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"Spontaneous and evoked electrical activity of neurons in leech Hirudo medicinalis studied by a new generation of voltage sensitive dyes."

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Note

The described in this dissertation was carried out at the International School for Advanced Studies, Trieste, between November 2009 and October 2013. All work reported here has not been submitted, as in whole or in part, to any other university or institute.

Majid Moshtagh Khorasani

Abstract

By using the newly developed voltage sensitive dye VF2.1.Cl invented by Miller and colleagues (Miller et al. 2012), I monitored simultaneously the spontaneous electrical activity of approximately 80 neurons in a leech ganglion, representing around 20% of the entire neuronal population. Neurons imaged on the ventral surface of the ganglion either fired spikes regularly at a rate of 1-5 Hz or fired sparse spikes irregularly. In contrast, neurons imaged on the dorsal surface, fired spikes in bursts involving several neurons. The overall degree of correlated electrical activity among leech neurons was limited in control conditions but increased in the presence of the neuromodulator serotonin. The spontaneous electrical activity in a leech ganglion is segregated in three main groups: neurons comprising Retzius cells, Anterior Pagoda , Leydig and Annulus Erector motoneurons firing almost periodically, a group of neurons firing sparsely and randomly, and a group of neurons firing bursts of spikes of varying durations. These three groups interact and influence each other only weakly.

I was able to obtain long optical recordings for several minutes. I studied, also, the evoked response of nervous system by stimulating mechanosensory neurons. This work paves the way for further studies of multicellular networks using the new voltage sensitive dye.

Introduction

The aim of this project was to study the spontaneous and evoked electrical activity of the entire population of neurons in a leech ganglion. For this purpose a technique was devised for imaging the electrical activity of the population of neurons by use of a new voltage sensitive dye (VSD) developed by Miller (Miller et al. 2012).

Of particular interest is the spontaneous activity that is continually on going in virtually all immature and mature nervous systems. The following questions arise: what role does spontaneous activity play in development and does it influence integrative mechanisms and behavior? How does it arise? At present the cellular mechanisms for its initiation and synchronization are not known. It is not easy to study the spontaneous activity in mammalian brain because of the vast numbers of neurons and connections. The nervous system is simpler in an invertebrate like the leech that contains only hundreds of neurons.

Leeches have been studied to understand the nervous system and the neuronal bases of behavior (Retzius 1891, Nicholls 1968, Muller et al. 1981, Kristan et al. 2005). Leeches are annelids and have a distributed nervous system. The central nervous system consists of a head brain, tail brain and 21 mid-body ganglia. These ganglia are distributed along the nerve cord and enclosed within a blood sinus and each ganglion contains about 400 neurons. The neurons are located on two sides of the ganglion, ventral and dorsal. The

neurons are monopolar and their cell body diameters range from 10μm to 60μm (Muller et al. 1981). There are 4 packets of neurons on the dorsal side which contains motoneurons, and interneurons. On the ventral side there are 6 packets of neurons that contains interneurons, mechanosensory neurons and motoneurons.

In the neural network of the leech Hirudo medicinalis, cells produce continuous spontaneous activity. It is interesting to establish which neurons are causing this activity and what their connections are. The interaction between these neurons shapes the nervous system's functions. To know what is happening between neurons in spontaneous activity could help us to understand better the mechanism of different kinds of behaviors. Local bending, swimming, crawling, shortening, searching with the head sucker are some of these behaviors of the leech. We can aim to understand how the animal makes a decision, how a behavior begins, how an animal switches from one behavior to another one.

A second problem approached in this dissertation is to determine how many cells and which cells are activated or inhibited by sensory impulses from pressure and nociceptive sensory cells. What happens when a mechnosensory neuron sends impulses into the ganglion from the periphery? Which interneurons are involved and which motoneurons are stimulated?

