Age-Dependent Occurrence of an Ascending Axon on the Omega Neuron of the Cricket, *Teleogryllus oceanicus*

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ABSTRACT
The omega neurons (ON1s) are a mirror-symmetrical pair of identified prothoracic auditory interneurons of crickets which have been previously described as intraganglionic. Using intracellular techniques we stained ON1s of female *Teleogryllus oceanicus* and found that many ON1s have axons which project anteriorly out of the prothoracic ganglion. The ascending axon arises contralateral to the soma at the most anterolateral bend of the bow-shaped process of an otherwise "archetypical" ON1 and travels up the neck connective in a ventral position just inside the connective tissue sheath. The occurrence of the ascending axon is age-dependent. Seventy-five percent of ON1s stained in late nymphal stages and in young adults had an ascending axon while only 30% of ON1s in older adults had an ascending axon. Evidence is presented to show that ON1s having ascending axons are developmental variants of the "archetypical" ON1 and do not represent a separate neuron type. The two morphological types of ON1s are not distinguishable on the basis of their responses to sound stimuli having carrier frequencies of 3.5–60 kHz. Although we know that the ascending axon conducts action potentials, its target and terminal morphology are not yet known.

Key words: postembryonic, development, morphogenesis, auditory, degeneration

Numerous behavioral experiments have demonstrated that female crickets are able to recognize and localize biologically significant sound patterns (see Elsner and Popov, '78, for a review). In an attempt to understand how the cricket’s nervous system processes sound, several neurons that respond to acoustic stimuli have been identified and characterized. One of these identified interneurons has been termed the omega neuron (ON1). This neuron occurs as one of a mirror-symmetric pair in the prothoracic ganglion and has been studied in several species (Casaday and Hoy, '77; Popov et al., '78; Wohlers and Huber, '78, '82; Wieland, '80; Wiese, '81; Atkins et al., '84).

The ON1 has been described as a local, intraganglionic interneuron that branches extensively in the auditory neuropiles of both the left and right sides of the prothoracic ganglion. The two regions of branching are joined by a bow-shaped process. On the basis of the relative amplitudes of synaptic potentials and action potentials recorded at different sites, it has been suggested that the soma-ipsilateral branches are the input region of the cell and that its output is in the soma-contralateral neuropile (Wohlers and Huber, '78, '82).

In this paper we report that in *Teleogryllus oceanicus* some ON1s have an axon that ascends to the head. Furthermore, the presence of this ascending axon is age-dependent.

MATERIALS AND METHODS
Adult female *Teleogryllus oceanicus* crickets were collected from a laboratory colony on the day of their final molt. Last and second-to-last instars were staged based on wing and ovipositor characteristics. All experimental animals were provided continuously with shelter, food (rabbit chow and carrots), and water and were maintained at 28°C, with a 12:12 light/dark cycle.

Recording and staining
Meso- and metathoracic legs and wings were removed and the cricket was fastened to a holder, ventral side up.

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The prothoracic coxae were moved away from the midline and fixed to the ventro-anterior edge of the pronotum. The femur was then flexed against the tibia and both segments were fastened with warm wax to the ventral edge of the pronotum. As a result, the posterior tympana (located on the tibiae) were facing laterally away from the midline of the cricket. The position in which the front legs were fixed is similar to that to which they adopt during flight (Bentley and Hoy, '70). The prothoracic ganglion was exposed by cutting away the overlying cuticle. For greater stability the anterior portion of the gut was removed and several muscles in the neck region were cut. The ventral nerve cord was severed just posterior to the metathoracic ganglion in order to minimize movements due to respiration. A silver platform was positioned under the prothoracic ganglion for stability and also acted as the indifferent electrode. Exposed tissue was kept moist with physiological saline (Field, '60) throughout the experiment.

