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Ultrasound sensitive neurons in the cricket brain

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Summary. 1. The aim of this study was to identify neurons in the brain of the cricket, *Teleogryllus oceanicus*, that are tuned to high frequencies and to determine if these neurons are involved in the pathway controlling negative phonotaxis. In this paper we describe, both morphologically and physiologically, 20 neurons in the cricket brain which are preferentially tuned to high frequencies.

2. These neurons can be divided into two morphological classes: descending brain interneurons (DBINs) which have a posteriorly projecting axon in the circumesophageal connective and local brain neurons (LBNs) whose processes reside entirely within the brain. All the DBINs and LBNs have processes which project into one common area of the brain, the ventral brain region at the border of the protocerebrum and deutocerebrum. Some of the terminal arborizations of Int-1, an ascending ultrasound sensitive interneuron which initiates negative phonotaxis, also extend into this region.

3. Physiologically, ultrasonic sound pulses produce 3 types of responses in the DBINs and LBNs. (1) Seven DBINs and 6 LBNs are excited by ultrasound. (2) Ongoing activity in one DBIN and 5 LBNs is inhibited by ultrasound, and (3) one cell, (LBN-ei), is either excited or inhibited by ultrasound depending on the direction of the stimulus.

4. Many of the response properties of both the DBINs and LBNs to auditory stimuli are similar to those of Int-1. Specifically, the strength of the response, either excitation or inhibition, to 20 kHz sound pulses increases with increasing stimulus intensity, while the response latency generally decreases. Moreover, the thresholds to high frequencies are much lower than to low frequencies. These observations suggest that the DBINs and LBNs receive a majority of their auditory input from Int-1. However, the response latencies and directional sensitivity of only LBN-ei suggest that it is directly connected to Int-1.

5. The response of only one identified brain neuron, DBIN8, which is inhibited by 20 kHz sound pulses, is facilitated during flight compared to its response at rest. This suggests that suppression of activity in DBIN8 may be associated with ultrasound-induced negative phonotactic steering responses in flying crickets. The other DBINs and LBNs identified in this paper may also play a role in negative phonotaxis, and possibly in other cricket auditory behaviors influenced by ultrasonic frequencies.

Key words: Cricket – Negative phonotaxis – Ultrasound – Descending interneurons – Local neurons – Brain

Abbreviations: DBIN descending brain interneuron; *LBN* local brain neuron

Introduction

An insect's brain processes sensory information and integrates sensory inputs with higher order motor centers to elicit specific behaviors (Schürmann 1987; Huber 1974). In the cricket, the brain has been shown to contain higher order motor centers for initiating stridulation and locomotion (Huber 1974; Bentley 1977; Otto and Weber 1982). Furthermore, the brain of crickets appears to integrate sensory and motor inputs to release two complex auditory behaviors – positive and negative phonotaxis (Schildberger 1986; Hoy and Nolen 1987; Brodfuehrer et al. 1988; Brodfuehrer and Hoy 1989).

In crickets, positive phonotaxis is released by species specific calling song, while negative phonotaxis is induced by ultrasonic stimuli (Thorson et al. 1982; Moiseff et al. 1978). Experiments suggest that the recognition of calling song is controlled by neural networks in the brain which act like temporal filters tuned to the conspecific calling song pattern (Schildberger 1985, 1986). In fact, in *Gryllus bimaculatus* specific sets of neurons in the brain have been identified whose activity is maximal in response to the correct temporal pattern of the calling song (Schildberger 1984). These neurons, tuned to the

temporal pattern of the calling song, may activate descending interneurons which then control the motor program for walking in the thoracic segments such that the cricket walks in the direction of the correct calling song pattern (Schildberger 1985, 1986). In negative phonotactic behavior, descending interneurons in the brain have been hypothesized to act as neural 'AND' gates, combining inputs from ultrasound-sensitive, sensory interneurons and motor activity from the flight central pattern generator. The output of these descending interneurons then drives the flight steering mechanism in the thoracic and abdominal segments to produce the appropriate steering response directing the cricket away from the source of the ultrasound (Hoy and Nolen 1987). In the Australian field cricket, *Teleogryllus oceanicus*, reception of ultrasonic stimuli in the central nervous system is mediated by a pair of interneurons, Int-1, which transmit this information to the brain (Moiseff and Hoy 1983). Recently, it has been shown that in *T. oceanicus* ultrasound sensitive descending interneurons are located in the brain and their response to ultrasound is facilitated during flight. Moreover, a reduction in the amount of ultrasound sensitive descending activity is correlated with a decrease in the magnitude of the negative phonotactic response in the abdomen during flight (Brodfuehrer et al. 1988; Brodfuehrer and Hoy 1989). However, the precise identity of ultrasound-sensitive descending interneurons that are modulated by the flight rhythm in the cricket brain is presently unknown.

In this paper we describe, both morphologically and physiologically, 20 neurons in the cricket brain which are preferentially tuned to high frequencies.

Materials and methods

All experiments were performed on adult, female crickets, *Teleogryllus oceanicus*, 2–6 weeks after the adult molt. These animals were originally collected in Hawaii, and have been raised for approximately 3 years in our laboratory. All crickets were maintained on a reversed light cycle (10 h light:14 h dark) and all experiments were performed during the subjective night (May et al. 1988). This procedure increased the reliability of eliciting sustained flight activity in response to wind puffs directed at the abdominal cerci in dissected preparations. In addition, 10^{-6} mol l^{-1} 3-isobutyl-1-methylxanthine (IBMX) bath was applied to the exposed nervous system of ventral flight preparations (see below) to facilitate initiating flight in response to wind (G.S. Boyan, personal communication, and P.D. Brodfuehrer and R.R. Hoy, personal observation).

Experimental preparations. Two preparations were employed to record from ultrasound sensitive neurons located in the cricket brain. In one preparation the cricket was oriented dorsal surface up, and attached with wax by its head to a metal stand. To do this, we first immobilized the mouthparts on the ventral surface of the head with wax and then attached the ventral surface of the head to the metal stand with additional wax. Next, a wax well was built around the head and anterior portion of the pronotum. The mesothoracic and the metathoracic pairs of legs and wings were removed, but the prothoracic legs were left intact and free to move. The brain was exposed by first removing a piece of cuticle extending from slightly dorsal to the median ocelli down to the epistomal suture, and between the eyes and around the antennal sockets. Following removal of this cuticle, the tracheae, fat, and connective tissue overlying the brain were removed. The esophagus, which runs between the circumesophageal connectives and gut were removed by severing the attachment points of the esophagus to the mouth and pulling the gut out of the cricket through a slit in the abdomen. After cutting away the clypeus and labrum, a pin was placed between the circumesophageal connectives to stabilize the brain for intracellular recording. Lastly, the sheath covering

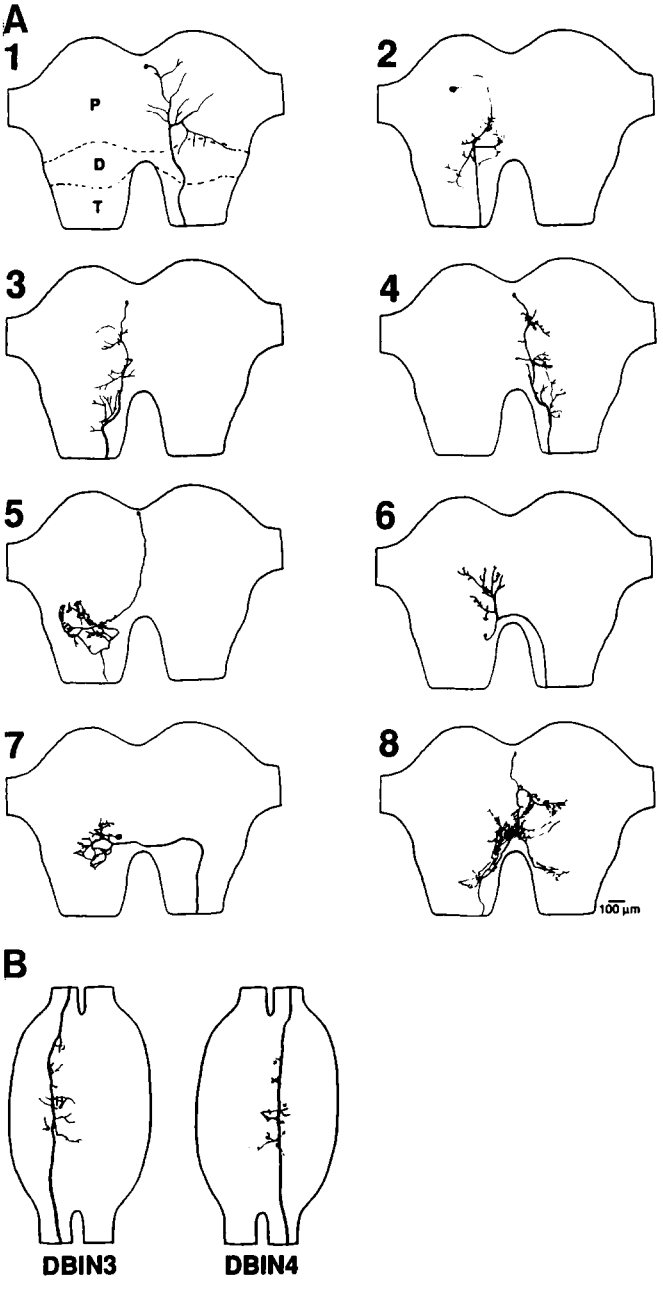

Fig. 1A, B. Morphology of descending brain interneurons (DBINs). **A** Camera lucida drawings of wholemount Lucifer Yellow fills, drawn within the approximate boundaries of the brain. In the text, the DBINs are referred to by the number in the upper left of each drawing. Dashed lines in DBIN1 indicate approximate boundaries between the 3 fused segments which comprise the brain – protocerebrum (P), deutocerebrum (D), and tritocerebrum (T). **B** Camera lucida drawings of the branching pattern of DBIN3 and DBIN4 in the subesophageal ganglion. Approximate boundaries of the subesophageal ganglion are drawn. Scale bar pertains to **A** and **B**