Microelectrodes are used to record the electrical activity of neurons but they have the limitation that can one only record from a few neurons. Usually two or four microelectrodes are the maximum that can be used at once. This means simultaneously we can record the electrical activity of only four neurons. It would obviously be a great advantage to record from all the cells at once in a single preparation. And this was the plan for the present experiments. The method is to record activity by measuring optical signals.

One technique for this is calcium dye imaging to study a population of neurons The problem with calcium indicators is that it is not possible to observe fast electrical events with them because their response time is slow, about hundred milliseconds- and Also they show depolarization but they do not indicate hyperpolarization well, and it takes a longer time for these indicators to go back to the resting potential (Thomas et al. 2000; Stosiek et al. 2003; Homma et al. 2009).

One cannot record a burst of spikes optically with calcium indicators because slow time response do not allow to detect action potentials, instead one observes only an envelope of the real fast signals (Lohr 2003; Stosiek et al. 2003).

Because of these reasons I chose voltage sensitive dye imaging.
The use of VSD to record electrical activity of neurons has progressed year by year. They do not have the limitations of microelectrodes and calcium

indicators yet one can still view the whole or larger part of the neural network (Cohen et al. 1989, Cacciatore et al. 1999, Briggman et al. 2005, Briggman and Kristan 2006, frost et al. 2007, stein et al. 2011, Hill et al. 2012, Städele et al. 2012). The main limitations of earlier VSDs were slow kinetics and photo-toxicity which are addressed in new VSDs (Miller et al. 2012).

The VSD used for this study has a fast time response to the change of the voltage of the membrane of the neurons in the range of <<140μs (Miller et al. 2012).

By using the new VSD and imaging techniques I was able to observe the electrical activity of 50-60 neurons at once on the dorsal side of the leech ganglion and 80-90 neurons on ventral side of the ganglion and I was able to record the action potentials and slow changes of the voltage in the membrane. I was able to observe electrical couplings between neurons. I also studied the effect of serotonin on the population of neurons.

I stimulated pressure mechanosensory neuron (P cell) and nociceptive mechanosensory (N cell) neuron to observe evoked electrical activity in the population of neurons and then compared VSD optical imaging recordings with previous studies done by electrophysiological recording using microelectrodes and prove that the VSD is not toxic.

In chapter one I present some background information about neurobiology of the leech and VSD used in this study. The imaging method is described in chapter two. In chapter three I present the results of imaging of the spontaneous activity of the ganglion and also in the presence of serotonin. In chapter four I present results obtained when mechanosensory neurons were stimulated and finally in the discussion I summarize the main conclusions of my PhD dissertation.

Chapter 1

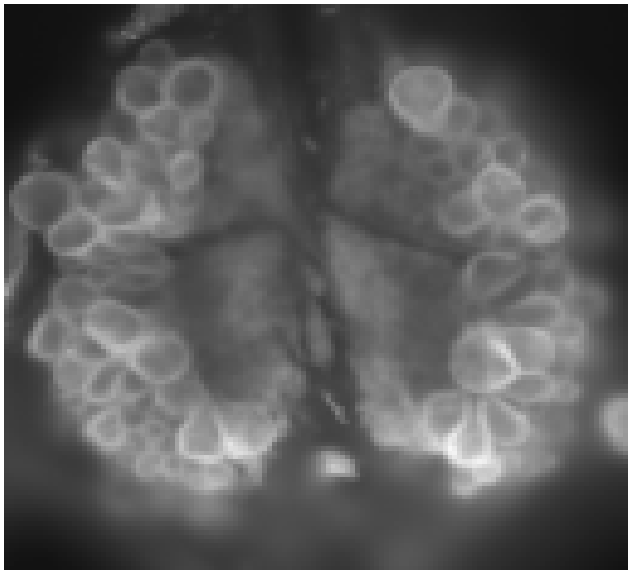
Background to the research

Neurobiology of the leech

Leeches are annelids. The leech has a distributed nervous system; the main parts of its nervous system are Head brain, tail brain and mid-body ganglia. There are 21 mid-body ganglia (Fig 1.1.). These ganglia are distributed along the nerve cord covered with blood sinus and each ganglion has almost 400 neurons inside. The neurons are located on two sides of the ganglion, ventral side and dorsal side. There are 4 packets of neurons on the dorsal side (Fig .1.2A) which contains mostly motoneurons and also interneurons. On ventral side (Fig 1.2B) there are 6 packets of neurons that contain interneurons, mechanosensory neurons and motoneurons.

