Central Projections of Auditory Receptor Neurons of Crickets

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ABSTRACT

We describe the central projections of physiologically characterized auditory receptor neurons of crickets as revealed by confocal microscopy. Receptors tuned to ultrasonic frequencies (similar to those produced by echolocating, insectivorous bats), to a mid-range of frequencies, and a subset of those tuned to low, cricket-like frequencies have similar projections, terminating medially within the auditory neuropile. Quantitative analysis shows that despite the general similarity of these projections they are tonotopic, with receptors tuned to lower frequencies terminating more medially. Another subset of cricket-song-tuned receptors projects more laterally and posteriorly than the other types. Double-fills of receptors and identified interneurons show that the three medially projecting receptor types are anatomically well positioned to provide monosynaptic input to interneurons that relay auditory information to the brain and to interneurons that modify this ascending information. The more laterally and posteriorly branching receptor type may not interact directly with this ascending pathway, but is well positioned to provide direct input to an interneuron that carries auditory information to more posterior ganglia. These results suggest that information about cricket song is segregated into functionally different pathways as early as the level of receptor neurons. Ultrasound-tuned and mid-frequency tuned receptors have approximately twice as many varicosities, which are sites of transmitter release, per receptor as either anatomical type of cricket-song-tuned receptor. This may compensate in part for the numerical underrepresentation of these receptor types. J. Comp. Neurol. 493:439 – 447, 2005.

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2001). The remaining receptors are divided nearly equally between the mid-frequency and ultrasound receptors. The receptor neurons project to the prothoracic ganglion where they interact with a number of identified central neurons, the anatomy of which has been thoroughly described (Wöhlers and Huber, 1985; Atkins and Pollack, 1987a; Stiedl et al., 1997).

Within the ear, receptor neurons are organized tonotopically (Oldfield et al., 1986). However, the anatomy and organization of their projections to the central nervous system have been reported only briefly (Eibl and Huber, 1979; Esch et al., 1980; Imaizumi and Pollack, 2001). In this article we present a detailed description of the afferent auditory projection, as studied using single-cell intracellular staining and confocal microscopy.

MATERIALS AND METHODS

*Teleogryllus oceanicus* were raised in the laboratory on a diet of Purina cat chow and water. Adult virgin females, aged 14–23 days after the final molt, were used. Cold-anesthetized animals were secured with wax to a platform ventral side up after removing the wings and mid- and hindlegs. The forelegs, which bear the ears, were held flexed against the sides of the pronotum in a position similar to that adopted during flight. The prothoracic ganglion was exposed by ventral dissection, bathed in modified TES ringer (Strausfeld et al., 1983), and supported on a metal platform.

Axons of receptor neurons were penetrated in the leg nerve, near its entrance into the prothoracic ganglion, with microelectrodes, the tips of which were filled either with 2–4% Lucifer Yellow CH (Aldrich, Milwaukee, WI; resistance, 100–300 MΩ) or with 2–2.5 MΩ Alexa 488 or 568 hydrazide (Molecular Probes, Eugene, OR; resistance 150–1,000 MΩ); electrode shafts were filled with 2 M LiCl. Interneurons were impaled in their dendritic processes within the prothoracic ganglion. Neurons were stained by injecting negative current (1–10 nA). Ganglia containing dye-filled neurons were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 10–30 minutes followed by 2–4% formaldehyde in methanol for 1 hour, then washed with 100% ethanol for 15 minutes. Ganglia were cleared in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 10–30 minutes followed by 4% formaldehyde in methanol for 1 hour, then washed with 100% ethanol for 15 minutes. Ganglia were cleared in methyl salicylate, mounted on a depression slide, and viewed with confocal microscopy (Leica CLSM or Zeiss LSM510). Selected specimens were embedded in Spurr’s (1 hour), and 100% Spurr (overnight), all at room temperature. Ganglia were then transferred to fresh Spurr and incubated at 60°C (≥19 hours). Sections were viewed and photographed with standard or confocal fluorescent microscopy and drawn by tracing the image of a negative projected with a photographic enlarger.

