ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol. Exp. 1998, 58: 277-281

Clock-spiking cells not only in the eye of the fly, but also in the antenna!

Sulochana D. Moro[1] and **Matti Huotari**[2]

[1]Department of Biology (Division of Genetics), Oulu University, FIN-90570 Oulu, Email: sulochan@cc.oulu.fi; [2]Department of Physics (Division of Biophysics), Oulu University, FIN-90570 Oulu, Finland

Abstract. During the course of single cell olfactory recordings from the funicular part of the antenna of *Drosophila virilis* we encountered a pair of cells firing synchronously and consistently at a rate of about 9 to 14 spikes per second. Every spike was seen to consist of a spike complex made up of two separate biphasic components thought to originate from two separate cells. The larger action potential, appearing first, had a peak-to-peak (ptp) amplitude of up to 200 μV followed closely by a smaller spike with an amplitude of about 60 μV. The repetitive firing pattern was not affected by air or odour puffs. This kind of consistent spontaneous spiking activity of two closely associated cells resembles remarkably closely the clock-spikes hitherto known only from the eyes of flies. Our encounter with such cells in a sense organ other than the eye poses many new questions and could lead to a renewed effort to understand the role(s) of the clock-spiking cells as possible oscillatory components of the dipteran pacemaking system in particular and the insect nervous systems, generally.

Short communication

Key words: clock-spikes, antenna, Diptera, Drosophila, oscillator , pace-maker, biorhythms, eyes

It came as a considerable surprise and led to a publication in "Nature", when vision researchers more than 30 years ago discovered "clock-spikes" in the optic lobes of *Calliphora erythrocephala* (Kuiper and Leutscher-Hazelhoff 1965, Leutscher-Hazelhoff and Kuiper 1966). It took 5 more years before the next recordings of clock-spikes were reported by Burtt and Patterson (1970), again from the eye of a fly. Characterized by their extreme repetitiveness and regularity, clock-spikes and their possible function(s) became a focus of attention. More recordings were obtained in the following years from the visual systems of other insects like, for instance, *Musca* (Henstenberg 1971) and the spikes became known as "C-spikes". However, the precise nature of the cells and more importantly their function remained enigmatic. That temperature had an effect on the firing frequency of these "C-spikes" was demonstrated by Hengstenberg (1971) in *Musca* and Patterson (1972) in *Calliphora*, but thermoreceptive units in insects possess totally different response characteristics (Altner and Loftus 1985) and the clock-spikes, therefore, clearly had to have another role. With no further progress in the understanding of the clock-spikes and the cells responsible for generating them forthcoming, the subject was laid aside.

In this short communication, we present our findings of an unexpected recording of a train of very consistent and regular spikes in a different arthropod sense organ, namely the antennal funiculus of *Drosophila virilis*. It was the intriguing similarity of the spikes to the "clock-spikes" already known from the optic lobes and the eye muscles of flies (Kuiper and Leutscher-Hazelhoff 1965, Hengstenberg 1971, Patterson 1972) which led us to publish this report on our finding of clock-spikes in a non-visual system. We also obtained shorter but similar recordings of clock-spikes from basically the same region of the funiculus in *D. montana* and the funicular tip of *C. vicinia*. However, we present here only the results obtained from *D. virilis*, as the recordings from this animal were the most stable and, with about 9 min of uninterrupted clock-spikes, gave us the longest record to analyse.

The 12 day old virgin female *D. virilis*, which provided us with the longest single unit extracellular recordings, was obtained, like all experimental specimens, from the fly cultures of the Department of Genetics (Oulu University). During the recordings, conducted at a room temperature of 24°C, the specimens were held immobile in a micropipette tip fixed on a stand with only the antenna being able to move freely. A conventional electrophysiological setup for extracellular single-cell recordings was used. The recording tungsten electrode was in contact with an area close to the arista on the proximal posterior aspect of the funiculus (cf. Stocker 1994), while the ground electrode was at the distal end rendering the antenna less mobile. The action potentials led off by a Grass P16 pre-amplifier (band pass filtering of 100 to 1,000 Hz) were monitored visually on the oscilloscope and stored on a DAT recorder (TEAC) at 11 kHz. The spikes recorded on the tape were filtered and sampled by a Multi Spike Detector (MSD) (Alpha Omega) at 30 kHz, to obtain inter spike intervals (ISIs) stored on Microsoft Excel. The acquisition of the spikes was based on the template matching of the larger spike in each spike complex. Microcalc Origin software was used to obtain histograms and distribution curves plotted on Word 6. The spikes on the DAT tape were also captured on a Signal Analyser (HP35665A) to obtain plots of the spike trains and their waveforms using Word 6.