Fig 1.1. Dorsal view of the nerve cord of the leech; the black arrow indicates one of the 21 mid-body ganglia located on the nerve cord of the Hirudo medicinalis leech

A



B

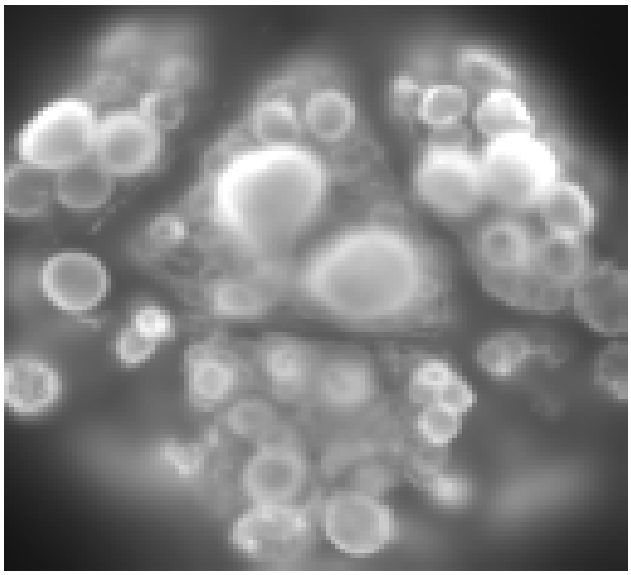


Fig. 1.2. Image of both sides of a desheathed ganglion (A) Image of the dorsal side of a desheathed ganglion. (B) Image of the ventral side of a desheathed ganglion.

Why the leech is a good preparation.

Each ganglion is similar to the others and has around 400 neurons which is a manageable number. Also many of these cells have been identified before. The preparation can be used for recording for 2-3 hours or for weeks in culture.

Background of optically imaging neural activity

The first to apply optical imaging to study the activity of the population of neurons in a ganglion were Cohen, Salzberg, and their colleagues (Salzberg et al. 1977, Zecevic et al. 1989). They were able to record from neurons during central pattern generator (CPGs) activation.

Cacciatore and colleagues (Cacciatore et al. 1999) also studied population of neurons during CPGs activation. They identified neurons active in swimming and their phase of activity in swimming period.

In the study done by Briggman and Kristan (Briggman and Kristan 2006) the imaging and the dye were both greatly improved. They optically recorded from the whole ganglion; the resolution of the imaging was increased in comparison to earlier results (Cacciatore et al. 1999). They could discriminate CPGs involved in two distinct behaviors.

Newly developed voltage sensitive dye VF2.1.Cl

VF2.1.Cl is the new VSD that I used in my study. It is a fluorescent voltage detector. This dye detects changes by modulation of photo-induced electron

transfer (PeT) from an electron donor through a synthetic molecular wire to a fluorophore (Miller et al. 2012).

This dye has bigger and faster response and less capacitance in comparison to previous dyes. The time response in the range of <<140μs (Miller et al. 2012). The action potential detected in rat hippocampal neurons and action potential were detected in leech Retzius neurons clearly with this VSD.

In next three chapters I present results of my study in which I developed the optically observing method of leech neurons electrical activity.

Chapter 2

Materials and Methods

Materials and Methods

Experiments were made with isolated single ganglia of the leech *Hyrudo medicinalis* (Ricarimpex Eysines, France). Leeches were maintained at 5ºC in dechlorinated water containing Instant Ocean salt (0.5g/liter; Aquarium Systems). Before an experiment, the leech was kept at room temperature for 24 hours.