Table 1. Physiological properties of Int-1 and descending brain interneurons (DBINs). Cell name of DBINs refers to Fig. 1. Best frequency compares the response elicited in each interneuron by 20 kHz and 5 kHz sound pulses. No spiking activity was elicited in DBIN1, DBIN2, and DBIN5 by 5 kHz sound pulses. Directional sensitivity: ipsilateral (I) and contralateral (C) refer to the neuron's soma side with respect to the direction of the sound source. Only two sensory modalities were examined, wind and sound. Duration of the long pulse is approximately 500 ms

Cell	Best freq. (20 kHz vs. 5 kHz)	Latency (at 20 K 90 dB)	Directional sensitivity	Multi-modal	Response to long pulses	Level of spont. activity
Int-1	20 kHz > 5 kHz	13–15 ms	I < C	no	phasic/tonic	< 1 Hz
DBIN1	20 kHz	25–30 ms	I > C	no	phasic/tonic	none
DBIN2	20 kHz	30–40 ms	I = C	yes	phasic	none
DBIN3	20 kHz ≫ 5 kHz	30–40 ms	I ≪ C	yes	phasic/tonic	1– 3 Hz
DBIN4	20 kHz ≫ 5 kHz	35–40 ms	I > C	yes	phasic/tonic	10–20 Hz
DBIN5	20 kHz	25–35 ms	I > C	yes	phasic	5–10 Hz
DBIN6	20 kHz ≫ 5 kHz	25–30 ms	I = C	yes	phasic/tonic	3– 5 Hz
DBIN7	20 kHz > 5 kHz	20–25 ms	I < C	yes	phasic/tonic	1 Hz
DBIN8[a]	20 kHz > 5 kHz	30–40 ms	I = C	yes	phasic	20 Hz

[a] Inhibited by ultrasound

All data presented in the table was measured while the cricket was at rest

the brain was removed with fine scissors. Following these manipulations, the cricket was able to assume flight posture and fly, as indicated both by the movement of its wing stubs and electromyographic recordings (EMG; see below) from wing muscles. In this preparation we recorded intracellularly from processes of ultrasound sensitive neurons directly in the brain neuropil.

In the other preparations the cricket was attached, ventral side up, with its head and pronotum waxed onto a small platform, and its prothoracic legs fixed in a natural flight posture with wax. Both the mesothoracic and metathoracic pairs of legs and wings were removed. With the cricket waxed in this position, the metathoracic wing stubs were free to move during flight. A large wax well extending from the pronotum around the head was built. The soft cuticle covering the cervical connectives and all the mouth parts were removed to expose the subesophageal ganglion. The gut was removed by cutting it just posterior the subesophageal ganglion and pulling it out of the cricket through a slit in the abdomen. The connective sheath covering the subesophageal ganglion was removed and a small metal platform was put under the subesophageal ganglion for support. With this preparation, we recorded intracellularly in the neuropil of the subesophageal ganglion from axons of descending brain interneurons whose somata where located in the brain. Of the 8 descending brain interneurons we found, only two were located while recording intracellularly from the subesophageal ganglion.

Physiological recording and staining procedures. Intracellular recording of activity from ultrasound sensitive neurons was made using conventional recording techniques. All intracellular recordings were made using glass microelectrodes filled at the tip with 5% Lucifer Yellow and the shank backfilled with 1 *M* LiCl (Moiseff and Hoy 1983; Nolen and Hoy 1984). The morphology of brain neurons was determined by iontophoresing Lucifer Yellow into the cells with constant hyperpolarizing current (3–5 nA for at least 5 min). At the end of the experiment, Lucifer Yellow was allowed to diffuse for at least 1 h. The brain and subesophageal ganglion were removed, processed for wholemount fluorescence microscopy, photographed, and drawn using a Leitz camera lucida drawing tube. In some animals, the nervous system from the brain through the metathoracic ganglion was removed and processed. After visualization of some wholemount preparations, the tissue was embedded in Ladd's medium and horizontally sectioned (20 μm) (Brodfuehrer and Hoy 1988) to determine the relationship between neuronal branches and specific brain regions. Most of the ultrasound sensitive brain neurons were penetrated at a depth of 150 μm to 300 μm from the anterior-ventral surface of the brain.

EMG recordings from a metathoracic wing depressor muscle (muscle 129a; Furukawa et al. 1983) were made using 50 μm copper wire insulated except at the tip. All electrical activity was recorded on a Vetter FM tape recorder and analysed later. Permanent physiological records were made using a Tektronix 2230 digital oscilloscope connected to an HP plotter.

Auditory stimulus presentation. Acoustic stimuli used in this study were generated electronically. The carrier frequency of the sound pulses was generated with a B & K Precision function generator, shaped by a custom built trapezoid shaper (symmetrical sound pulse with rise and fall times of approximately 5 ms), amplified by a Nikko amplifier, and delivered through a pair of Motorola piezoelectric tweeters. The speakers were placed approximately level with the cricket's ears and 90 ° to the left and right of its longitudinal body axis. Peak sound pressure levels (SPL) are expressed in dB rel. to 20 μPa. All harmonics were at least 30 dB lower than the fundamental carrier frequency of the stimulus, as measured by a Nicolet (444A) real-time spectrum analyser.

Results

Ultrasound sensitive descending brain interneurons and local brain neurons

In the brain, we recorded from more than 50 neurons whose response was greater to 20 kHz (ultrasound) than 5 kHz sound pulses. The morphologies of 20 of these ultrasound sensitive brain neurons were determined from whole-mount Lucifer Yellow fills (Figs. 1, 6, 7 and 11 A). We divided these ultrasound sensitive neurons into two general morphological classes, descending brain interneurons and local brain neurons. Descending brain interneurons (DBINs) all had axons which projected posteriorly in the circumesophageal connective, while the processes of local brain neurons (LBNs) remained entirely within the brain. The anatomy of the DBINs and LBNs is based only on one fill of each neuron, except for DBIN3 and DBIN6 which were filled twice, one in each hemisphere of the brain. Nevertheless, we believe that all the neurons described in this study are distinct cells (see Discussion).

Descending brain interneurons – morphology. We have identified 8 different DBINs based on their morphology in the brain (Fig. 1A). Five of the DBINs, DBIN1–DBIN5, have axons that exit the brain ipsilateral to their soma and processes, while DBIN6–DBIN8 have axons which project posteriorly contralateral to their soma and processes in the brain. In general, the main neurites of DBIN1–DBIN5 and DBIN8 transverse the entire brain just lateral to the midline, and in all but DBIN5, the processes of the DBINs extend only a short distance from the main neurite in this region. In addition, the processes of DBIN6 also arborize just lateral to the midline of the brain. The main neurite of DBIN6 and DBIN7 crosses the midline of the brain, and the processes of both DBIN5 and DBIN7 arborize mainly in the deutocerebrum. Furthermore, the processes of the DBINs do not cross the midline of the brain, except for DBIN8. From sectioned fills, we also observed that none of the DBINs had processes which projected into the central body region of the brain, although some processes did appear to surround and extend into the region of the mushroom bodies (not shown).

The morphology of the DBINs in the subesophageal ganglion was clearly visible in only 3 interneurons. Two of these DBINs (DBIN3 and DBIN4) are shown in Fig. 1B. In all 3 DBINs only a few processes projected from their axons in the subesophageal ganglion and, for the most part, did not project across the midline of the subesophageal ganglion. Moreover, the axons of all 3 DBINs projected posteriorly in the connectives at least as far as the mesothoracic ganglion, and only a few branches extended a short distance into the prothoracic ganglion.