Receptor neurons were classified physiologically on the basis of their responses to 30-ms sound pulses, presented twice per second. Sound frequency ranged from 3–40 kHz and intensity ranged from 40–100 dB SPL. Our experimental protocol was to hold intensity constant while scanning through the range of test frequencies. We repeated this for as many intensity levels as recording duration (mean duration = 3 minutes) permitted. A neuron’s characteristic frequency (CF) was defined either as the frequency at which threshold was lowest or (when recordings could not be held long enough for complete determination of the neuron’s threshold curve) as the frequency that elicited the largest number of action potentials at the lowest suprathreshold intensity tested. Stimuli were produced by National Instruments (Austin, TX) digital-analog boards driven by custom software. See Imaizumi and Pollack (1999) for further details.

Quantification of anatomy

We quantified a neuron’s projection pattern by mapping the positions of varicosities on its axons terminals in confocal optical sections. Ultrastructural studies show that varicosities are rich in synaptic vesicles, and thus are good markers for presynaptic sites (Hardt and Watson, 1999). Optical horizontal sections were first made at low power (6.3× objective) to visualize the entire projection, as well as the borders of the ganglion. A second set of sections was then made at higher power (25× water-immersion) at intervals of 1.5–2 μm, overlapping the fields of view when required to encompass all of a neuron’s varicosities. Arrows (or portions of them) were reconstructed by collapsing series of sections into a single plane.

Counts of varicosities were made directly on the monitor screen. An acetate sheet was fixed on the screen and individual varicosities were marked and counted section-by-section, using the reconstructed arbors for guidance.

We mapped the positions of varicosities in the horizontal plane on confocal, optical sections using the programs Scion Image for Windows (http://www.scioncorp.com) or UTHSCA Image Tool (http://ddsdx.uthscsa.edu). When necessary, neighboring fields of view were aligned with reference to landmarks common to both of them. Positions of varicosities were normalized with respect to the length and width of the hemiganglion (see Fig. 2). We estimate that errors introduced by the position of the ganglion along the curvature of the cavity of the depression slide were less than 0.6%; therefore, we did not correct for them.

Series of confocal sections were collapsed into single images by setting the value of each pixel in the final image to the maximum value for the same x-y coordinates in the contributing sections, using the software native to the confocal microscope systems (Leica CLSM or Zeiss LSM510). Image brightness and contrast were adjusted using Gimp 2.2, running on a Linux workstation. The image in Figure 5 was additionally sharpened using a deconvolution filter.

RESULTS

The results presented are based on intracellular fills of 146 auditory receptors (98 low-frequency, 22 mid-frequency, and 26 ultrasound receptors). Forty-eight receptors, for which intracellular penetration could be maintained long enough for excellent staining, were selected for quantitative analysis (32 low-frequency, 7 mid-frequency, and 9 ultrasound receptors). The remaining 98 receptors provided additional supporting evidence.

Two anatomically distinct receptor types

Horizontal and transverse reconstructions of axon terminals are illustrated in Figure 1. Axonal arbors are of two types. One type terminates near the midline of the
prothoracic ganglion (medial termination; MT), and the other terminates more laterally, with large, bifurcating branches (bifurcating; BC). BC types occur only among low-frequency receptors, whereas MT types occur among all three frequency classes (Fig. 1A). For convenience, in this work we use the designations BC and MT to refer only to the two types of low-frequency receptor.

MT-type, mid-frequency, and ultrasound receptors terminate in the anterior part of the auditory neuropil (medial ventral association center, mVAC). The projections of these receptors are also similar in transverse sections (Fig. 1B). The mVAC contains the dendrites of several identified auditory interneurons (Wohlers and Huber, 1985), and is a site of synaptic contact between receptors and interneurons (Hardt and Watson, 1984; Watson and Hardt, 1996; Hirtz and Wiese, 1997). The anterior branches of BC-type receptors enter the lateral part of the mVAC. Their posterior branches terminate in a region posterior to the mVAC.