ON1s were penetrated with aluminosilicate glass micropipettes (Frederick Haer & Co.) filled either with 3% Lucifer Yellow CH (Aldrich) dissolved in 0.01 M LiCl (resistance 60–120 MΩ) (Stewart, '78, '81) or with 0.1 M Co(NH3)6Cl3 (resistance 30–60 MΩ) or with 0.25 M NiCl2 (resistance 30–60 MΩ) (Delcomyn, '81). Lucifer Yellow was iontophoresed with \(-1\) to \(-20\) nA of DC current, while Co(NH3)6Cl3 and NiCl2 were iontophoresed with 1–5 nA 100-ms pulses. Ganglia containing Lucifer Yellow-filled cells were fixed in formaldehyde, dehydrated in ethanol, cleared in methyl salicylate, and viewed with a fluorescence microscope (Stewart, '81). When Co(NH3)6Cl3 or NiCl2 was used ganglia were placed in H2S-saturated saline for 10 minutes, fixed in Carnoy's fixative, silver intensified (Bacon and Altman, '77), dehydrated in ethanol, cleared in methyl salicylate, and viewed with brightfield illumination. Transverse 10-μm sections were made of selected ganglia with a steel knife following embedment in soft Spurr's epoxy resin (Spurr, '69).

In order to determine whether the ascending axon conducts action potentials, extracellular recordings were made with a suction electrode placed on the ventral surface of the neck connective while intracellular potentials were recorded simultaneously from the processes of ON1. A洛克威 AIM-65 concomputer triggered by the intracellularly recorded action potential averaged the extracellular recording to determine whether a time-locked action potential occurred in the neck connective.

**Acoustic stimuli**

Sound stimuli consisted of 30-ms pulses, with rise and fall times of 5 ms, presented at a repetition rate of 2 pulses/second. Sound was played from one of a matched pair of loudspeakers (Realistic 40-1310) situated to the left and right of the midline of the cricket, 45 cm from the cricket. Sound intensities were measured with a one-quarter-inch Bruel and Kjaer 4135 condenser microphone and Bruel and Kjaer 2610 measuring amplifier. Reflective surfaces inside the recording chamber were covered with fiberglass insulation so that echoes measured at the position of the cricket were at least 30 dB less intense than the main signal. Twenty-three discrete carrier frequencies ranging from 3.5 to 60 kHz were used. Stimuli of each frequency were separately attenuated by a custom-built 23-channel attenuator network, thus ensuring that the frequency response of the sound delivery system was flat (± 3 dB).

**RESULTS**

**Anatomy**

One hundred seventy-two ON1s were stained with Lucifer Yellow. Ninety-three of these had an axon ascending to the head, while 79 did not. Figure 1 (A,B) shows examples of ON1s with and without ascending axons. Both types of ON1 were also revealed with Co(NH3)6Cl3 (seven with an ascending axon, three without) (Fig. 1C) and with NiCl2 (three with an ascending axon, two without). No consistent anatomical difference could be found between the two classes of ON1 other than the presence or absence of an ascending axon, although individual ON1s often differed slightly in their fine branching.

We considered the possibility that all ON1s have ascending axons but that we sometimes failed to detect them because of poor or incomplete staining. However, many cells that we subjectively judged to be poorly stained clearly revealed an ascending axon, while many other cells that we judged to be well stained failed to do so.

A more objective evaluation of the detectability of neuronal structure was developed by measuring the contrast...
Age-dependent occurrence of ascending axons

ON1s were stained in crickets whose age was known to within a day. Approximately 75% of ON1s in adults less than 2 weeks of age had ascending axons, while only 25–35% of ON1s in animals older than 28 days had ascending axons (Fig. 4).

Although the cricket's ear is not fully developed until the last molt, auditory receptor cells arise and grow into the central nervous system at earlier stages (Ball and Young, '74), and auditory neurons of late nymphal stages can be excited by sufficiently intense sound stimuli (Ball and Hill, '78). By using stimulus intensities between 90 and 110 dB we were able to locate and stain ON1s of last and second-to-last nymphal instars. Aside from being somewhat smaller than their adult counterparts, ON1s of late nymphs were essentially identical in structure to those of adults (Fig. 5).

The rate of occurrence of ascending axons was similar to that found for young adults (Fig. 4).

It is possible that, as a result of unknown genetic or physiological effects, the age-dependence we have observed might be due to earlier mortality among crickets with axon-bearing ON1s. However, fewer than 2% of our crickets died between the ages of 1 and 42 days. Thus, differential mortality cannot explain our findings.