When the extracellular recording electrode made contact with the particular cell-couplet, the spike train sounded unusually different from regular olfactory cells on the audiomonitor and resembled a fast firing, regular volley of bullets. While we had been trying to get responses from olfactory cells, this cell couplet was not affected by the odour puffs of the particular chemical (tiglic acid ethyl acetate) we were using for our olfactory recording experiments. There was also no obvious change in the firing pattern to air puffs used during the delivery of the odour. The best recording remained stable for about 9 min. This is considerably longer than so-called injury discharges, known to occasionally arise as small, positive potentials from partially injured axons. Furthermore, when we examined the spike records, they (unlike injury discharges) appeared unusually regular and consistent in the AC-mode as well as in the superimposed DC-recordings throughout the duration of the recording. We noticed the similarity to the "clock-spikes" known from the eyes of some other Diptera (Kuiper and Leutscher-Hazelhoff 1965, Hengstenberg 1971, Patterson 1972) and decided to thoroughly analyse the clock-spike recordings we had obtained from the antennal funiculus.

Figure 1 shows an example of part of the recording of the continuous train of spikes, appropriately filtered. The DC-recording of the spikes as seen in Fig. 1A displays the summed complex in each spike, while the AC-recording in Fig. 1B shows that every spike in the record actually consists of 2 very closely apposed spikes firing

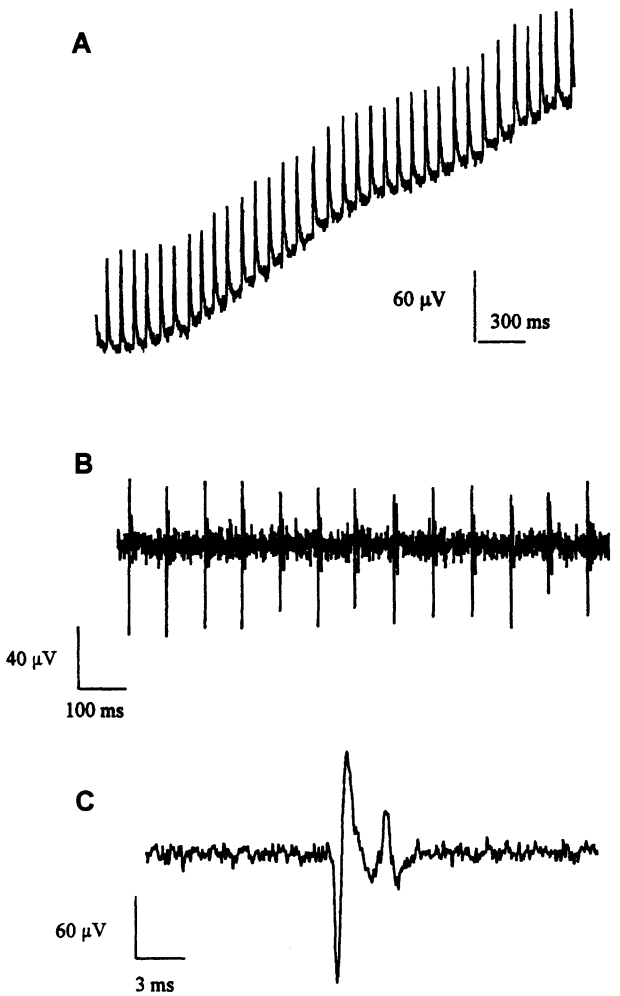

Fig. 1. A, part of a DC-recording of the clock-spikes, showing a highly consistent train of spikes superimposed on the fluctuating DC baseline. B, part of an AC-recording of a spike train, showing typical clock-spike action potentials. C, an average of 4 spike complexes, showing details of the waveforms of the two action potentials in every single spike-complex.