Many receptor neurons have small branches within the root of nerve 5, lateral to the mVAC, where several central neurons (ON1, ON2, AN2, DN1) also have processes (Wohlers and Huber, 1985; Atkins and Pollack, 1987a; Hardt and Watson, 1994; Watson and Hardt, 1996). These
small branches are most prevalent on BC-type, mid-frequency, and ultrasound receptors.

Quantification of axonal arbors

The axon terminals of receptor neurons bear many varicosities (see Fig. 1A), and ultrastructural studies have shown that these are sites of synaptic output (Hardt and Watson, 1999). We quantified the anatomical projections of receptor neurons by mapping the positions of their varicosities in the horizontal plane. Figure 2 illustrates the coordinate system. The position of each varicosity was expressed as the percentage along the length of the anterior–posterior (A-P) and medial–lateral (M-L) axes of the hemiganglion. Figure 3 shows the positions of varicosities of representatives of each of the four receptor types. BC types differ strikingly from the other receptors in that they terminate more laterally. We compared this difference quantitatively for the two anatomical types of low-frequency receptors. The M-L coordinate of the most medial varicosity of BC types was 18.0 ± 2.3% (mean ± SD); the corresponding value for MT types was 2.3 ± 2.1% (P < 0.0001, t-test).

Tonotopy

We first examined the possibility of tonotopy of the afferent projection by summarizing the distribution of varicosities of all receptors, including both MT and BC types, along the A-P and M-L axes as the means of the coordinate

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Fig. 2. Coordinate system for quantification of varicosity positions. A: Length of the anterior–posterior (A-P) axis defined as the distance from the anteriormost to the posteriormost portion of the midline. Length of the medial–lateral (M-L) axis defined as the distance from the midline to the posterior edge of the entry point of nerve 5. B: In 25 (of 48) ganglia, the midline (readily apparent by its autofluorescence) was shifted laterally, probably due to asymmetric shrinkage of the ganglion during fixation/dehydration. In these cases the M-L axis was defined as the distance from a line tangent to the most lateral point of the midline to the junction between the ganglion and the posterior edge of nerve 5. The shift shown in B is an extreme case. Scale bar = 200 μm in A (applies to A,B).

Fig. 3. Positions of varicosities in the horizontal plane. Each panel shows three representative receptors. A: BC types; B: MT types; C: mid-frequency receptors, and D: ultrasound receptors. The CF of each receptor is indicated.
values of all of their varicosities. When all varicosities are considered, there is no significant relationship between CF and their mean positions, along either the A-P or M-L axis (linear regression, \( n = 48 \), data not shown). However, of particular interest is the organization within the mVAC, in which extensive interactions occur between receptors and central neurons (see above). It is difficult to define the mVAC in our confocal reconstructions because the internal structure of the ganglion is not visible. However, varicosities are densely packed in a region extending from 35–50% along the A-P axis and from 0 – 40% along the M-L axis (Fig. 3). We operationally take these boundaries as delimiting the mVAC. To compute mean termination position within the mVAC we included only varicosities located within these boundaries. The anatomical differences between BC types and the other three types, considered together with the anatomy of central neurons (Wohlers and Huber, 1985; Hardt and Watson, 1994; Watson and Hardt, 1996), suggest that the target neurons of BC types may differ from those of the other types. In particular, BC types are unlikely to make extensive, direct connections with the three central neurons that have been most directly implicated in auditory behavior, namely, ON1, AN1, and AN2 (Atkins et al., 1984; Nolen and Hoy, 1984; Schildberger and Hörner, 1988; see below). To examine whether the axonal projections onto a common set of potential central targets might be tonotopic, therefore, we excluded BC types from this analysis. Figure 4A shows that, for this restricted set of receptor neurons, the projection to the mVAC is indeed tonotopic along the M-L axis, with receptors that are tuned to higher frequencies terminating more laterally (\( n = 29 \), \( r^2 = 0.252, P = 0.005 \)). Although statistically significant, the magnitude of the difference in mean position is only 4% of the M-L width, corresponding to \( \approx 20 \) \( \mu \)m. There is no significant relationship along the A-P axis (Fig. 4B; \( n = 29 \), \( r^2 = 0.054, P = 0.225 \)). Nor is there a significant relationship between CF and M-L position within any of the three receptor populations (low-frequency receptors, \( n = 14 \), \( r^2 = 0.06, P = 0.4 \); mid-frequency receptors, \( n = 7 \), \( r^2 = 0.239, P = 0.219 \); ultrasound receptors, \( n = 8 \), \( r^2 = 0.239, P = 0.219 \)).