Descending brain interneurons – physiology. Of the 8 DBINs identified, 7 (DBIN1–DBIN7) were excited by ultrasound and in one, DBIN8, ongoing spontaneous activity was inhibited by ultrasound. The response properties of the DBINs to auditory stimuli are summarized in Table 1, and are similar to the response properties of Int-1 in several ways. First, the strength of the response (number of spikes per stimulus) in all 7 DBINs excited by ultrasound increased with increasing stimulus intensity (Fig. 2A). Similarly, the amplitude and duration of the inhibitory response evoked by 20 kHz sound pulses in DBIN8 also increased with increasing stimulus intensity, especially when the cricket was flying (Fig. 2B, C). Second, all the DBINs had lower thresholds for 20 kHz sound pulses than for 5 kHz, with thresholds for ultrasound ranging from approximately 55 dB to 75 dB. In addition, 20 kHz sound pulses always produced stronger responses in the DBINs than 5 kHz pulses. This is illustrated in the frequency response curve of DBIN3, where three times as many spikes were elicited by high frequency sound pulses (16, 20 and 30 kHz) than low frequency sound pulses (3, 5, and 10 kHz) (Fig. 3A). The amplitude and duration of the inhibitory response in DBIN8 was also greater to 20 kHz sound pulses than 5 kHz pulses (Fig. 3B). Third, 5 of 8 DBINs were activated throughout long duration (500 ms) sound

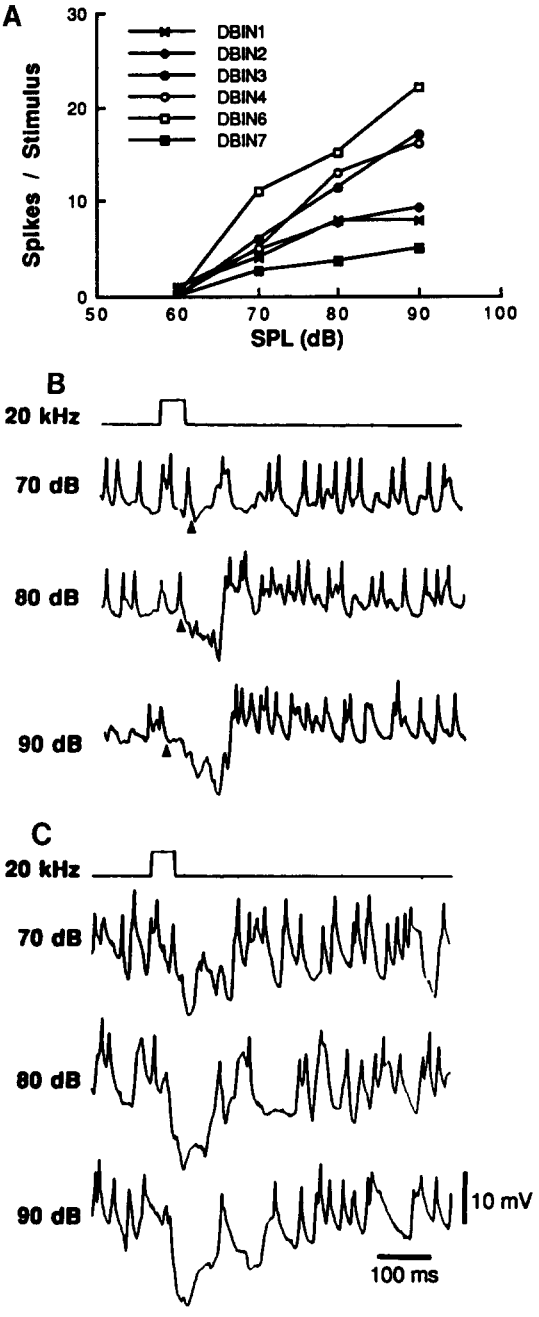

Fig. 2A–C. Graded response of the DBINs. **A** Number of spikes elicited in each DBIN, except DBIN5, in a 100 ms following a 20 kHz sound as a function of stimulus intensity (SPL). As stimulus intensity increases the number of spikes elicited in each DBIN increases. **B** and **C** Graded nature of the inhibitory response in DBIN8. As stimulus intensity increases, the amplitude and duration of the inhibitory response in DBIN8 increases, while the latency decreases (onset of inhibition in **B** indicated by arrowhead). In addition, note that ultrasound-induced inhibition in DBIN8 is greater during flight **C** than at rest **B**. In **B** and **C**, top trace: timing of auditory stimulus; bottom trace(s): intracellular recording. Scale bars in **C** also pertain to **B**

pulses. The response in these DBINs to long duration sound pulses consisted of an initial phasic burst of spikes followed by a lower level of tonic activity (Fig. 4A). The other three DBINs showed only a phasic response to long duration ultrasonic sound pulses (Fig. 4B). Fi-

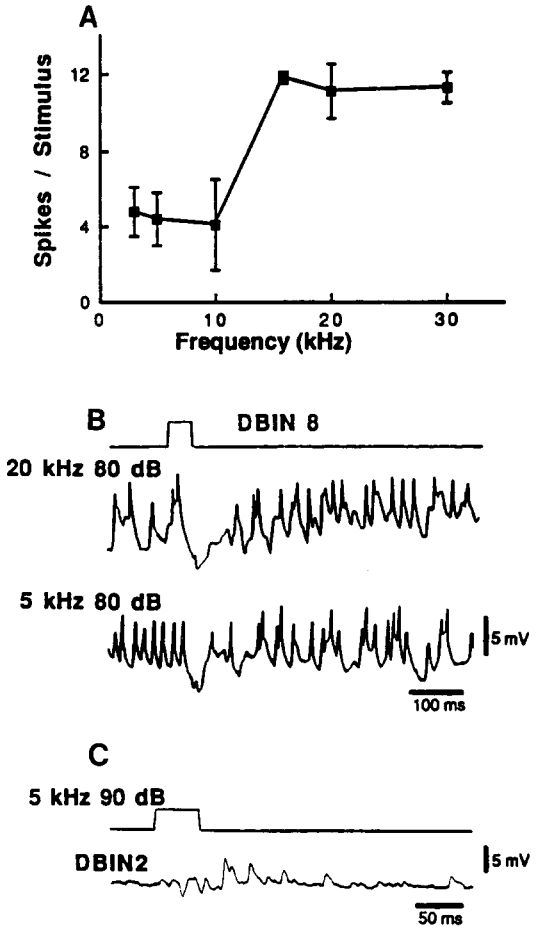

Fig. 3A–C. Best frequency. **A** Frequency response curve of DBIN3. The average number of spikes (±SEM) evoked within a 100 ms following an 80 dB sound pulse by high frequency (16, 20 and 30 kHz) sound pulses is greater than by low frequency (3, 5, 10 kHz) sound pulses. The frequency response curve of DBIN3 was determined in two preparations, and 5 stimulus trials were presented at each frequency. **B** Strength of the response in DBIN8 to a 20 kHz and a 5 kHz sound pulse. The amplitude and duration of the inhibitory response in DBIN8 is greater to a 20 kHz (80 dB) sound pulse than a 5 kHz (80 dB) sound pulse. **C** A 5 kHz sound pulse does not elicit spiking activity in DBIN2. In **B** and **C**, top trace: timing of auditory stimulus; bottom trace(s): intracellular recording

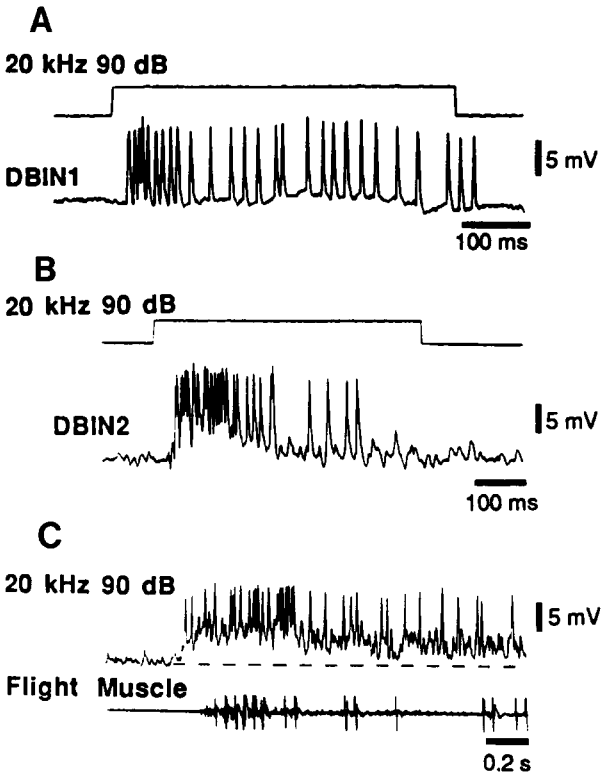

Fig. 4A–C. Long duration sound pulses. **A** Spiking activity in DBIN1 is elicited throughout a long duration (approx. 500 ms) 20 kHz 90 dB sound pulse. **B** and **C** DBIN2 is only phasically activated by a long duration 20 kHz sound pulse, but responds in a phasic/tonic manner to a long duration wind puff (dashed line). In **A** and **B**, top trace: timing of auditory stimulus; bottom trace: intracellular recording. In **C**, top trace: intracellular recording; bottom trace: EMG recording from wing depressor flight muscle

nally, the response latencies of the DBINs were generally greater to 5 kHz sound pulses than 20 kHz, and in some DBINs, the response latencies decreased with increasing stimulus intensity (Fig. 2B, C). We found that response latencies in all the DBINs to 20 kHz 90 dB sound pulses were greater than 20 ms, which was approximately 5 ms longer than Int-1's response latency recorded in the brain (Table 1).