nearly together as a single complex at a frequency of 9 to 14 Hz. Figure 1C clearly shows the waveform of the spike complex separable into 2 diphasic components. The first and larger spike exhibited amplitudes reaching 200 µV, followed closely by the second, smaller spike of about 60 µV. The time-delay between 1st and 2nd spike of the complex, averaging 1.8 ms as in Fig. 1C, actually varied between 1.6 ms and 2.2 ms when longer stretches of the recordings were analysed. The duration of the larger spike was 3.6 ms, that of the second and smaller spike was 1.5 ms and that of the entire waveform complex 7 ms (Fig. 1C).

To acquire the inter-spike-intervals (ISIs) by the MSD, we chose to use the template of the larger spike throughout. Figure 2 shows an example of the distribution of ISIs in the interval histogram of the spikes for over 1,000 consecutive spike-complexes. The Gaussian distribution of the ISIs brought the data under close to a normal curve with an average ISI of 87.5 ms. Although the firing is not of the same high precision for the ISIs to fall under a perfectly normal curve as first seen for the eye of the blowfly (Kuiper and Leutscher-Hazelhoff 1965), the proximity of the Gaussian distribution to a normal curve as well as the uninterrupted repetitiveness of the firing would categorise these spikes under the term "C-spikes" first used by Hengstenberg (1971). We plotted ISI histograms (not included here) for adjoining olfactory cells in the antenna and established that unlike the firing of the C-spiking cells under discussion here the olfactory cell outputs did not fit any normal curve, but rather obeyed a Poisson distribution of a cell firing spontaneously in the usual irregular way (Moro et al., in preparation).

The consistent firing of 2 close cells together reveals for the first time the existence of such cells in a sense organ previously not envisaged to have them. We consider this new finding to be very significant with regard to the possible functions of these clock-spiking cells and whether the latter are autonomously active or form part of a more centrally-controlled pace-maker system in the insect body. With two places now known to harbour clock-spiking cells, the speculation of the possible existence of autonomous circadian oscillators and pacemakers in different regions of the insect (Hall 1995, Plautz et al. 1997) has received a boost.

The firing frequency of the clock-spikes in the slower-moving *Drosophila* is lower than that known from the eye of the faster *Calliphora*, concurring with Kuiper and Leutscher-Hazelhoff s (1965) interpretation of such differences. In terms of time constants, amplitudes, and other typical waveform characteristics, our recordings of the funicular spikes show a remarkable similarity to the clock spike complexes recorded from the fly eyes by Kuiper and Leutscher--Hazelhoff (1965) and Patterson (1972). A closer comparison, however, between the spike complexes recorded by Kuiper and Leutscher-Hazelhoff (1965) from the fly optic lobe and Patterson (1972) from the eye muscle reveals minor differences in polarity. Our spikes, in which the larger and slower component appeared just before the smaller and faster one, resembled most close-