Dissection

Animals were anesthetized by chilling with ice for 15-20 minutes. They were then immersed in 150-200 ml chilled normal ringer solution (in mM: 115 NaCl, 1.8 $CaCl_2$, 4 KCl, enriched with 10 glucose and buffered with 10 Tris-maleate pH 7.4 with NaOH), and were pinned to a board with fine needles. Ganglia from segments 8-16 were dissected and pinned in Petri dishes coated with a silicone elastomer (Sylgard 184, World Precision Instruments).The ganglion was desheathed and stained with VSDs. Neurons in these stained ganglia were visible and could be identified as usual.

Dye loading and imaging with VSD

The voltage sensitive dye VF2.1.Cl was diluted with 5 µl of 20 % solution of Pluronic F127 in DMSO and 1ml saline solution to a final concentration of 400-500 nM as mentioned in (Miller et al. 2012, SI appendix).The solution containing the dye was flowed past the desheathed ganglion by a peristaltic pump for 20-30 minutes. After dye loading, the ganglion was rinsed with saline and transferred to a dish. The ganglion rested on a 35 mm diameter coverslip #0(from Thermo Scientific) sealed onto the bottom of the petri dish. Before imaging, the silicone layer on which the ganglion was pinned was turned upside-down inside the glass bottom dish containing saline solution so that the ganglion faced the objective of the inverted microscope. To press the ganglion surface against the glass surface weights were placed on the inverted silicon layer. Fig. 2.1. shows this process.

The inverted microscope was used because of the curved shape of the surface of the ganglion. In addition electrphysiological recording is simpler on an inverted microscope while imaging from the other side face to the glass.

I used a 20x objective with 0.75 numeral aperture designed for excitation around 488 nm and emission around 510 nm which is also the excitation and emission wavelength of the VSD used in this study. CFI Super Fluor Series objective lenses have improved signal-to-noise ratio.