Receptor terminals might, in principle, also be distributed systematically in the dorsoventral plane, as occurs in tettigoniids (Römer, 1983). However, as shown in Figure 1B, receptors of all types terminate at approximately the same depth within the neuropil. Figure 5 shows projections from an MT-type receptor (red) and an ultrasound receptor (green) within the same, 10.2-\( \mu \)m-thick region of neuropil. Varicosities from both receptors are intermingled. Co-occurrence of varicosities from the two receptors occurs throughout the entire depth of their projections (not shown). Thus, although we cannot rule out the possibility of a subtle segregation of receptor terminals along the dorsoventral axis, their projections overlap considerably in depth.

**Overlap between receptor projections and interneuron dendrites**

We examined possible patterns of connectivity between receptor neurons and central neurons by staining both in the same individual (Fig. 6). Figure 6A,B shows low-power views of the interneurons ON1 and DN1, respectively. ON1’s medial dendrites occur in the same restricted re-
gion as the projections within the mVAC of two other identified neurons, AN1 and AN2 (Wohlers and Huber, 1985), and thus serve as a marker for the medial dendrites of these neurons. DN1’s dendrites lie mainly outside of this region. Figure 6C illustrates the overlap between MT types and the medial dendritic tuft of ON1. Thus, MT receptors are anatomically well suited to provide extensive direct input to all three of these interneurons (although probably with carrier-frequency-specific connectivity). Figure 6D shows that the same is true of ultrasound receptors. By contrast, as a consequence of the more lateral and posterior projections of BC-type receptors, there is little or no overlap between their terminal branches and ON1’s medial dendrites (Figs. 3A, 6E). Absence of overlap applies as well to the short processes arising from the axons of BC-type receptors, which are posterior to the lateral process of ON1 (not shown). Thus, BC types probably make few, if any, direct connections with ON1, AN1, or AN2 (Pollack and Imaizumi, 1999). This observation raises the question: what are the targets of BC types? DN1 has extensive dendritic processes in the same region as the branches of BC receptors (Atkins and Pollack, 1987a), and is thus a potential postsynaptic target of BC types. Figure 6F indeed confirms that the terminal branches of BC receptors overlap with DN1’s dendritic processes.

**DISCUSSION**

**Sound frequency-specific anatomical differences**

Systematic relationships between the peripheral positions of the cell bodies of sensory neurons and the locations of their central terminals have been described for several insect sensory systems, for example, mechanosensory hairs (Mücke and Lakes-Harlan, 1985; Johnson and Murphey, 1985; Newland, 1991) and chordotonal sensilla (the sensillum type of auditory receptors) (Nishino, 2000, 2003; Nishino and Field, 2003).
In auditory systems, the position of a receptor neuron’s cell body within the ear is related to its frequency sensitivity, as well as to the location of its terminations in the central nervous system. Tonotopy has been described in both orthopteran insects. Both in tettigoniids (Römer, 1983; Stöltzing and Stumpner, 1998) and in acridids (Hallex et al., 1988; Jacobs et al., 1999), receptor neurons that are tuned to different sound frequencies project systematically to different regions of auditory neuropil. Although our quantitative analysis did reveal central tonotopy of cricket auditory receptors (Fig. 4), this was rather minor compared to other orthopterans. There is considerable overlap, for example, between the projection areas of MT types, mid-frequency, and ultrasound receptors.