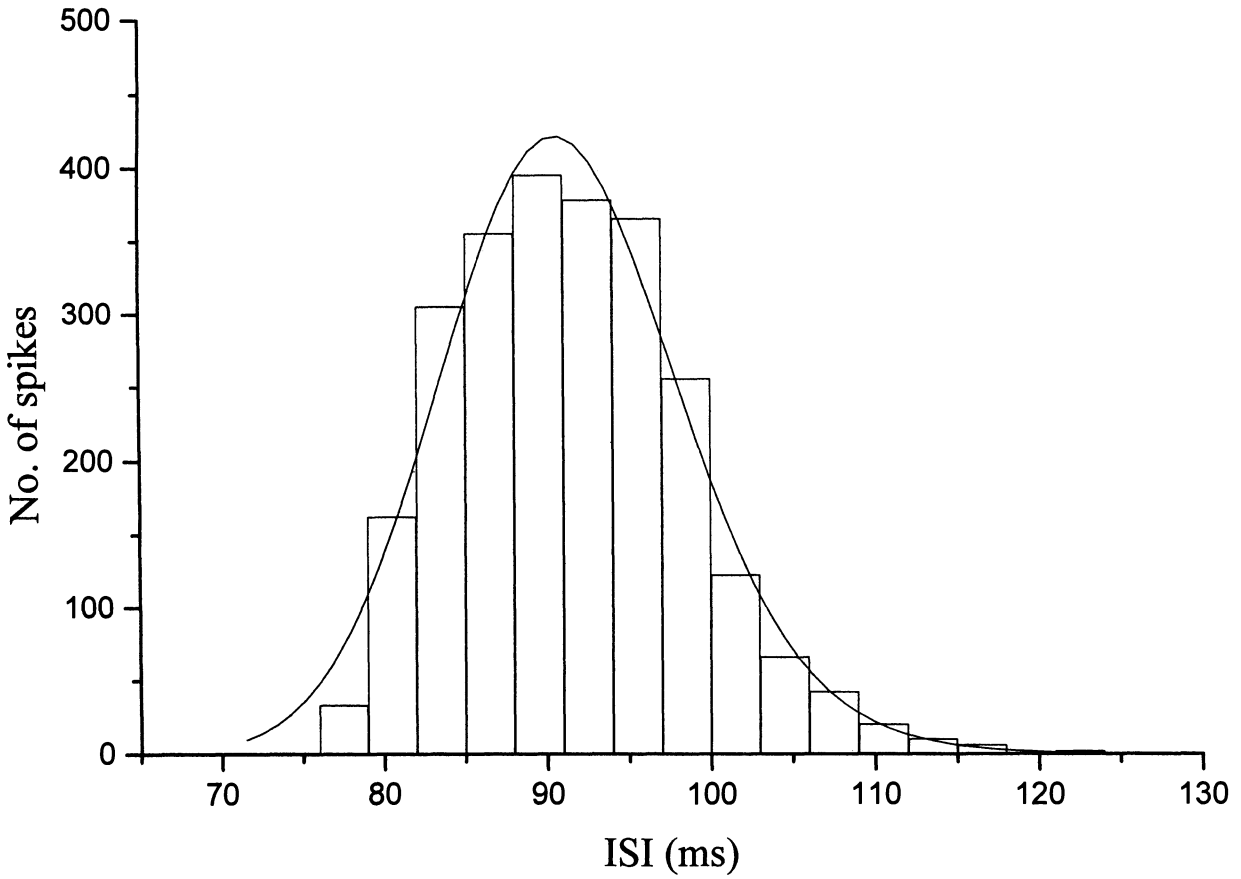

Fig. 2. An inter-spike-interval (ISI) histogram with the normal distribution curve added for a randomly chosen part of the clock--spike recordings. ISI, average = 87.5 ± 6.5. Total number (*n*) of spikes analysed = 1203.

ly those of the optic lobes of *Calliphora* rather than its eye muscle.

Polarity, spike height and shape can, of course, depend on the recording electrode and its position in the tissue. Nevertheless, the question arises as to whether the complex waveform in our recording might not contain a component from a motor neurone. Muscle fibres are, however, not known to exist in the more distal antennal segments of pterygote insects and in particular have never been found in the *Drosophila melanogaster* funiculus (Venkatesh and Singh 1984, Shanbag et al. 1995, Singh, personal communication), the most distal of all the antennal segments! Are the cells giving off the clock-spikes, then perhaps connected in any way to the eye muscle of the *Drosophila* eye *via* the antennal nerve? Studies in this direction would involve tedious and patient anatomical, immunocytochemical, and neurophysiological work, but such research has the potential of being immensely rewarding. A demonstration of a connection between the eye and antennal clock-spiking cells would be significant in terms of possible coordinating networks between two different sense organs.

