; Müller and Bicker, 1994).

In addition, NADPH-diaphorase reaction products were also detected in the central body complex, the major neuropil involved in the cephalic control of grasshopper stridulation. Whole mount brain preparations (Fig. 3C) revealed that at least part of the central body staining originated from a pair of neurons located laterally from the upper division. Axons emerging from these cell bodies projected between the upper and lower divisions of the central body and seemed to connect the outer layers of the central body upper division and the lateral accessory lobes, both of which were labeled by punctuate staining. Transverse histological sections of the protocerebrum demonstrated that the NADPH-diaphorase staining pattern seemed to be associated with the columnar structure of the central body upper division (Fig. 3E). In addition, the most intense labeling appeared in a distinct layer extending beneath the ventral and lateral surface of the upper division (Fig. 3F). Sections through posterior portions of the central body (Fig. 3G) revealed additional punctuate NADPH-diaphorase staining in both the brain region adjacent to the dorsal border of the central body and the lateral accessory lobes.

To confirm the specificity of the NADPH-diaphorase staining, NOS immunocytochemistry using anti-universal NOS antiserum was applied to transverse sections of *Ch. biguttulus* brains. NOS-associated immunofluorescence and NADPH-diaphorase staining patterns appeared to be very similar in antennal and optic lobes (not shown in figure) and in the upper division of the central body (Fig. 3H,I). NOS immunoreactivity was largely restricted to a peripheral layer of the central body upper division extending beneath its ventral and lateral border. Inner layers of the upper division and the entire lower division contained