A more likely explanation for the age-dependence demonstrated in Figure 4 is that the ascending axon degenerates or becomes disassociated from the rest of ON1. We asked, therefore, whether the axon is physiologically active when it is present in the adult. One-for-one correspondence between spikes recorded extracellularly from the neck connective and intracellularly from ON1 could not be discerned from visual inspection of electrophysiological records, even when Lucifer Yellow injections demonstrated the presence of an ascending axon. However, action potentials which were time-locked to the intracellularly recorded ON1 spike could be detected in extracellular records after signal averaging in five of eight preparations in which the ON1 was shown anatomically to have an ascending axon. Figure 6A shows the averaged action potential from one of

Fig. 1. Photographs of wholemounts showing (A) an "archetypical" ON1 and (B and C) ON1s with ascending axons. A and B were stained with Lucifer Yellow whereas C (a montage of photographs taken in different planes of focus) was stained with Co(NH3)6Cl3 followed by sulfide precipitation and silver intensification. Calibration bar represents 200 μm.

between the stained ON1 and the surrounding tissue (see Materials and Methods for a complete description). Contrast measurements for 45 ON1s having ascending axons ranged from 1.23 to 12.29 (median = 3.38) whereas for 35 ON1s lacking ascending axons the measurements ranged from 1.27 to 16.36 (median = 5.42). Although the medians of the two groups differ significantly (Mann-Whitney U test, P < .05), the direction of the difference is opposite to that expected if the failure to detect ascending axons were due to poor contrast between the axon and its surroundings.

The ascending axon, when it occurs, arises on the soma-contralateral side slightly posterior and lateral to the point where the major process bends posteriorly after crossing the midline (Fig. 1B,C, 2A). The axon travels anteriorly to the soma-contralateral neck connective (Fig. 2A,B). In the neck connective, the axon maintains an extremely ventral position, just inside the connective tissue sheath (Fig. 2C).

Figure 3A shows a cross section through a neck connective where both the ascending axon of an ON1 and the axon of another identified auditory interneuron, interneuron-1 (Moiseff and Hoy, '83), were stained. The axon of ON1 is consistently more ventral and lateral than that of interneuron-1 and is much smaller in diameter.

Thus far we have only been able to stain the ON1 as far anterior as the base of the suboesophageal ganglion.

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Fig. 2. Photographs (A–C) of transverse sections through a ganglion (ventral side up) containing a stained ON1 having an ascending axon. Planes of sections are identified in sketch (D). For clarity, enlargements are shown (A', B') of the area of the sections in A and B that contains the ascending axon. Calibration bar represents 200 μm in A–C and 100 μm in A' and B'.
Fig. 3. A. Ascending axon of ON1 in the neck connective, together with the ascending axon of another identified ascending auditory interneuron, interneuron-1, for comparison. B. Wholemount preparations showing interneuron-1. Calibration bar represents 100 μm in A and 300 μm in B. V, ventral; M, medial; D, dorsal; L, lateral; A, anterior; P, posterior.

Fig. 5. Examples of ON1s with (A) and without (B) ascending axons from second-to-last instar nymphs. Calibration bar represents 100 μm.

**A single identified cell type has two morphological variants**

There are at least two possible explanations for our findings. (1) There is only a single pair of ON1s per prothoracic ganglion; these neurons have ascending axons which occur in an age-dependent manner. According to this possibility, the occurrence of ascending axons on some ON1s but not on others represents variability of structure of a single, identified cell type. (2) There are two distinct pairs of ON1s per prothoracic ganglion, one pair having ascending axons, and the other not. According to this hypothesis, the two types coexist in the same animal and, for unknown reasons, their relative penetrability with microelectrodes is age-dependent. The following evidence suggests that the first possibility is correct.