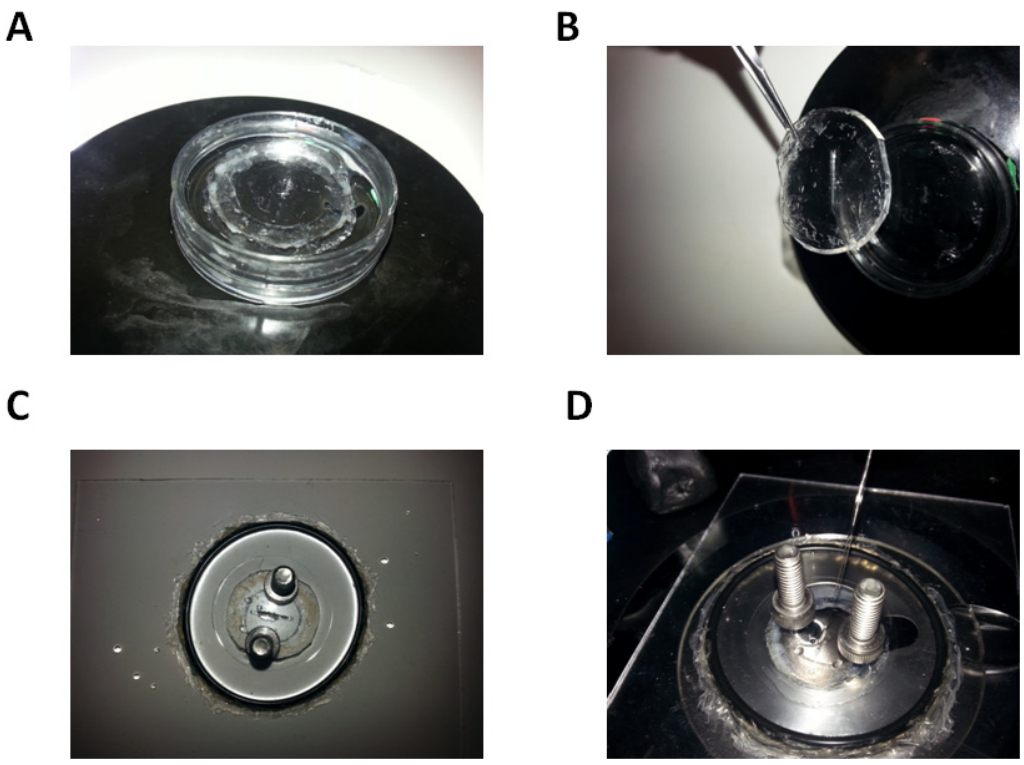


Fig. 2.1. Dye loading and transferring ganglion for imaging (A) Ganglion pinned in the middle of the dish that contains dye solution. There is a sylgard ring around the ganglion to reduce the volume of the dish (B) After dye loading and rinsing with the normal solution the sylgard on which ganglion is pinned cut out and moved to the dish shown on C (C) In the dish used for recording, the sylgard layer is upside down therefore the ganglion touches bottom of the dish covered with the coverslip (D) There is a slot on the sylgard through which the microelectrode can pass for intracellular recording.

Imaging was obtained by using an Electron Multiplier CCD Camera C9100-13 from Hamamatsu Photonics equipped with the Wasabi software. The camera is coupled with a 0.7x lens to increase the field of view of the camera and to image the entire ganglion surface. Images were acquired at a sampling rate of 94 to 170 frame/s and at a spatial resolution of 128 × 128

pixels. Theoretically higher framing rate would provide a better sampling rate to detect spikes according to Nyquist theory. However the light capture would be worse, the optimum framing rate I found was 94 frame/s for detecting spikes specially in small neurons. A Nikon Eclipse Ti inverted Microscope with the CFI Super Fluor 20x/0.75 objective with 1 mm working distance was used for imaging and the sample was illuminated with a 488 nm light, emitted by a HBO 103 W/2 mercury short arc lamp from Osram. The light intensity could be attenuated by the Nikon neutral density filters, ND4 and ND8. Emission was collected with a 505 nm long pass filter passing through a 490 nm dichroic\beamsplitter.

Optical recordings lasting 30 or 60 s were interspersed by 1 or 2 minutes of darkness, during which the ganglion and the dye could adjust to photobleaching. Reliable optical recordings could be obtained for approximately 10 minutes.

There was no significant difference in the firing rate of Retzius cells, Annulus erector (AE) and Anterior Pagoda (AP) neurons, in control experiments (16 experiments) and in the presence of the VSD (22 experiments) , suggesting limited toxicity of the dye during our optical recordings.

Electrical recordings

Suction electrodes were made from borosilicate glass capillaries (World precision instruments, Germany) pulled by a conventional puller (P-97, Sutter Instruments Co., USA). The electrode tip was cut using a diamond sharpened tip mounted on an appropriate manipulator under a stereoscopic microscope (Olympus SZ40, Europe). Electrodes with a final internal diameter between 80 and 150 μm, were polished using an incandescent filament under a 20x objective mounted on an upright microscope (Zeiss, Germany). Suction electrodes filled with normal ringer leech solution were connected to an extracellular recording amplifier (Pinato et al. 2000; Pinato and Torre 2000). Extracellular signals were digitized at 10 kHz by an A/D converter (model digidata-1322, 16 bit converter; Axon, molecular Devices, U.S.) and data were transferred and stored on a PC computer.