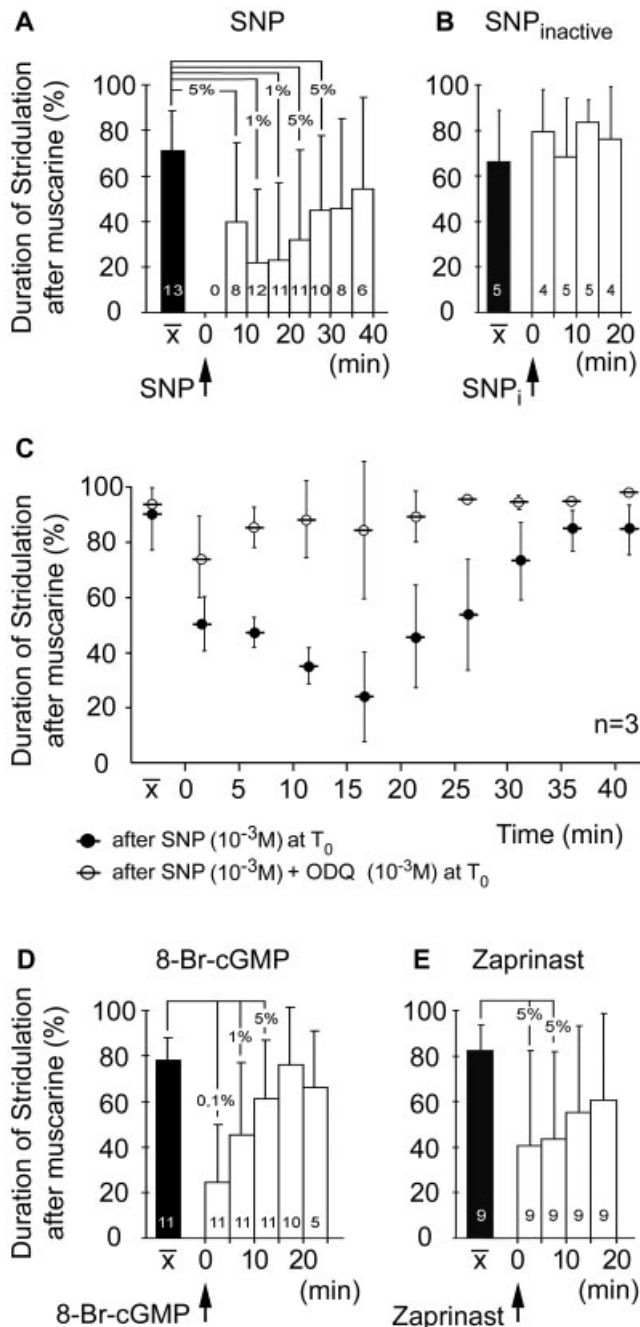


Fig. 2. Activation of NO/cGMP signaling in the central protocerebrum inhibits muscarine-stimulated stridulation in male *Ch. biguttulus*. **A:** The duration of stridulation stimulated by 10<sup>-4</sup> M muscarine was reversibly reduced by a single injection of freshly dissolved sodium nitroprusside (SNP; 10<sup>-3</sup> M). **B:** In contrast, depleted SNP, from which NO has presumably evaporated, had no inhibitory effect on the muscarine-stimulated behavior. **C:** The inhibitory effects of SNP on muscarine-stimulated stridulation (filled circles) were suppressed by co-injection of both SNP and [<sup>3</sup>H]-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ 10<sup>-3</sup> M, open circles). **D,E:** Accumulation of cGMP by injection of both the membrane-permeable analog 8-Br-cGMP (10<sup>-2</sup> M, D) and the phosphodiesterase inhibitor Zaprinast (10<sup>-2</sup> M, E) resulted in a reversible suppression of muscarine-stimulated stridulation. In each animal, the longest duration of all songs stimulated with muscarine at a particular injection site was set as 100%, and calculated relative values of durations were used for the statistical analysis. Black columns represent the average relative duration of muscarine-stimulated songs calculated from three consecutive trials. White columns represent the average relative duration of muscarine-stimulated songs after a single injection of the test substance. Numbers in columns indicate the number of experiments conducted in different animals. Percentages refer to the confidence interval for statistical significance.

no detectable label. Cell bodies located laterally to the upper division, like those that were stained by NADPH-diaphorase products (Fig. 3C), were not detected with the NOS antiserum label.

### NO-induced accumulation of cGMP in the central body

To identify potential target neurons that may be influenced by endogenously released NO, dissected brains were exposed to the NO donor SNP in the presence of the

phosphodiesterase inhibitor Zaprinast (each  $10^{-3}$  M) and subsequently analyzed for cGMP immunoreactivity. With this method, cGMP was exclusively detected by intense immunofluorescence in two cell groups, each consisting of approximately 75 cell bodies situated in the pars intercerebralis region in the rostral and ventral part of the brain and the tracts formed by their axons (not shown). No accumulation of cGMP could be detected in the central body or brain regions known to contain sGC such as the antennal and optic lobes. In contrast, all three brain regions showed cGMP-associated immunoreactivity after NO exposure in the presence of YC-1. Most importantly for our studies, cGMP could be detected in the central body. As shown in horizontal brain sections (Fig. 3J), a layer in the outer region of the lower division bordering the inner part of the upper division of the central body showed the most intense staining of fine neuronal arborizations. Separated by a smaller layer, from which cGMP immunofluorescence was essentially absent, the inner portion of the lower division seemed to contain diffuse but rather weak labeling. In addition, distinct cGMP-immunoreactive neurites appeared to project through all layers of the upper division of the central body (Fig. 3K). No particular staining, indicative of extensive synaptic contacts in one of the layers, could be detected.

After control incubations in saline, no cGMP-associated immunofluorescence was detected anywhere in the *Ch. biguttulus* brain (not shown).

## DISCUSSION

In acoustically communicating grasshoppers such as the species *Ch. biguttulus* used in our studies, the central