Activity in the DBINs also differed from Int-1 in several ways. First, all the DBINs except DBIN1, were activated both by sound and wind. In fact, many cells were activated more strongly by a continuous wind puff to their cerci than by a long duration ultrasonic stimulus (compare Fig. 4B, C). Second, spiking activity was elicited in DBIN1, DBIN2 and DBIN5 only by high frequency sound pulses, but not by 5 kHz sound pulses (Fig. 3C). Third, unlike Int-1 which was generally not spontaneously active, 6 of 8 DBINs had a level of spontaneous activity greater than 1 Hz. Finally, the response in most DBINs outlasted short duration (50 ms), high intensity sound pulses (Figs. 2B, 5A, B). In fact, the response evoked by ultrasound in DBIN3 and DBIN6 could last up to several 100 ms following the end of a stimulus pulse (Fig. 5A), which never occurs in Int-1.

To determine if the strength of the response in the DBINs was directionally dependent, ultrasonic stimuli were presented both ipsilateral and contralateral to the soma and arborizations of the DBINs in the brain. We found that DBIN4 and DBIN5 were preferentially activated by ipsilateral stimuli, while DBIN3 (Fig. 5A) and DBIN7 were more strongly activated by contralateral stimuli. In contrast, the response in DBIN1, DBIN2 (Fig. 5B), DBIN6 and DBIN8 was similar independent of the direction of the stimulus.

Local brain neurons – morphology. The LBNs consisted of 3 morphological types (Figs. 6, 7 and 11A). Half of the LBNs (6 of 12) had a characteristic morphology consisting of bilateral fields of innervation in both hemispheres of the brain connected by a neurite which

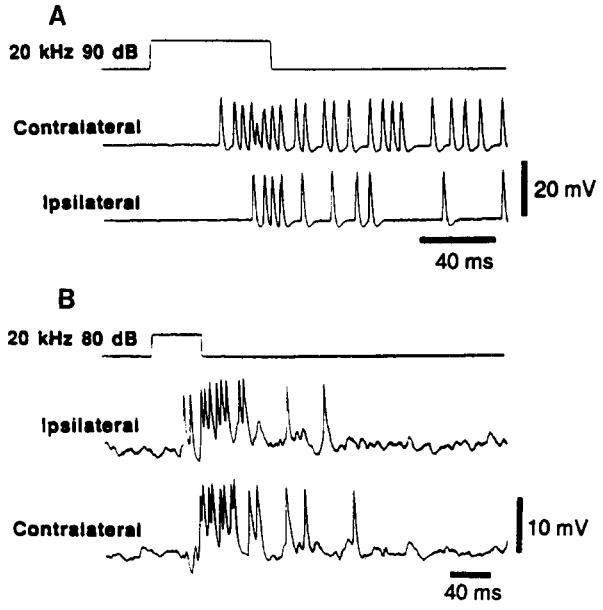

Fig. 5A, B. Directional sensitivity. **A** An ultrasonic stimulus presented contralaterally to the soma and processes of DBIN3 in the brain elicits a greater response than an ipsilateral stimulus. **B** The strength of the response evoked by 20 kHz 90 dB sound pulse presented either ipsilateral or contralateral to DBIN2's processes in the brain is similar. In **A** and **B**, top trace: timing of auditory stimulus; bottom traces: intracellular recording. Activity in DBIN3 was recorded intracellularly from its axon in the subesophageal ganglion

crossed the midline of the brain ventral to the central body and did not branch. The bilateral fields of these LBNs were similar in the number of branches, but were clearly not symmetrical. Four of the LBNs also had fields of innervation on both sides of the brain, but in these LBNs, one side had many fewer branches compared to the other side. Finally, two LBNs arborized entirely within one cerebral hemisphere of the brain.

Local brain neurons – physiology. Three different types of physiological responses were observed in the LBNs in response to ultrasonic stimuli. (1) In 5 LBNs ongoing spiking activity was inhibited (LBNs-i). (2) In 6 LBNs spiking activity was activated or increased (LBNs-e), and (3) in one LBN, it was either excited or inhibited depending on the direction of the stimulus (LBN-ei). In this section we will only describe the physiological properties of the LBNs-i and LBNs-e.

The LBNs-i and LBNs-e have 4 physiological properties in common with the DBINs. First, the strength and latency of the response were graded with increasing stimulus intensity. Both the amplitude and duration of inhibition in the LBNs-i, and the number of spikes elicited in LBNs-e by ultrasound increased with increasing stimulus intensity, while the response latencies decreased (Fig. 8). Second, the strength of the response was greater to 20 kHz sound pulses than 5 kHz sound pulses. An example of this response property is shown in Fig. 9

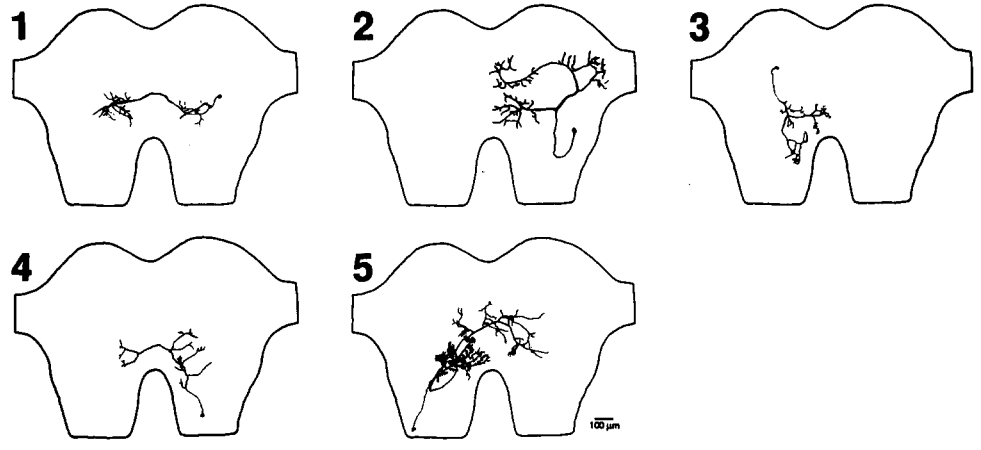

Fig. 6. Morphology of local brain neurons inhibited by ultrasound (LBN-i). Camera lucida drawings of whole-mount Lucifer Yellow fills, drawn within the approximate boundaries of the brain. In the text, the LBNs-i are referred to by the number in the upper left of each drawing

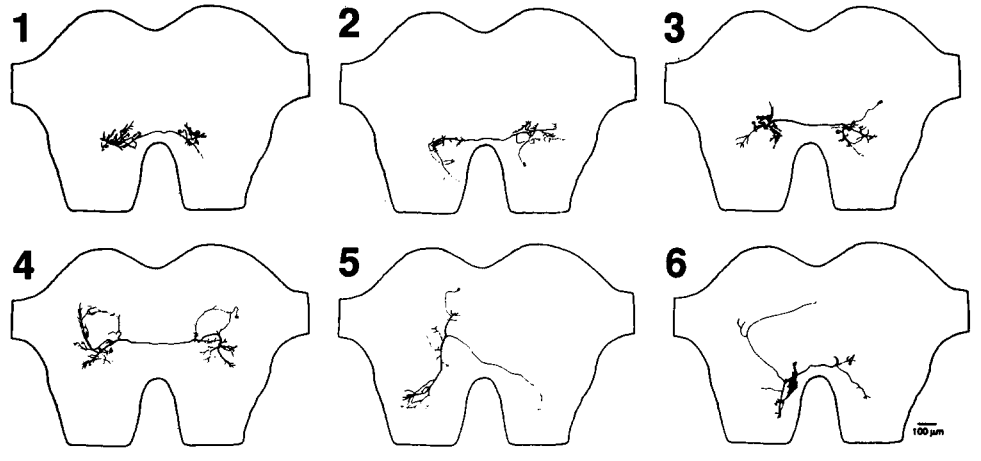

Fig. 7. Morphology of local brain neurons excited by ultrasound (LBN-e). Camera lucida drawings of whole-mount Lucifer Yellow fills, drawn within the approximate boundaries of the brain. In the text, the LBNs-e are referred to by the number in the upper left of each drawing