Neurons were impaled with sharp intracellular microelectrodes (30 MΩ filled with 4 M potassium acetate because synaptic potentials were to observed and to prevent the reversal of inhibitory postsynaptic potentials (IPSPs) that resulted from diffusion of Cl- into the cell), as described previously (Nicholls and Baylor 1968). Currents of 1-2.5 nA were injected to stimulate neurons. A stereoscopic microscope (Olympus SZ40, Europe) on aforementioned inverted Nikon microscope that I used for imaging to have electrophysiological recording simultaneously. There was a small slot on the silicon layer which ganglion was pinned on it to insert electrodes through it and impaling the cell. Intracellular signals were digitized at 10 kHz by an A/D converter (model digidata-1322, 16 bit converter; Axon,

molecular Devices, U.S.) and data were transferred and stored on a PC computer. Signals were recorded and visualized using respectively Clampex v.8.1 and Clampfit v.9.2 software (Molecular Devices, USA). Because I am using and inverted microscope for imaging and the ganglion has two dorsal and ventral sides therefore I was able to impale neuron at the margin of the side I was imaging or impaling the contradictory side of the ganglion that I was imaging from and see the response of stimulation on the other side.

Processing and analysis of optical recordings

The initial processing of video images was performed using the Wasabi software, provided by Hamamatsu. Image sequences were also processed and analyzed by software developed in the lab. For analysis I used a program written in MATLAB version 7.11. Neurons were imaged and an appropriate region of interest (ROI) around the neuron cell body was selected. The time course of the fluorescence intensity $I_f(t)$ in this ROI was displayed on the computer screen and the extent of its decay - consequence of dye bleaching – was evaluated. To compensate photo bleaching, the decay of $I_f(t)$ was fitted with an exponential function or with polynomial or cubic splines. The compensation based on the exponential function fitted the decay of fluorescence intensity $I_f(t)$ with the single exponential function :

$$Y(t)=ae^{-bt} \quad (1)$$

When the decay of $I_f(t)$ was not well fitted by the single exponential function I used either a third order polynomial:

$$Y(t)=at^3+bt^2+ct+d \quad (2)$$

or cubic splines interpolating $I_f(t)$ at 10 or 20 points. The function Y(t) fitting $I_f(t)$ was then subtracted to the original optical signal – to compensate for dye bleaching - and the fractional optical signal was taken as $(Y(t)-I_f(t))/I_f(0)$, where $I_f(0)$ is the fluorescence intensity at the beginning of the recording.

Computation of raster plots

Spikes in the optical traces were detected in two ways. First, when the spikes were clearly evident, as those from Retzius cells and mechanosensory neurons , a threshold Th was set and the spikes were identified to occur at the times t_i corresponding to the crossings of Th. These times were stored as a file. In optical recordings from neurons known to produce spikes with amplitude of less than 10 mV (APs, AEs, motoneurons and interneurons) the standard deviation σ_o of the optical trace was estimated and upward deflections of the trace larger than $5\sigma_o$ were identified as spikes. Upward deflections lasting longer than 10 msec, i.e. for two successive frames, were not considered to be spikes. Detected spikes were also visually controlled by

the experimenter. These times t_i were represented in conventional raster plots.

Comparison of optical and electrical recordings

All of the neurons identified based on the approximate localization according to the atlas in (Muller et al. 1981). Neurons were impaled with a fine intracellular microelectrode, while simultaneously acquiring optical signals. As shown in (Fig 2.2A), the optical trace (red line) superimposed well onto the electrical recording (blue line) obtained with an intracellular electrode. When an N mechanosensory neuron was impaled, it generated spikes with an amplitude up to 100 mV that were clearly seen in the optical signal. The signal to noise ratio (S/N) increases with the number of pixels composing the ROI corresponding to the cell body of the neuron: for large Retzius cells the ROI was composed of 100-200 pixels and for the smaller neurons, such as an N cell, the ROI was composed of 80-120 pixels. The amplitude of spikes in Retzius and N cell are 30 mV and 80 mV respectively and are detected optically with a signal to noise of 5 and 6 respectively and the fractional change of measured fluorescence was approximately 1% per 100 mV. By using an acquisition rate of 100 frames per second I detected spikes from Anterior Pagoda (APs), Annulus Erector (AE) neurons, which have an amplitude of only 4-6 mV (Fig. 2.2B). Under these conditions, I could reliably record optical signals also caused by slow changes of the membrane potential and when the heart motoneuron HE was

imaged (Fig. 2.2C) I optically recorded slow waves that had a period of about 5 sec, during which small spikes could be seen riding on top of positive deflections. These optical traces had time courses very similar to those obtained with intracellular recordings (Kristan et al 2005) where the membrane voltage undergoes cyclic oscillations with an amplitude of about 10 mV. These results indicate that the dye VF2.1.Cl is able to detect changes of the membrane potential with an amplitude of 5 mV or less with a S/N larger than 2-3.