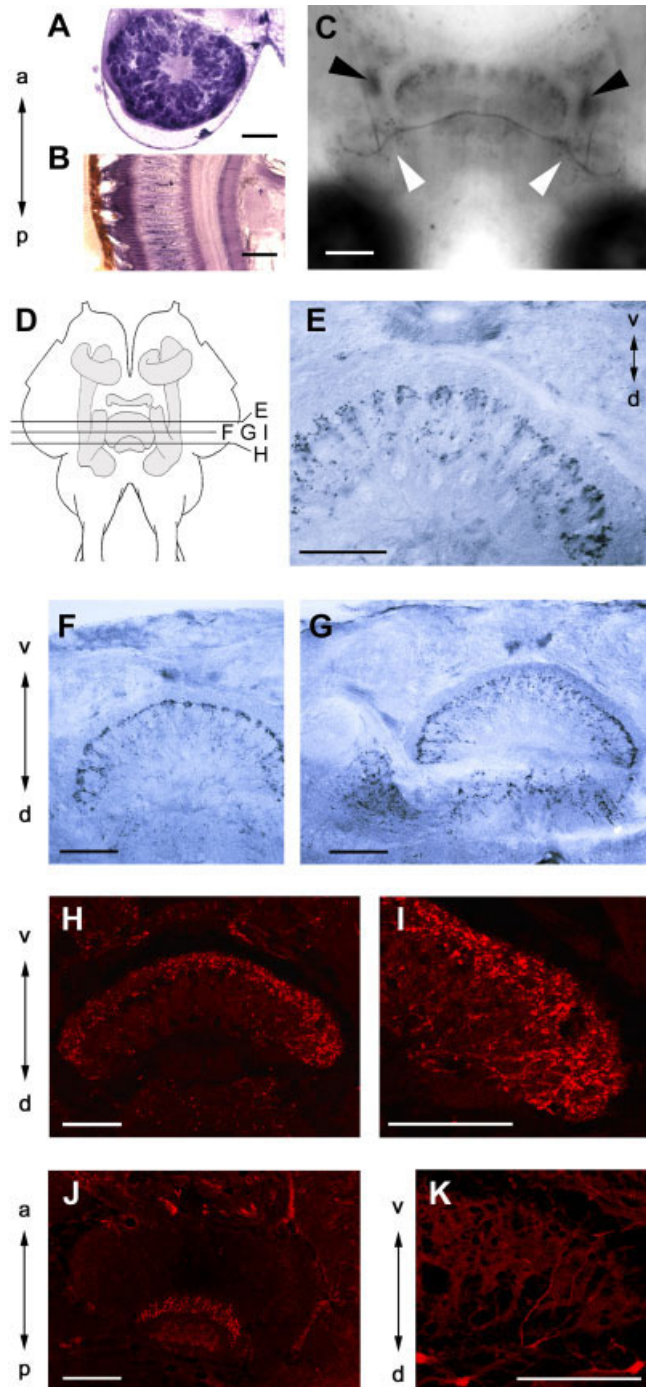


Fig. 3. NADPH-diaphorase staining and NOS immunocytochemistry in the brain of *Ch. biguttulus*. **A,B**: The most intense staining developed in the antennal lobes (A, Vibratome section, horizontal). Distinct diaphorase staining was also seen in the optic lobes (B, Vibratome section, horizontal). **C**: Whole mount preparations (ventral view) revealed specific NADPH-diaphorase staining in the central body, predominantly in its upper division. At least part of the labeling originated from a pair of cell bodies located just laterally to the upper division of the central body (black arrowheads; cell bodies located ventrally to the plane of focus). Their axons projecting between the two divisions of the central body seemed to be responsible for the punctuate staining of both the central body upper division and the lateral accessory lobes (white arrowheads). **D**: Schematic drawing of a horizontal view of a grasshopper brain, including the mushroom bodies and the central body complex and the approximate planes of transversal sections shown in E–I. **E–G**: Transversal sections through the central body complex revealed NADPH-diaphorase staining in outer layers of the upper division that seemed to be associated with its columnar organization pattern (best seen in E). **G**: More posterior transversal sections show additional staining both in the region situated dorsally to the central body and in the lateral accessory lobes (only one accessory lobe shown in this figure). **H,I**: Transversal section of the central body showing anti-NOS immunofluorescence in outer layers of the upper division. The right border of the upper division shown in H has been enlarged in I to reveal details of the staining pattern and its similarity with the diaphorase staining shown in E. **J,K**: Anti-cGMP immunocytochemistry after NO stimulation in the presence of YC-1. **J**: Horizontal section showing distinct labeling in a layer between the upper and lower division of the central body and diffuse staining in the lower division. **K**: Transverse section through the lateral part of the central body upper division with immunoreactive neurites. The terms horizontal and transverse are relative to the neural axis of the ventral nerve cord. A, anterior; p, posterior; v, ventral; d, dorsal. Scale bars = 50  $\mu$ m.