The difference in degree of tonotopy between crickets and tettigoniids is particularly notable because the peripheral organization of auditory receptors is similar in these groups (Oldfield, 1982; Oldfield et al., 1986). In both cases, receptors are arrayed along the proximodistal axis of the tibia, with proximal receptors tuned to low sound frequencies, and distal receptors to high frequencies. Despite anatomical similarity, the receptor arrays differ physiologically. In tettigoniids, there is a relatively gradual shift in tuning from receptor to receptor (Oldfield, 1982; Römer, 1983; Stöltzing and Stumpner, 1998), whereas in crickets the representation of sound frequency by the receptor array is discontinuous, with receptors falling into three distinct groups based on frequency tuning (Imaizumi and Pollack, 1999). This physiological organization in crickets coincides with behavioral tests that demonstrate that crickets perceive sound frequency categorically, rather than as a continuous range of frequencies (Wytenbach et al., 1996).

At least one identified interneuron, ON1, receives input from both low-frequency and ultrasound receptors; this neuron has a W-shaped tuning curve, with enhanced sensitivity in both of these frequency ranges (Atkins and Pollack, 1986). Faulkes and Pollack (1999) showed previously that ultrasound input to ON1 is monosynaptic but, based on several electrophysiological observations, they proposed that low-frequency input might be mainly polysynaptic. However, our double fills suggest strongly that MT types provide direct input to ON1 for processing of low-frequency sounds (Fig. 6). This is supported by simultaneous paired intracellular recordings from MT types and ON1 (G.S. Pollack, G. Marsat, P. Sabourin, unpublished observation).

Our earlier work demonstrated the numerical imbalance between low-frequency and ultrasound receptors (Pollack and Faulkes, 1998; Imaizumi and Pollack, 1999, 2001). However, despite the underrepresentation of ultrasound at the receptor-neuron level, ultrasonic stimuli evoke robust responses in central neurons, including ON1, in part because unitary, ultrasound-elicited EPSPs, recorded in ON1’s large, dendritic integrating segment, are relatively large (Pollack, 1994; Faulkes and Pollack, 2001). One possible explanation for this is that the termination sites of ultrasound receptors are electronically more favorable than those of low-frequency receptors, resulting in less attenuation of ultrasound inputs. The present findings suggest that this is unlikely; both MT types and ultrasound receptors terminate in approximately the same region. Indeed, the slight difference in location, with ultrasound receptors terminating more laterally than low-frequency receptors, would favor increased attenuation of ultrasound-elicited EPSPs. Our counts of varicosities suggest another factor that might contribute to the large sizes of ultrasound-induced unitary EPSPs. Ultrasound receptors (and mid-frequency receptors) have approximately twice as many varicosities per receptor as low-frequency receptors, suggesting that they may make more contacts with their target neurons.

**Segregation of low-frequency information at the primary-neuron level**

The most striking anatomical distinction among the physiologically different receptors occurs between BC and MT types. BC types project more laterally and posteriorly than MT types. This difference is particularly interesting in view of the fact that both anatomical types are tuned to cricket-like sound frequencies.