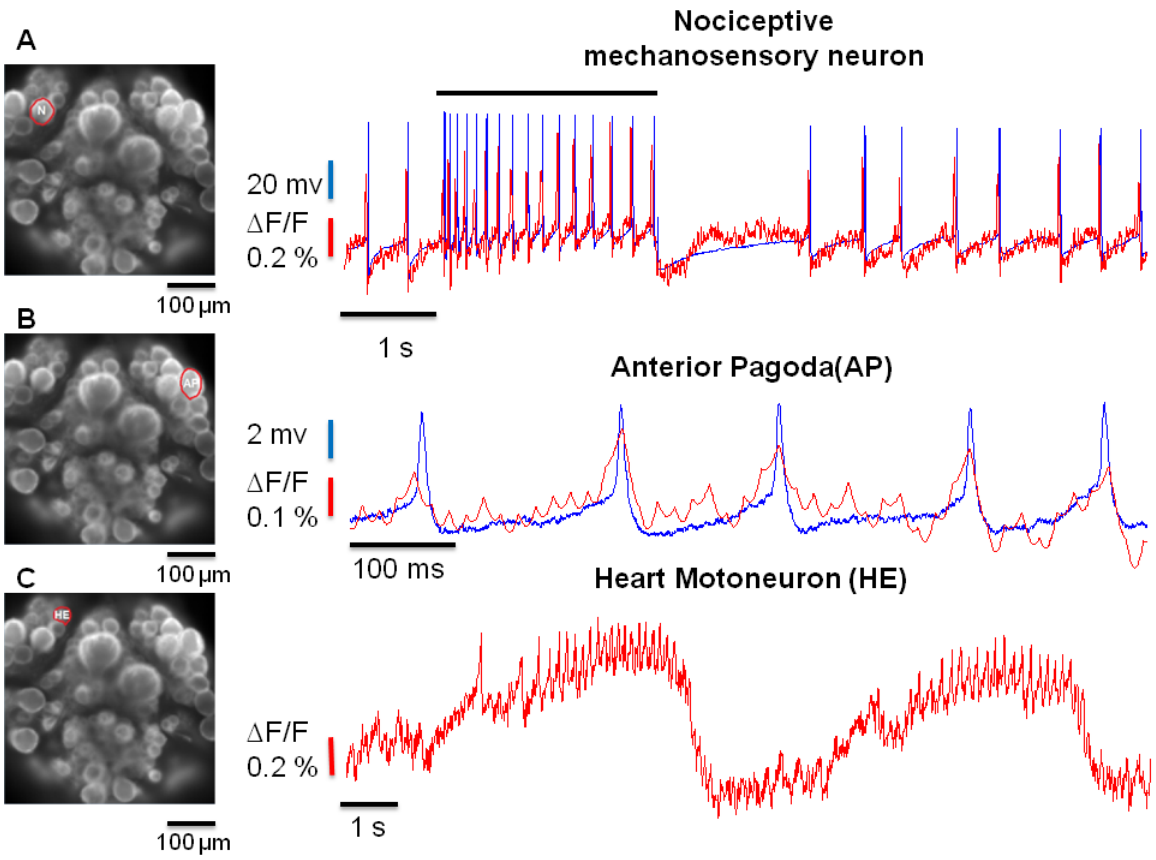


Figure 2.2. Comparison of optical (red) and electrical (blue) recordings and optical recordings from the heart motoneuron A: Simultaneous recordings from the mechanosensory neuron encircled in red in the panel on the left. Images acquired at 100 Hz. Optical and electrical recordings have the same time course, but the peak of the spike of the mechanosensory neuron has variable amplitude because of the limited sampling rate of image acquisition. Optical recordings follow very precisely the undershoot of about 10 mV following the spike. B: as in A, but during an electrical recording from the Anterior Pagoda neuron encircled in the panel on the left. The spike amplitude of Anterior Pagoda neurons is approximately 4 mV and is clearly recorded also during the optical recording. C: Optical recording from the Heart Motoneuron encircled in the panel on the left. The optical recording exhibits an oscillatory behaviour with a period of 5-10 sec typical of these neurons (Kristan et al.2005) during which the membrane potential changes by about 10 mV. There is no simultaneous intracellular recording because it is a small neuron and it is not easy to find it from the opposite side of the ganglion.

Computation of cross-correlation of occurrence of spikes

The cross-correlation $\sigma_{AP_{ij}}$ of the firing of neuron i and neuron j was computed in the following way. The total recording time T_{tot} was divided into N intervals (1,..,n,…,N) of a duration Δt and if f_{in} and f_{jn} are the number of spikes fired by neuron i and neuron j in the time interval Δt_n, then

$$\sigma_{AP_{ij}} = \frac{\Sigma_n f_{in} f_{jn}}{\sqrt{(\Sigma_n {f_{in}}^2)(\Sigma_n {f_{jn}}^2)}} \quad (3)$$

so that $\sigma_{AP_{ij}}$ depends on Δt and varies between 0 and 1. The range of explored values of Δt ranged from 10 to 1000 msec.

Computation of cross-correlation of slow signals