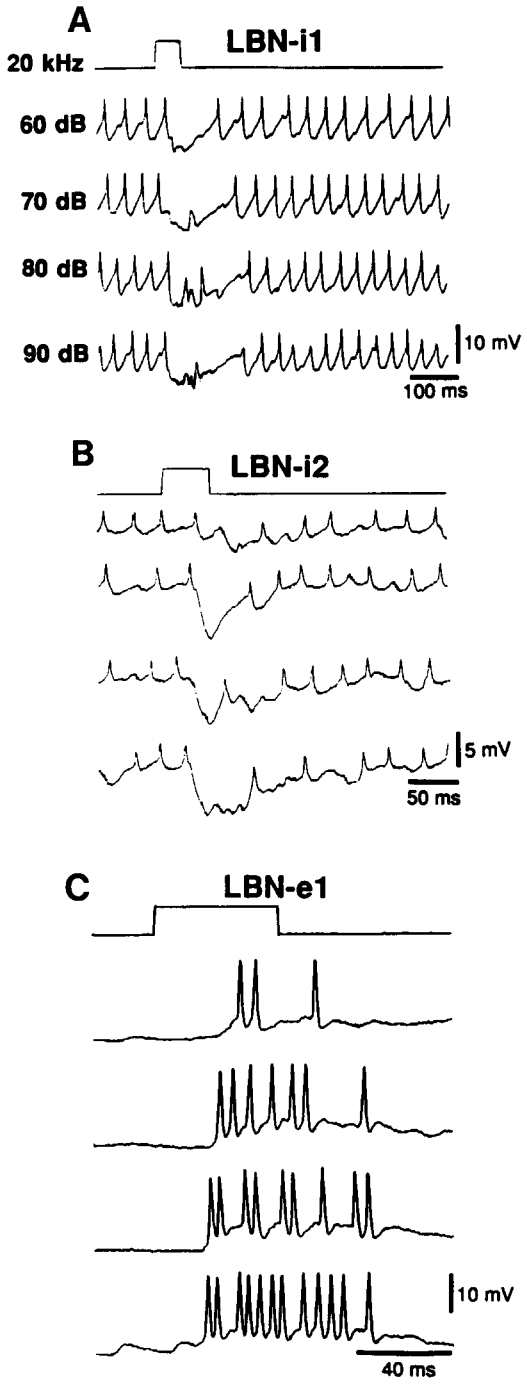

Fig. 8A–C. Graded response in LBNs. **A** and **B** Graded nature of the inhibitory response in LBN-i1 and LBN-i2 as a function of stimulus intensity. As the intensity of a 20 kHz sound pulse increases both the amplitude and duration of the inhibitory response increases. **C** Graded nature of the excitatory response in LBN-e1 as a function of stimulus intensity. As stimulus intensity increases, the number of spikes elicited in LBN-e1 by a 20 kHz sound pulse increases. In **A–C**, top trace: timing of auditory stimulus; bottom traces: intracellular recording

for LBN-e1. Third, the minimum response latency evoked by 20 kHz 90 dB sound pulses was approximately 20 ms. Finally, the duration of the evoked response generally outlasted the duration of 50 ms sound pulse (Fig. 8).

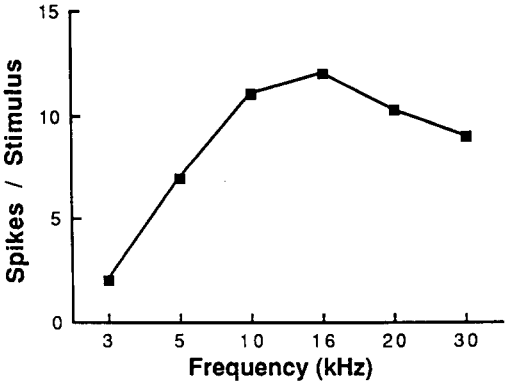

Fig. 9. Frequency response curve of LBN-e1. LBN-e1 is preferentially activated by high frequency sound pulses (>10 kHz). Each point represents the average number of spikes (3 stimulus trials) evoked within a 100 ms following an 80 dB sound pulse at each frequency

The response properties of the LBNs-i were, however, not identical to the LBNs-e. The LBNs-i generally had lower thresholds for ultrasonic stimuli than LBNs-e. In fact, in most LBNs-i, a 20 kHz 60 dB sound pulse was sufficient to stop ongoing spiking activity (Fig. 8A, B), while the threshold for activation of several LBNs-e by 20 kHz sound pulses was often greater than 70 dB. Moreover, the response latencies of LBNs-i were generally much shorter than those in LBNs-e, which could be as great as 70 ms. Finally, only LBNs-i1 was inhibited throughout a long duration (500 ms) ultrasonic pulse, while several LBNs-e remained excited throughout long duration pulses.

Since many of the LBNs have processes that innervate both hemispheres of the brain, we investigated how the direction of the stimulus affected the strength of the response in the LBNs. In all the LBNs-e and LBNs-i which have bilateral fields of innervation, except LBN-i4, stimuli presented from the ipsilateral soma-side evoked a stronger response than from the contralateral side (Fig. 10A). The response in LBN-i4, on the other hand, was equally inhibited by stimuli from either side. The response in LBN-i3, whose soma and processes are contained within one hemisphere, was also stronger to stimuli from the ipsilateral soma-side (Fig. 10B), while LBN-i2 was equally inhibited by stimuli from either side (Fig. 10C).

Response properties of LBN-ei. In LBN-ei, the type of response, either excitation or inhibition, elicited by ultrasonic sound pulses was dependent on the direction of the stimulus. Ultrasonic stimuli presented ipsilateral to the soma-side of LBN-ei evoked an excitatory response followed by slight inhibition (Fig. 11B). On the other hand, contralateral stimuli sometimes elicited weak excitation, one spike, followed by strong inhibition (Fig. 11C). Unlike the other LBNs, the response latency of LBN-ei to ipsilateral sound pulses (20 kHz 90 dB) was approximately 15 ms, while the response latency to contralateral stimuli was 18–20 ms. In addition, trains of 20 Hz ultrasonic pulses presented ipsilateral to LBN-ei elicited only a prolonged spiking activity and masked

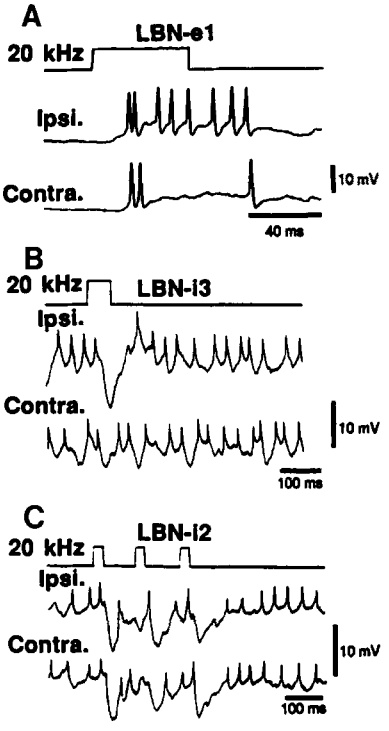

Fig. 10A–C. Directional sensitivity. **A** A 90 dB 20 kHz sound pulse presented ipsilateral (Ipsi.) to the soma of LBN-e1 in the brain elicited a greater response than a contralateral (Contra.) sound pulse. **B** Only ultrasonic stimuli presented ipsilateral to LBN-i3's soma inhibit its ongoing activity. **C** The inhibitory response is approximately equal to 20 kHz sound pulses presented both ipsilateral and contralateral to the soma and processes of LBN-i2 in the brain. In **A–C**, top trace: timing of auditory stimulus; bottom traces: intracellular recording

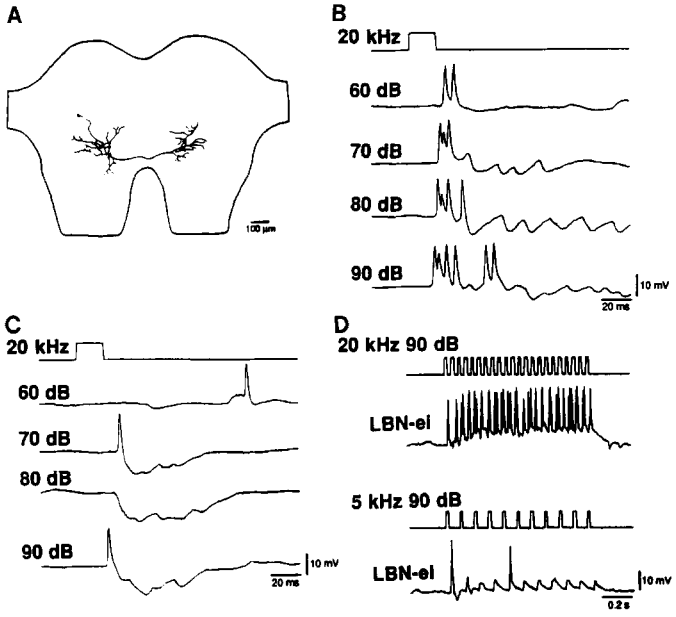

Fig. 11A–D. Morphology and physiology of LBN-ei. **A** Camera lucida drawings of the major processes of LBNs-e drawn within the approximate boundaries of the brain. **B** and **C** Directional sensitivity. Ultrasonic stimuli presented ipsilateral to LBN-ei's soma in the brain elicit spiking activity followed by inhibition, while contralateral stimuli elicit predominantly an inhibitory response. Note that in **B** and **C**, the excitatory and inhibitory responses, respectively, are graded with increasing stimulus intensity. **D** LBN-ei follows trains of ipsilateral 20 kHz sound pulses, but not trains of 5 kHz sound pulses. In **B** and **C**, top trace: timing of auditory stimulus; bottom traces: intracellular recording. In **D**, first and third traces: timing of auditory stimulus; second and fourth traces: intracellular recording

the inhibitory response (Fig. 11D). Activity in LBN-ei was similar to the LBNs-i and LBNs-e in that the predominant response, either excitation and inhibition, of LBN-ei was graded with increasing stimulus intensity (Fig. 11B, C), responded to a greater extent to 20 kHz sound pulses than 5 kHz sound pulse (Fig. 11D), and outlasted the duration of the stimulus pulse (Fig. 11B, C).