Projections similar to those of MT and BC types have been described previously. Esch et al. (1980) described receptors in the cricket *Gryllus bimaculatus* that resemble BC and MT types, and were tuned to the sound frequency of the species’ song (4–5 kHz). However, in their illustration (their fig. 7B), both types terminate at similar locations along the M-L axis. It is not possible to determine from their results whether the BC-like morphology was restricted to low-frequency-tuned receptor neurons. Mason et al. (1999) also described receptors with MT-type and BC-type morphology in the haglaid *Cyphoderris monstrosa*, a primitive member of the suborder Ensifera, which includes crickets. The two types were similar in minimum threshold and best frequency but differed in bandwidth; receptors with BC-type morphology responded relatively poorly to the higher sound frequencies used for communication.

Double fills of receptor neurons and ON1 suggest that MT-type and BC-type receptors may interact with different central pathways. MT types are anatomically well suited to provide direct input to AN1, an ascending, low-frequency-tuned interneuron that plays an important role in behavioral responses to cricket song (Wohlers and Huber, 1982, 1985; Schildberger and Hörner, 1988). MT types can also influence ascending information indirectly through their connections with ON1, which provides contralateral inhibition to AN1 (Faulkes and Pollack, 2000). AN1 appears to be the main source of afferent input to brain circuits that filter stimulus temporal pattern (Schildberger, 1984), a stimulus feature that is crucial for signal recognition.

By contrast, BC types make few if any direct connections with ON1 or, by extension, with AN1, and thus may have little or no influence on ascending auditory information. They are, however, a possible source of input to DN1, a descending neuron that relays information about cricket songs to more posterior ganglia (Wohlers and Huber, 1982; Atkins and Pollack, 1987b). Consistent with this, in *T. oceanicus* both BC types and DN1 have relatively high minimum thresholds (BC types: 62.3 ± 7.3 dB SPL, Imaizumi and Pollack, 2001; DN1: 65–70 dB SPL, Atkins and Pollack, 1987b) compared to MT types (52.2 ± 8.3 dB SPL; Imaizumi and Pollack, 2001) and ON1 (44 ± 3.3 dB SPL, Atkins and Pollack, 1986) or AN1 (46.1 ± 4.2 dB SPL, Marsat and Pollack, 2005).

A possible function of descending auditory information is to provide input to motor circuitry that accomplishes phonotactic steering. This circuitry has access to temporally detailed information about sound stimuli (Pollack
and Hoy, 1981; Hedwig and Poulet, 2004). Recent experiments (Hedwig and Poulet, 2004) suggest that phonotaxis might be accomplished by reflex-like (though not direct) circuitry between auditory afferents and motor neurons, gated by descending inputs from the brain reflecting recognition of the stimulus temporal pattern (Pollack and Hoy, 1981; Doherty, 1991; Hedwig and Poulet, 2004). Two physiological features of the BC types are interesting in this respect. First, although threshold for phonotaxis is similar to that of MT types (Doolan and Pollack, 1985; Schmitz, 1985), behavioral performance improves with increasing intensity, reaching a plateau at ~60 dB SPL, similar to the intensity at which BC types and DN1 would come into play (Pollack and Plourde, 1982; Schmitz, 1985). Second, intensity-response functions of BC types are steeper than those of MT types (Imaiizumi and Pollack, 2001), perhaps facilitating their coding of stimulus amplitude at each ear, which is the dominant cue for sound direction (Pollack, 2003). Our results raise the intriguing possibility that the neural substrates for two different components of acoustic behavior, signal recognition and phonotactic steering, are segregated from the earliest stage of processing, with input for the former carried mainly or exclusively by MT-type receptors, and that for the latter by BC-type receptors.

The relative numbers of BC and MT types are similar (of 98 low-frequency receptors stained in the present study, 53 were BC types and 45 were MT types). Although much attention has been paid to how acoustic signals are represented and processed by ON1 and by ascending neurons, relatively little is known about the descending pathway (Wohlers and Huber, 1982; Atkins and Pollack, 1987b). Our finding, that more than a third of the entire receptor population may interact mainly, if not exclusively, with descending interneurons, suggests that this component of auditory circuitry warrants further investigation.

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