complex constitutes the major central nervous neuropil responsible for the selection and coordination of sound production in the context of the behavioral situation encountered. Several signaling systems, including transmitters and their ionotropic or metabotropic receptors, have been shown to contribute to the balance of excitatory and inhibitory synaptic input that determines the motivation to sing and the selection of the appropriate song pattern (Heinrich et al., 1997, 1998; Wenzel et al., 2002).

The aim of this study was to demonstrate a functional contribution of NO/cGMP signaling to the cephalic control of grasshopper sound production. Pharmacological experiments were performed with restrained but functionally intact grasshoppers whose species-typical singing behavior was used to monitor the level of excitation that resulted from drug application into the central body. Although pharmacological studies on intact and behaving preparations face certain limitations not shared with studies on isolated cells or on tissue preparations (e.g., identification of directly stimulated neurons and determination of exact drug concentration at its site of action), they offer the opportunity to select those physiological mechanisms relevant for the control of a certain behavior from a larger number of measurable but rather irrelevant reactions induced by a particular drug. Grasshoppers require certain conditions for performing stridulation, such as appropriate temperature, illumination, and the absence of visual, auditory, and vibratory sensory input that may signal the presence of predators. Whereas conditions that favor singing should excite cephalic control centers, probably by activating muscarinic AChRs (Heinrich et al., 2001b), conditions indicating that stridulation is not an appropriate behavior should prevent excitation in circuits that control sound production. Earlier studies identified  $\gamma$ -aminobutyric acid (GABA) and glycine as transmitters involved in the rapid and short-lasting interruption of ongoing stridulatory activity by activation of ionotropic receptors (Heinrich et al., 1998). Because second messenger-dependent signaling mechanisms usually mediate slower and more persistent changes, the NO/cGMP system could in contrast represent a signaling pathway acting on a longer time scale that relays sensory information about general inappropriateness of stridulation to song control circuits in the protocerebrum.

### **NO-mediated inhibition of sound production involves soluble guanylyl cyclase activation and accumulation of cGMP**

Typically, NO synthesized by NOS-expressing neurons diffuses through rather large volumes of neuropil and causes the accumulation of cGMP in cells containing soluble guanylyl cyclase (Bredt and Snyder, 1992; Garthwaite and Boulton, 1995). Pharmacological perturbation of each of these processes in the central body provided strong evidence for the participation of this pathway in the control of grasshopper stridulation. Suppression of muscarine-stimulated sound production was achieved by liberation of NO from SNP (Figs. 1, 2) and by increasing cytosolic levels of cGMP through both the application of the membrane-permeable cGMP analog 8-Br-cGMP (Fig. 2D) and inhibition of cGMP-specific phosphodiesterase with Zaprinast (Fig. 2E). In addition, NO-mediated suppression of stridulatory behavior was blocked in the presence of ODQ, an inhibitor of soluble guanylyl cyclase (Fig.

2C). A specific action on the desired molecular targets and processes in the brain of *Ch. biguttulus* can be assumed because all drugs used in our study had previously been shown to exert their specific effects in various other insect nervous tissues (e.g., Müller, 2000; Müller and Hildebrandt, 2002; Wildemann and Bicker, 1999).