If there were two distinct pairs of ON1s in each prothoracic ganglion it should be possible, at least occasionally, to demonstrate members of both pairs in the same ganglion. The laterality of an ON1 can be determined from its physiological responses. Under “closed field” stimulus conditions, the ON1 is excited by sound played from the soma-ipsilateral side and inhibited by sound played from the soma-contralateral side (Wohlers and Huber, '82). Under
the "free field" conditions used in the present work, the
neuron can be excited by sound played from either side, but
is 15–30 dB more sensitive to soma-ipsilateral sound. Using
criteria such as laterality and frequency sensitivity (see
below) we were able to identify an ON1 with high reliabil-
ity on physiological grounds alone. In 20 animals, we re-
corded from and stained a neuron believed, on the basis of
physiological characteristics, to be an ON1 of particular
laterality. We then withdrew the electrode and again pene-
trated and stained a neuron with physiological character-
istics (including laterality) similar to those of the first cell.
If two ON1s of the same laterality existed (one with an
ascending axon and one without) we might have expected,
in some of these experiments, to reveal both of them. How-
ever, in all 20 cases we stained only a single ON1.

In 28 other preparations two ON1s of opposite laterality
were stained. In 15 such cases both ON1s had ascending
axons, in 11 neither had an ascending axon, and in two one
had an ascending axon while its partner did not. If two
pairs of ON1s existed in an individual cricket, we would
have expected one-half of these 28 double stains to have
shown one ON1 of each type. However, this situation oc-
curred only twice, further supporting our conclusion that
only a single pair of ON1s exists in the prothoracic gan-
glion. The two sets of unmatched ON1s were found in 21-
day-old crickets—an age when the occurrence of ascending
axons is decreasing rapidly (Fig. 4)—and might be ex-
plained by a slight asynchrony in the disappearance of the
axons on the left and right sides.

The preceding evidence demonstrates that ON1s can
display considerable morphological variability. We considered
whether this morphological variability was accompanied
by variability in physiological responses. Specifically, we
compared the sensitivity and frequency-tuning of ON1s
with and without ascending axons. As Figure 7 demon-
strates, the two groups of ON1s were indistinguishable on
these grounds. Both groups showed best sensitivity and
sharpest tuning to carrier frequencies in the range of 4.5–5
kHz, which is similar to the carrier frequency of the species
calling song. A second, less sharply tuned, peak of sensitiv-
ity was found at carrier frequencies from 14 to 35 kHz.

DISCUSSION

In the present study stains were introduced into neurons
through intracellular microelectrodes. The possibility ex-
ists, however, that some of the structures stained belonged
not to the penetrated neuron, but rather to one that was
dye-coupled to it. Many neuronal markers, including Luci-
er Yellow, cobalt chloride, and horseradish peroxidase, are
known to cross neuronal membranes and to travel from one
cell to another (Politoff et al., '74; Strausfeld and Ober-
mayer, '76; Stewart, '78; Fredman and Jahan-Parwar, '80;
Nassel, '82). For this reason we cannot rule out the possibil-
ity that the ascending axon which we have described be-
longs not to ON1 but rather to a dye-coupled cell. This
question will not be answered unambiguously until ultra-
structural studies are performed. For the present, we as-
sume that the ascending axon is indeed part of ON1 and
not the result of dye-coupling, and that the age effect we
have described is a manifestation of the resorption or de-
generation of this axon, rather than of its uncoupling.

In the only previous report of the morphology of ON1 in
T. oceanicus, Casaday and Hoy ('77) described this neuron
as intraganglionic. Their description was based on seven
preparations, some of which were incompletely stained. It
is therefore perhaps not surprising that they did not ob-
servve ascending axons in any of their preparations. The fact
that the ascending axon of ON1 has not been reported for
other cricket species (Popov et al., '78; Wohlers and Huber, '78, '82; Wieland, '80; Wiese, '81; Atkins et al., '84) might be explained by differences in the ages of crickets tested or in the timing of the disappearance of the axon, or it may be that the ascending axon never exists in some species.

The ability of the ascending axon of ON1 to conduct spikes (Fig. 6) demonstrates that ON1, in its interganglionic form, can be considered as a direct pathway for transmitting acoustic information from the prothoracic ganglion to higher processing centers. In previous descriptions of ON1 it was reasoned that it could only influence higher auditory centers indirectly via other ascending interneurons (Wohlers and Huber, '78). It is now clear that for a limited time period information from ON1 may be relayed directly to the head as well.