Future studies on the question of clock-spiking cells as possible rhythmic oscillators or part of some pace-making oscillatory unit for a particular organ should focus on whether they are autonomous or controlled by a central pacemaker. Sassone-Carol (1998) suggests that clocks could exist in distinct anatomical structures which may overlap to regulate the output and one wonders if the molecular dynamics of firing of such cells might not possibly act as "gears" of clocks independently working in different parts of the insect body. With the discovery now of clock-spiking units in a second region of the insect body it no longer seems impossible to find the overlap and coordination between the clock-spiking cells in the dipteran antenna and optic lobe. Whether the clock--spiking cells in the antenna are under the direction of a "central clock" and to what extent the system is able to compensate for temperature fluctuations known to affect any biological clock (Pittendrigh 1993), is something for

neurophysiologists to work out and to present to molecular geneticists like Sassone-Carol (1998), Hall (1995), and others involved in the search of the molecular clock.

Finally, if clock-spiking cells are all generally affected by temperature as shown by the earlier work of Patterson (1972) as well as Hengstenberg (1971) and respond to physiologically unacceptably high temperatures with increased firing, these cells may be involved in monitoring and signalling thermally-induced cell deaths of the organ or tissue they are associated with. In conclusion, the mystery of the clock-spiking cells, for the time being, remains, but with the discovery of now a second anatomical site from which these enigmatic cells can be monitored, the chances to cast light on their role(s) have certainly improved. May therefore a new round of investigations commence and solve the "riddle of the clock-spikes".

We are indebted to Dr. V.B. Meyer-Rochow of Oulu University, Section Animal Physiology, for enlightening us on the subject of clock-spikes and for his help in critically reading the MS. We are very thankful to Dr. Esa Hohtola for giving us his valuable time in discussing statistical matters. SDM feels deeply grateful to Dr. Anneli Hoikkala of the Department of Genetics of Oulu University for helping her through to make the paper possible. We owe our thanks to Dr. P.Mela for use of the equipment in the Biophysics Section of the Department of Physical Sciences. Finally, SDM wishes to thank the Finnish Academy for the financial support received through Dr. Hoikkala for this project.

Altner H., Loftus R.(1985) Ultrastructure and function of insect thermo- and hygroreceptors. Ann. Rev. Entomol. 30 : 273-295.

Burtt E.T., Patterson J.A. (1970) Internal muscle in the eye of an insect. Nature 226: 183-184.

Hall J.C. (1995) Tripping along the trail to the molecular mechanisms of biological clocks. TINS 18: 230-240.

Hengstenberg R. (1971) Das Augenmuskelsystem der Stubenfliege *Musca domestica*. I. Analyse der "clock-spikes" und ihrer Quellen. Kybernetik 2: 56-77.

Kuiper J.W., Leutscher-Hazelhoff J.T. (1965) High-precision repetitive firing in the insect optic lobe and a hypothesis for its function in object location. Nature (Lond.) 206: 1158-1160.

Leutscher-Hazelhoff J.T., Kuiper J.W. (1966) Clock-spikes in the *Calliphora* optic lobe and a hypothesis for their function in object location. In: The functional organization of the compound eye (Ed. C.G. Bernhard). Pergamon, Oxford, p. 483-492.

Patterson J. (1972) The eye muscle of *Calliphora vomitoria* L. I. Spontaneous activity and the effects of light and dark adaptation. J. Exp. Biol. 58 : 563-583.

Pittendrigh C.S. (1993) Temporal organization: reflections of a Darwinian clock-watcher. Annu. Rev. Physiol. 55: 17-54.

Plautz J.D., Kaneko M., Hall J.C., Kay S.A. (1997) Independent photoreceptive circadian clocks throughout *Drosophila*. Science 278: 1632-1635.

Sassone-Carol P. (1998) Molecular clocks: mastering time by gene regulation. Nature 392: 871 - 374.

Shanbhag S.R., Singh K., Singh R.N. (1995) Fine structure and primary sensory projections of sensilla located in the sacculus of the antenna of *Drosophila melanogaster*. Cell Tissue Res. 279:

Stocker R.F. (1994) The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res. 275: 3-26.

Venkatesh S., Singh R.N. (1984) Sensilla on the third antennal segment of *Drosophila melanogaster* Meigen (Diptera : Drosophilidae). Int. J. Insect Morphol. Embryol. 13: 51- 63.

Received 27 July 1998, accepted 24 September 1998