Modulation of ultrasound sensitive brain neurons during flight

It has been shown that the amount of ultrasound evoked activity descending from the cricket brain is greater during flight than at rest, and that these ultrasound sensitive descending units are most likely involved in negative phonotaxis (Brodfuehrer et al. 1988; Brodfuehrer and Hoy 1989). To determine if ultrasound-induced activity in the DBINs was enhanced during flight, we compared the number of spikes elicited by ultrasonic sound pulses both during flight and at rest for all the DBINs except DBIN6. No comparison was performed on DBIN6 because we were unable to elicit flight in this preparation while recording from DBIN6.

Flight activity did not increase the responsiveness of any of the DBINs to ultrasound compared to their response at rest. For example, in DBIN2 approximately the same number of spikes were elicited by a 20 kHz sound pulse during flight and at rest at each stimulus intensity tested (Fig. 12). Moreover, there was no consistent change in response latency in DBIN2 at rest and during flight. The strength of the response in 3 LBNs-e tested (LBN-e1, LBN-e2, and LBN-e4) was also not enhanced by flight activity.

Flight activity did appear to affect the responsiveness of DBIN8. The changes that occurred in the response of DBIN8 to ultrasound during flight compared to that at rest are shown in Figs. 2 and 13. During flight, the amplitude and duration of the inhibitory response elicited by short duration 20 kHz sound pulses was greater than at rest at all stimulus intensities (compare Fig. 2B, C). In addition, at rest the firing frequency in DBIN8 increased slightly following release from inhibition compared to before the onset of the stimulus (Figs. 2B and 13B), while no increase in the firing frequency of DBIN8 was apparent following release from inhibition during flight (Figs. 2C and 13D). The threshold of the inhibitory response also appeared to decrease slightly during flight. At rest, only a slight inhibitory response was observed in DBIN8 to a 20 kHz 70 dB sound pulse, but during flight, spontaneous activity in DBIN8 was clearly

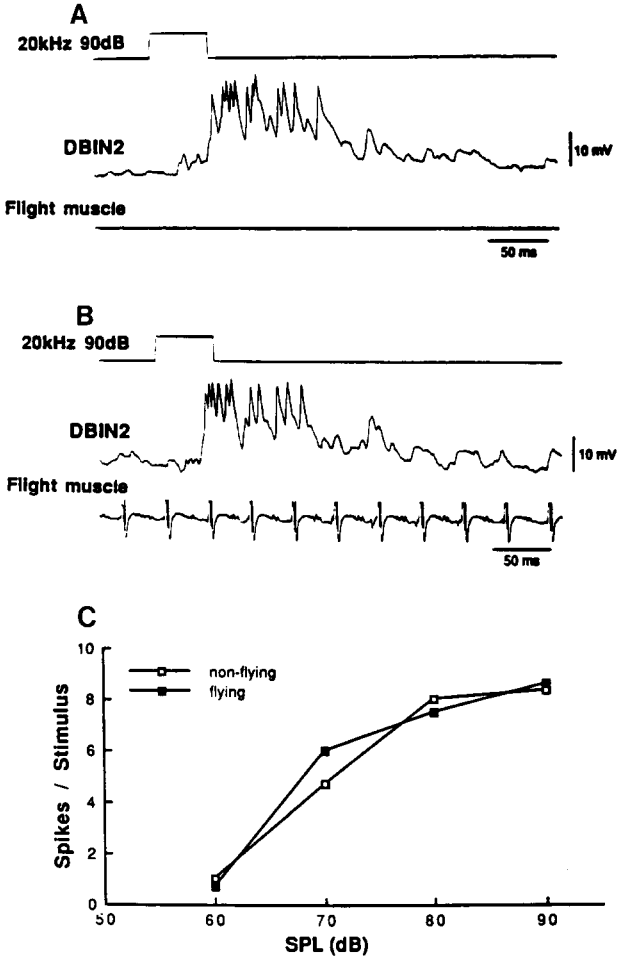

Fig. 12A–C. Effect of flight activity on DBIN2. **A** Preparation at rest (non-flying). **B** Preparation flying. Flight activity did not increase the response, either the number of spikes (see part **C**) or duration of spiking activity, evoked by a 20 kHz 90 dB sound pulse. Top trace: timing of auditory stimulus; middle trace: intracellular recording; bottom trace: EMG recording from wing depressor flight muscle. **C** Graph of the number of spikes elicited by 20 kHz sound pulses as a function of stimulus intensity (SPL). Each point in **C** represents average of 3 stimulus trials at each intensity

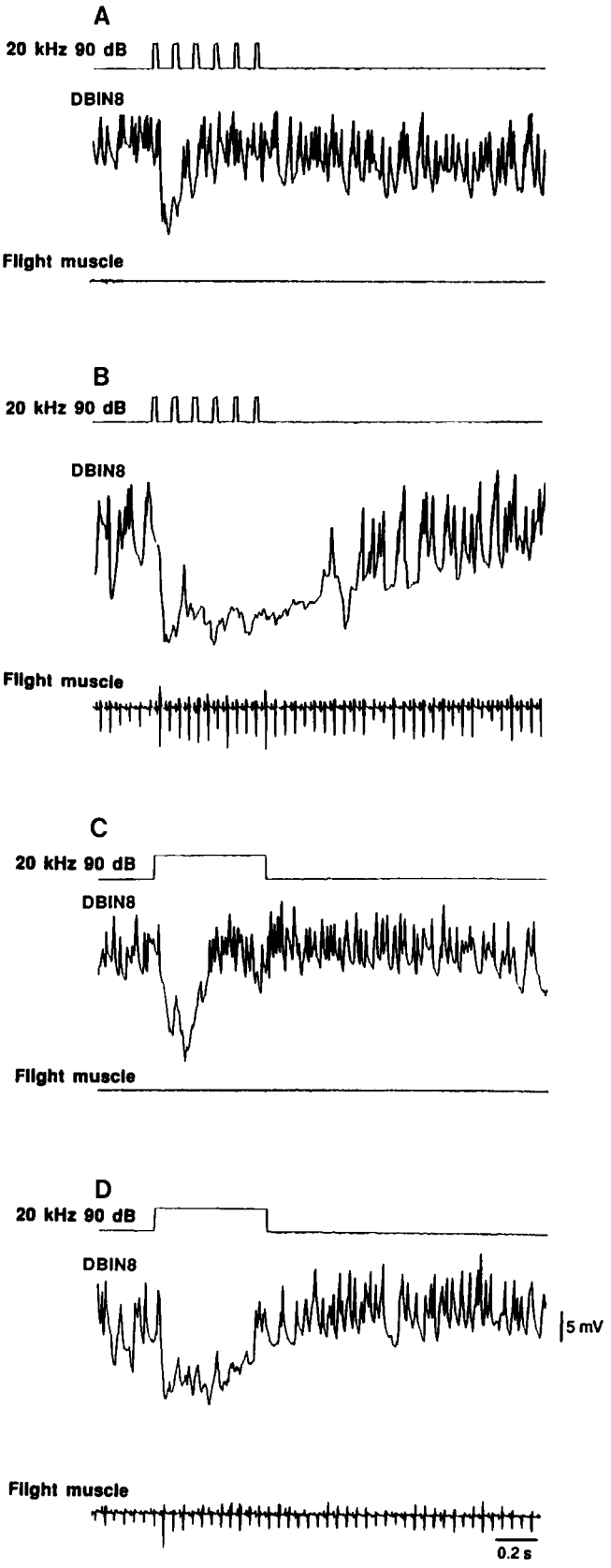

Fig. 13A–D. Effect of flight activity on DBIN8. **A** and **C** Preparation at rest. **B** and **D** Preparation flying. At rest, both trains and long duration 20 kHz sound pulses only phasically inhibit ongoing activity in DBIN8, while during flight the same stimuli inhibit activity in DBIN8 throughout the duration of the stimulus

inhibited by this same stimulus. Furthermore, at rest both trains and long duration ultrasonic pulses elicited only phasic inhibitory responses in DBIN8 (Fig. 13A, C). During flight these same stimuli, inhibited ongoing activity in DBIN8 throughout the duration of the stimulus.