Unfortunately, we have no indication as to what the functional significance of this information might be. We have not attempted to correlate the behavioral performance of individuals with the presence or absence of ascending axons on their ON1s. Indeed, the functional role of ON1 itself is as yet not clear. Wiese ('78) and Wiese and Elits-Grimm ('85) have suggested that ON1 may be important for recognition of the species-specific temporal pattern of the calling song, and Casaday and Hoy ('77), Popov et al., ('78), and Wohlers and Huber ('78, '82) have proposed that ON1 is important for sound localization. However, Atkins et al. ('84) showed that crickets could recognize and localize a model of the species calling song even after one or both ON1s were destroyed by photoactivation.

Comparison of ON1 development to other insect neurons

Examination of the embryonic and postembryonic development of several identified neurons, such as the DCMD of grasshoppers (Bently and Toroian-Raymond, '81), the DUM cells of grasshoppers (Goodman and Spitzer, '79), the MGI of crickets (Shankland and Goodman, '82), and the G, B1 and B2 auditory interneurons of locusts (Boyan, '83; Pearson et al., '85) have yielded similar conclusions about the general pattern of neuronal morphogenesis in hemimetabolous insects. Essentially all of the morphological characteristics of the adult neurons are established by the end of embryogenesis and postembryonic changes are restricted to allometric growth and, in some cases, small increases in the density of neuropile processes. ON1 departs from this pattern. As Figure 4 shows, the disappearance of the ascending axon from ON1 usually occurs a few weeks after the imaginal molt.

It should be emphasized that it is not the loss per se of the ascending axon which is unusual, but rather its timing. For example, the Hcell of grasshoppers grows one interganglionic axon early in embryogenesis which degenerates later in embryogenesis after four new interganglionic axons emerge (Goodman et al., '81). Degeneration of the original axon of the H-cell is completed by the end of embryogenesis before the cell plays a role in behavior, whereas degeneration of the ascending axon of ON1 can occur as late as several weeks after the adult molt. Behavioral experiments (Sakaluk and Cade, '80; Sakaluk, '82) have shown that auditory behavior of crickets begins within a week after completion of the imaginal molt and continues for several weeks thereafter. It seems clear then that the ascending axon disappears at a time when the ON1 is functioning in the auditory system.

Although neuronal morphology of hemimetabolous insects is usually very stable throughout postembryonic development and adult life, gross postembryonic changes can be artificially induced. The MGI of adult crickets, for example, has been shown to sprout and retract processes from its soma, axon, and dendrites in response to lesions (Roe-derer and Cohen, '83). Similar induced postembryonic changes have been demonstrated in other neurons of insects and other invertebrates (Murphey et al., '75; Clark, '76; Müller and Carbonetto, '78; Murphy and Kater, '80), including interneuron-1, another identified auditory interneuron of T. oceanicus (Hoy et al., '78). These artificially induced changes demonstrate that morphological rearrangements can occur during postembryonic life; however, the disappearance of ON1's ascending axon is unusual because it occurs without surgical intervention.

Gross morphological changes do occur in neurons of holometabolous insects during the normal course of development. For example, in the lepidopteran, Manduca sexta, some identified motor neurons innervate newly formed muscles in the adult, and presumably subserve different functions than they did in the larva. In addition to changing target muscles during the pupal stage, these motor neurons undergo massive reorganization of their dendritic processes (Truman and Reiss, '76; Levine and Truman, '82; Levine, '84). However, these morphological changes occur during the pupal stage, which is a transition between two different functional periods, and not while the neurons are involved in specific larval or adult behavior.

In summary, the age-dependent loss of the ascending axon from ON1 represents a novel pattern of neuronal development of hemimetabolous insects. First, a gross morphogenetic change occurs during the functional life of a neuron, when its activity is presumably behaviorally relevant. Second, this change occurs without surgical intervention. It seems unlikely, however, that this developmental pattern will be found to be unique to ON1, as we have found that at least one other identified interneuron, ON2 (Wohlers and Huber, '82), also exhibits an ascending axon in an age-dependent manner seemingly analogous to that of ON1 (Atkins, unpublished results).

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