### **Possible cGMP-initiated mechanisms that mediate the inhibition of sound production**

Cyclic GMP can theoretically mediate effects via cGMP-dependent protein kinase (PKG), cGMP-regulated phosphodiesterases, or cyclic nucleotide-gated channels. A synergistic action of cGMP and cyclic adenosine monophosphate (cAMP) leads to the formation of long-term memory in honeybees that is apparently based on a direct cGMP-mediated activation of protein kinase A (Müller, 1996; Müller and Hildebrandt, 2002). However, grasshopper stridulation can be stimulated by activation of a muscarinic AChR-initiated signaling cascade that involves cAMP formation and protein kinase A function (Heinrich et al., 2001b; Wenzel et al., 2002). In contrast to their synergy in long-term memory formation in honeybees, cAMP and cGMP act as functional antagonists on the performance of sound production in grasshoppers. Antagonistic effects of NO-mediated accumulation of cGMP and the cAMP/protein kinase A pathway have been described in locust embryos, in which the cellular distribution of F-actin and neuronal migration is regulated in an opposite fashion (Haase and Bicker, 2003). A direct interference of cAMP and cGMP signaling pathways also regulates the biosynthesis of ecdysteroids in blowfly ovaries (Maniere et al., 2003). Examples of differential regulation of excitability and/or synaptic efficacy by opposite actions of cAMP and cGMP in the same insect neuron are, to our knowledge, lacking. Although cGMP-activated protein kinase G has been shown to hyperpolarize *Drosophila* neurons through phosphorylation of  $K^+$  channels (Renger et al., 1999), it remains questionable whether NO directly inhibits stridulation by elevating cGMP in brain neurons involved in the command chain for the activation of sound production in *Ch. biguttulus*.

Alternatively, and more likely, suppression of stridulation may be mediated through activation of brain neurons that synaptically inhibit those neurons that, if sufficiently excited, induce the performance of stridulation. Brain neurons that tonically suppress sound production through picrotoxin-sensitive chloride channels whenever grasshoppers are not stridulating have been described in several grasshopper species, including *Ch. biguttulus* (Heinrich et al., 1998). Modulation of their activity through sensory input may regulate the general arousal in the cephalic control circuits for stridulation. NO has been demonstrated to increase motoneuron spike activity in *Manduca sexta* (Qazi and Trimmer, 1999) and promote vesicle release at *Drosophila* neuromuscular junctions (Wildemann and Bicker, 1999). A similar mechanism may contribute to the regulation of the activity of stridulation-inhibiting interneurons.

### **Sources and targets of NO in the central body**

To complement our physiological results, we identified potential sources and targets of NO in the brain of *Ch. biguttulus* (Fig. 3). Whole mounts and sections of brain tissue were processed for both NADPH-diaphorase activ-

ity and NOS immunocytochemistry. Labeling obtained by both procedures was largely the same in both the central body and other brain regions known to contain NOS (see below). Following the protocol of Ott and Elphick (2002, 2003) the NADPH-diaphorase staining of whole mounts was substantially improved through better penetration of staining reagents and reduced nonspecific staining of the perineural sheath. Compared with NOS immunocytochemistry, the NADPH-diaphorase staining seemed to be more sensitive and revealed more details of the labeled neural structures. As one example, the NADPH diaphorase-positive neurites connecting the central body with the lateral accessory lobes (Fig. 3C) were never seen in brains processed for NOS immunoreactivity. The lateral accessory lobes seem to represent integrative links among the central body, sensory pathways, and ventral nerve cord, and various neurons connecting the central body with the lateral accessory lobes have been described (Homberg, 1994).

NADPH-diaphorase reaction products and NOS immunoreactivity were detected in various neuropils including the antennal lobes, the optic lobes, and the central body. The antennal and optic lobes are known to express NOS in various insects (reviewed by: Müller, 1997; Bicker, 2001) suggesting a common function of NO in processing of olfactory and visual sensory information (Müller and Hildebrandt, 1995; Elphick et al., 1996; Bicker and Schmachtenberg, 1997). Although characteristic staining was detected in the central bodies of various insect species (Müller, 1997), its functional role in this neuropil remained unresolved. Our studies suggest a contribution of NO to the selection and control of sound production in grasshoppers because NO can be generated by NOS-containing neurons in the central body and has been shown to interfere with muscarine-stimulated stridulation when released into this neuropil. Studies on locusts and moths revealed a direct coupling of nicotinic AChR-mediated excitation to NO release (Müller and Bicker, 1994; Zayas et al., 2002), and all the NO-releasing neurons in the locust antennal lobe appeared to use GABA as an additional conventional transmitter (Seidel and Bicker, 1997). Both ACh and GABA have also been shown to contribute to the cephalic control of stridulation in grasshoppers (Heinrich et al., 1997, 1998), and a potential co-localization of NADPH-diaphorase activity with nicotinic receptors and GABA will be a subject of our future studies.