Discussion

In this study we stained and recorded intracellularly from 20 neurons in the cricket brain that were sensitive to high frequencies. Based on their morphology, these neurons were divided in two broad classes, descending brain interneurons (DBINs) and local brain neurons (LBNs). Three different types of physiological responses were observed in the DBINs and LBNs. These included (1) neurons (7 DBINs and 6 LBNs) which were excited

by high frequency sound pulses, (2) neurons (one DBIN and 5 LBNs) which were inhibited by high frequency sound pulses, and (3) one LBN which was either excited or inhibited by high frequency sound pulses depending on the direction of the stimulus with respect to its somaside in the brain. Although the physiological and morphological data for each neuron were obtained from single preparations, we believe that our data clearly indicates that each neuron is a distinct cell type. In only one case was the anatomical structure of two cells, DBIN3 and DBIN4, similar enough to suggest that they may be two fills of the same interneuron. However, clear differences in their response properties to ultrasonic stimuli (e.g. directional sensitivity) indicate that this is not the case, and that DBIN3 and DBIN4 are different cells. In addition, since DBIN3 and DBIN6 were filled twice, one in each hemisphere of the brain, it suggests that all the DBINs, like all auditory interneurons so far identified in the cricket prothoracic ganglion, occur as bilaterally symmetrical pairs of interneurons.

Auditory neurons in the cricket brain. In the cricket, 4 previous studies have identified both local and descending neurons in the brain that respond to high frequency sound pulses. Local neurons (our terminology) include the BNC1s (BNC1c and BNC1d; Schildberger 1984) and the UABNs and PABNs (Boyan 1980), while descending brain interneurons include cell DN (Schildberger 1985, 1986) and cells IDBN and CDBN (Boyan and Williams 1981). None of these previously identified neurons sensitive to high frequencies appear to be morphologically and physiologically identical to the DBINs and LBNs described in this study, except cell IDBN whose morphology is similar to DBIN2. Moreover, like DBIN2, cell IDBN is excited only by high frequency sound pulses and not by 5 kHz sound pulses.

All the local and descending neurons characterized in the cricket brain, except the UABNs, appear to have one anatomical feature in common. They all have branches which extend into the ventral brain region at the border between the protocerebrum and deutocerebrum. This is also one region of the brain where the processes of Int-1 terminate (Moiseff and Hoy 1983; Brodfuehrer and Hoy, personal observation). Thus the ventral brain region bordering the protocerebrum and deutocerebrum appears to be an area of the brain where extensive processing and integration of auditory signals likely occurs. Moreover, extensive searches of the prothoracic ganglion in several cricket species have only identified 4 neurons that are sensitive to ultrasonic frequencies: Int-1 (and its homologs) and cells TN2, 3 and 4 (Wohlers and Huber 1982; Casaday and Hoy 1977; Atkins and Pollack 1987), while over 30 ultrasound sensitive neurons have so far been characterized in the cricket brain. The large number of brain neurons tuned to high frequencies, in contrast to the relatively few thoracic interneurons tuned to high frequencies, also supports the notion that extensive sensory processing of auditory information and integration of auditory input with higher order motor centers occurs in the brain. Furthermore, our search of the cricket brain for neurons tuned to high frequencies was not exhaustive. We concentrated our search in only one area, the midline region bordering the protocerebrum and deutocerebrum, which is only one of the areas of the brain where Int-1 projects (Moiseff and Hoy 1983). Since Int-1 also terminates in two other brain regions, it would appear that potentially many more ultrasound-sensitive brain neurons remain to be found.

Relationship between Int-1 and ultrasound sensitive neurons. In *T. oceanicus* several sensory interneurons in the prothoracic ganglion, Int-1 and cells TN2, 3 and 4, have been shown to be preferentially tuned to high frequencies (>10 kHz), and have axons which carry this ultrasonic input to the brain (Moiseff and Hoy 1983; Atkins and Pollack 1987). The fact that many of the response properties of both the DBINs and the LBNs match those of Int-1 strongly suggests that these brain neurons receive their auditory input from Int-1, and not cells TN2, 3 and 4. In addition, the short response latencies of the DBINs and LBNs also eliminates cells TN2, 3 and 4 from being their major source of auditory input. Ultrasonic stimuli (at 100 dB) activate cells TN2, 3 and 4 in the prothoracic ganglion with latencies of greater than 30 ms (Atkins and Pollack 1987), while all of the DBINs and most of the LBNs have latencies equal to or less than 30 ms at ultrasonic stimuli 10 dB less intense. Our anatomical data, determined from wholemount Lucifer Yellow fills, suggest that Int-1 could be presynaptic to the DBINs and LBNs since Int-1's terminal branches in the brain and the arborizations of most of the DBINs and LBNs appear to project into the same brain regions. The overlap of their processes is especially evident in the brain area bordering the protocerebrum and deutocerebrum, just lateral to midline of the brain.

Two physiological results suggest that Int-1 may be directly connected only to LBN-ei, and not to the DBINs, LBNs-i and LBNs-e. First, in insects the synaptic delay for monosynaptic connections has been shown to be on the order of 1 ms (Weeks and Jacobs 1987; Pearson et al. 1976; Burrows 1975; Hennig 1988). In fact, in the cricket, *Teleogryllus commodus*, the synaptic delay between auditory receptors and AN2, a homolog of Int-1 in *T. commodus*, in the prothoracic ganglion is only 0.5 ms (Hennig 1988). We have shown that the response latencies for Int-1 in the brain and LBN-ei were approximately equal. Since these measurements were determined in two different preparations, it appears that the synaptic delay between Int-1 and LBN-ei is most likely less than 1 ms, and thus consistent with the connection being monosynaptic. However, the difference in response latency between Int-1 and the DBINs, LBNs-i and LBNs-e was greater than 5 ms. Second, in many of the DBINs and some of the LBNs, stimuli presented ipsilateral to their soma and processes did not produce the strongest response in these brain neurons. Since the strongest response evoked in Int-1 is by ultrasonic input ipsilateral to its terminal fields in the brain (Moiseff and Hoy 1983), only brain neurons whose processes overlap

the ipsilateral terminal fields of Int-1 and are more sensitive to ipsilateral stimuli could be directly connected to Int-1. LBN-ei again fits this criterion. Thus even though anatomically the processes of Int-1 and the DBINs and LBNs project into common brain regions, our physiological data indicates that only the connection between Int-1 and LBN-ei could be monosynaptic.

Brain neurons inhibited by ultrasound. The 6 LBNs and one DBIN inhibited by ultrasound represent a new class of auditory brain neurons in the cricket. In previous studies on auditory neurons in the cricket brain (Schildberger 1984, 1986; Boyan 1980; Boyan and Williams 1981), no neurons inhibited by auditory input were described. Ultrasonic frequencies, however, have been shown to have an inhibitory effect on brain neurons in both locusts and moths. For example in locusts, Römer and Seikowski (1985) demonstrated that the amount of ongoing activity recorded extracellularly from specific regions of the brain decreased in response to ultrasound. In the moth, activity in ultrasound sensitive units located in the brain increased initially with increasing stimulus intensity, but these units were then suppressed as intensity continued to increase (Roeder 1969).

The functional role of ultrasound-induced inhibition of auditory units in the locust and moth brains are presently unknown. Similarly, in the cricket we can only speculate on the functional role these neurons play in controlling acoustic behaviors. First, the morphology of DBIN8 and many of the LBNs-i suggest that they could be associated with localizing the direction of an ultrasonic sound source. However, their individual response properties to ultrasound do not support this hypothesis. In contrast, both the morphology and response properties of LBN-ei are consistent with it being involved in sound localization. Most likely, however, sound localization is the function of a network of cells in the brain, which may include many of the LBNs found in this study that have bilateral fields, and is not the specific function of any single cell. Second, neurons inhibited by ultrasound may simply be neurons whose activity is inappropriate for the acoustic behaviors released by ultrasound. For example, two behaviors in the leech, swimming and longitudinal body shortening, are incompatible. To ensure that shortening does not occur during swimming, the motor neurons controlling shortening are rhythmically inhibited by neurons involved in generating the swimming rhythm (Ort et al. 1974). Thus in order for ultrasound to induce negative phonotactic responses, it may be necessary to suppress activity in neurons involved in behaviors that conflict with negative phonotaxis. Such may be the case for DBIN8, since the extent of the ultrasound-induced inhibitory response in DBIN8 was enhanced during flight as compared to at rest. Finally, ultrasound appears to function in more than just negative phonotaxis (Latimer and Lewis 1986; Harrison et al. 1988; Hutchings and Lewis 1984; see below). Neurons that were inhibited by ultrasound, therefore, could be associated with other acoustic behaviors in the cricket.

Possible functions of ultrasound sensitive brain neurons. The neuronal pathway controlling negative phonotactic behavior in *Teleogryllus oceanicus* is known to originate in the prothoracic ganglion, ascend to the brain via Int-1, and descend to the thoracic and abdominal segments via descending interneurons that interact with the flight steering mechanism (Nolen and Hoy 1984; Hoy and Nolen 1987). Converging with this pathway is input from the flight central pattern generator since ultrasound only elicits negative phonotaxis in flying crickets (Hoy and Nolen 1987). Recently, we have shown that ultrasound input (Int-1 activity) and flight input converge on ultrasound sensitive units in the brain such that the response to ultrasonic stimuli in these descending units is facilitated during flight compared to at rest (Brodfuehrer et al. 1988; Brodfuehrer and Hoy 1989).