Due to its physical properties, NO is not suited for rapid or precise neuron-to-neuron transmission, rather, it acts like a gain control mechanism (Elphick et al., 1996), affecting populations of neurons in largely unpredictable areas around its site of release. NO is regarded as the most diffusible messenger currently known, and its diffusion length constant in nervous neuropils has been estimated at 90  $\mu\text{m}$  (Kasai and Petersen 1994). Because this distance represents approximately half the entire lateral extension of the central body in *Ch. biguttulus*, NO released within the central body probably affects neurons in adjacent areas that could mediate suppression of muscarine-stimulated behavior. However, muscarine-stimulated stridulation was also reduced by injections of 8-Br-cGMP and Zaprinast into the central body (Fig. 2D,E), and NO-induced inhibition of sound production was blocked by co-injection of ODQ (Fig. 2C). In contrast to NO, these drugs would not be expected to reach target

cells located far away from the injection site in sufficient concentration. The results of our pharmacological experiments therefore suggested that NO-responsive neurons in close proximity to the sites of injection within the central body mediate the inhibitory effects on stridulation.

Specificity of NO signaling in the nervous system is largely determined by expression of its principal target, soluble guanylyl cyclase, in responsive neurons. In addition, cGMP-independent effects of NO have been described (Stamler et al., 1997) suggesting that only a subset of neurons that in vivo are responsive to NO can be detected by specific antisera against cGMP (DeVente et al., 1987). Earlier immunocytochemical studies on adult locust brains revealed accumulation of cGMP upon NO stimulation in olfactory interneurons of the antennal lobe (Bicker et al., 1996), subsets of Kenyon cells (Bicker et al., 1996), and photoreceptor cells (Bicker and Schmachtenberg, 1997; Jones and Elphick, 1999). Recent studies by Ott and coworkers (2004) demonstrated that YC-1 strongly potentiates the NO-induced accumulation of cGMP in the insect brain and suggested that the number of cellular targets for NO may have been considerably underestimated in previous studies.

In *Ch. biguttulus*, cGMP immunoreactivity was only detectable in the central body when YC-1 was present during NO stimulation. The most intense staining resulted from neural branches within an outer layer of the lower division, and more diffuse labeling was present in the central areas of this neuropil (Fig. 3J). Accumulation of cGMP was also detected in neurites that appeared to cross all layers of the central body upper division (Fig. 3K). Subsets of these neurons in the upper and lower division of the central body that accumulated cGMP following exposure of entire brains to NO and YC-1 may also mediate the suppression of muscarine-stimulated stridulation through focal activation of NO/cGMP signaling pathways and may even contribute to the control of stridulation endogenous release of NO.

## CONCLUSIONS

NO/cGMP signaling in the central body complex has been demonstrated to contribute to the cephalic control of grasshopper sound production by decreasing arousal in neural circuits controlling stridulation, making this behavior less likely to occur. In contrast to muscarinic AChR signaling activated in situations that favor stridulation (e.g., hearing a conspecific female), the NO/cGMP pathway should be activated by sensory signals associated with situations in which stridulation appears to be inappropriate. Our study contributes further evidence that the central body plays a role in selecting behavior on the basis of integrated sensory information from various sources. Our present knowledge suggests that context-appropriate sound production in grasshoppers is influenced by multiple signaling pathways that either promote (nicotinic and muscarinic AChRs, proctolin) or inhibit (GABA, glycine, NO) the performance of stridulation.

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