The DBINs that were excited by ultrasound do not appear to be the same descending units whose excitatory response to ultrasound was facilitated during flight for two reasons. First, the strength of the excitatory response to ultrasound in the DBINs was similar during flight and at rest. Second, the response latency of ultrasound sensitive descending units recorded extracellularly in the cervical connective was approximately 20 ms (Brodfuehrer et al. 1988; Brodfuehrer and Hoy 1989), while the response latency in most of the DBINs was at least 5 ms greater. We did, however, record from some ultrasound sensitive brain neurons whose activity was enhanced slightly during flight as compared to at rest, but were unfortunately unable to successfully fill these neurons with dye to complete their identification.

The DBINs could, however, function during some aspect of negative phonotactic steering movements since this behavior need not be controlled entirely by descending units whose activity level is facilitated by flight input to the brain. The DBINs may be summating with flight CPG input directly on motor neurons and interneurons in thoracic and abdominal segments to affect cricket steering movements, similar to the mechanisms controlling visually guided steering corrections during locust flight (Rowell 1988; Reichert and Rowell 1985a, b, 1986). Moreover, the response latencies of the DBINs suggest that they could be involved in some aspect of the latter stages of the negative phonotactic steering maneuvers since behavioral studies have shown that negative phonotactic steering maneuvers begin approximately 30–35 ms following a high intensity ultrasonic sound pulse and far outlast the duration of the ultrasonic stimulus (May et al. 1988; Nolen and Hoy 1986). Thus, the response latencies of the DBINs, which range from 25 to 35 ms, are compatible with the time course of the latter stages of negative phonotactic steering maneuvers.

The DBINs and LBNs may also play a role in two other cricket auditory behaviors, positive phonotaxis and courtship behavior. The possible roles of the DBINs and LBNs in these auditory behaviors stem from the fact that several behavioral and physiological experiments have suggested a functional role of Int-1 in both positive phonotaxis and courtship behavior. For example, orientation accuracy improves in *T. oceanicus* to

calling song that includes high frequency harmonics (Latimer and Lewis 1986) and Int-1 reliably follows the temporal structure of courtship song which contains high frequency harmonics (Harrison et al. 1988; Hutchings and Lewis 1984). Since we have shown that the DBINs and LBNs most likely receive their ultrasonic auditory input from Int-1, it suggests that the DBINs and the LBNs could also be involved in controlling some aspect of positive phonotaxis and courtship behavior.

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References

Atkins G, Pollack GS (1987) Response properties of prothoracic, interganglionic, sound-activated interneurons in the cricket *Teleogryllus oceanicus*. J Comp Physiol A 161:681–693

Bentley D (1977) Control of cricket song patterns by descending interneurons. J Comp Physiol 116:19–38

Boyan GS (1980) Auditory neurons in the brain of the cricket *Gryllus bimaculatus* (De Geer). J Comp Physiol 140:81–93

Boyan GS, Williams JLD (1981) Descending interneurons in the brain of the cricket. Naturwissenschaften 68:486–487

Brodfuehrer PD, Hoy RR (1988) Effect of auditory deafferentation on the synaptic connectivity of a pair of identified interneurons in adult field crickets. J Neurobiol 19:17–38

Brodfuehrer PD, Hoy RR (1989) Integration of ultrasound and flight inputs on descending neurones in the cricket brain. J Exp Biol 145:157–171

Brodfuehrer PD, May ML, Hoy RR (1988) Ultrasonic neurons in the brain of crickets. Neurosci Abstr 14:311

Burrows M (1975) Monosynaptic connexions between wind stretch receptors and flight motoneurons of the locust. J Exp Biol 62:189–219

Casaday GB, Hoy RR (1977) Auditory interneurons in the cricket *Teleogryllus oceanicus*: physiological and anatomical properties. J Comp Physiol 121:1–13

Furukawa N, Tomioka K, Yanaguchi T (1983) Functional anatomy of the muscular and innervation of the neck and thorax in the cricket, *Gryllus bimaculatus*. Zool Mag 92:371–385

Harrison L, Horseman G, Lewis B (1988) The coding of the courtship song by an identified auditory neurone in the cricket *Teleogryllus oceanicus* (Le Guillou). J Comp Physiol A 163:215–225

Hennig RM (1988) Ascending auditory interneurons in the cricket *Teleogryllus commodus* (Walker): comparative physiology and direct connections with afferents. J Comp Physiol A 163:135–143

Hoy RR, Nolen TG (1987) The role of behavioral context in decision making by an identified interneuron in the cricket. In: Wise SP (ed) Higher brain functions: recent explorations of the brain's emergent properties. John Wiley and Sons, New York, pp 133–155

Huber F (1974) Neural integration (central nervous system). In: Rockstein M (ed) The physiology of insecta, vol. IV. Academic Press, New York, pp 3–100

Hutchings M, Lewis B (1984) The role of two-tone suppression in song coding by ventral cord neurones in the cricket *Teleogryllus oceanicus* (Le Guillou). J Comp Physiol A 154:103–112

Latimer W, Lewis DB (1986) Song harmonic content as a parameter determining acoustic orientation behavior in the cricket *Teleogryllus oceanicus* (Le Guillou). J Comp Physiol A 158:583–591

May ML, Brodfuehrer PD, Hoy RR (1988) Kinematic and aerodynamic aspects of ultrasound-induced negative phonotaxis in flying Australian field crickets (*Teleogryllus oceanicus*). J Comp Physiol A 164:243–249

Moiseff A, Hoy RR (1983) Sensitivity to ultrasound in an identified auditory interneuron in the cricket: A possible neural link to phonotactic behavior. J Comp Physiol 152:155–167

Moiseff A, Pollack G, Hoy RR (1978) Steering responses of flying crickets to sound and ultrasound: mate attraction and predator avoidance. Proc Natl Acad Sci 75:4052–4056

Nolen TG, Hoy RR (1984) Initiation of behavior by single neurons: the role of behavioral context. Science 226:992–994

Ort CA, Kristan WB Jr, Stent GS (1974) Neuronal control of swimming in the leech. II. Identification and connections of the motor neurons. J Comp Physiol 94:121–154

Otto D, Weber T (1982) Interneurons descending from the cricket cephalic ganglion that discharge in the pattern of two motor rhythms. J Comp Physiol 148:209–219

Pearson KG, Wong RKS, Fourtner CR (1976) Connexions between hair-plate afferents and motoneurons in the cockroach leg. J Exp Biol 64:251–266

Reichert H, Rowell CHF (1985a) Integration of non-phaselocked exteroceptive information in the control of rhythmic flight in the locust. J Neurophysiol 53:1201–1218

Reichert H, Rowell CHF (1985b) Course correction circuitry translates feature detection into behavioral action in locusts. Nature 315:142–144

Reichert H, Rowell CHF (1986) Neuronal circuits controlling flight in the locust: how sensory information is processed for motor control. Trends Neurosci 9:281–283

Roeder KD (1969) Brain interneurons in noctuid moths: differential suppression by high sound intensities. J Insect Physiol 15:1713–1718

Römer H, Seikowski U (1985) Responses of model songs of auditory neurons in the thoracic ganglia and brain of the locust. J Comp Physiol A 156:845–860

Rowell CHF (1988) Mechanisms of flight steering in locusts. Experientia 44:389–395

Schildberger K (1984) Temporal selectivity of identified auditory neurons in the cricket brain. J Comp Physiol A 155:171–185

Schildberger K (1985) Recognition of temporal patterns by identified auditory neurons in the cricket brain. In: Kalmring K, Elsner N (eds) Acoustic and vibrational communication in insects. Paul Parey, Berlin, pp 41–49

Schildberger K (1986) Acoustic communication in crickets: behavorial and neuronal mechanisms of song recognition and localization. In: Ali MA (ed) Nervous systems in invertebrates. NATO ASI Series A: Life Sciences, vol. 141, pp 603–619

Schürmann F (1987) The architecture of the mushroom bodies and related neuropils in the insect brain. In: Gupta AP (ed) Arthropod brain – its evolution, development, structure and functions. John Wiley and Sons, New York, pp 231–264

Thorson J, Weber T, Huber F (1982) Auditory behavior of the cricket. II. Simplicity of calling song recognition in *Gryllus*, and anomalous phonotaxis at normal carrier frequencies. J Comp Physiol 146:361–378

Weeks JC, Jacobs GA (1987) A reflex behavior mediated by monosynaptic connections between hair afferents and motoneurons in the larval tobacco hornworm, *Manduca sexa*. J Comp Physiol A 160:315–329

Wohlers DW, Huber F (1982) Processing of sound signals by six types of neurons in the prothoracic ganglion of the cricket, *Gryllus campestris* L. J Comp Physiol 146:161–173