Most neurons in the ganglion do not have an excitable cell body, Spikes are produced in the axons therefore it is useful to investigate also the cross-correlation among slow or graded signals, keeping in mind that the cross-correlation among slow/graded signals can be negative, while $\sigma_{AP_{ij}}$ varies only between 0 and 1. The cross-correlation $\sigma_{slow_{ij}}$ of slow signals obtained from neuron i and neuron j was computed in the following way. If s_{in} is the slow signal from neuron i at time t_n its mean value $<s_i>$ is computed as $\frac{\Sigma_n s_{in}}{N}$ where N is the total number of available samples.

$$\sigma_{slow_{ij}} = \frac{\Sigma_n (s_{in} - <s_i>)(s_{jn} - <s_j>)}{\sqrt{(\Sigma_n (s_{in} - <s_i>)^2)(\Sigma_n (s_{jn} - <s_j>)^2)}} \quad (4)$$

so that $\sigma_{slow_{ij}}$ varies between -1 and 1 and the typical interval on which

$\sigma_{slow_{ij}}$ was computed varied from 30 to 180 s. s_{in} was obtained by smoothing, using a time window of 100 msec, to remove spikes from the optical signal.

Chapter 3

Spontaneous electrical activity

The new VSD VF2.1.Cl allows the detection of spikes and also of slow changes of the membrane voltage, which cannot be resolved by calcium imaging (Grienberger & Konnerth (2012) or by multielectrode recordings of extracellular voltage signals (Takehara-Nishiuchi et al 2008; Solzbacher et al. 2009; Luczak et al 2013). The VSD VF2.1.Cl allows satisfactory optical recordings for up to 10-15 minutes. In order to obtain satisfactory optical recordings, it is necessary to desheathe the ganglia. The VSD VF2.1Cl allows the simultaneous recordings of electrical signals in leech ganglia with an unprecedented resolution and accuracy.

In the present investigation we analysed the spontaneous electrical activity of the leech nervous system, i.e. in the absence of sensory stimulation and when the animal is not engaged in a specific behavior, such as swimming or crawling. The characterization of the spontaneous activity is equivalent to the analysis of the background noise and it is a necessary step for a proper understanding of information processing in this nervous system.

Optical recordings from the ventral surface

We imaged the ventral surface of the ganglion, focusing on as many neurons as possible (Fig. 3.1A). The two largest neurons in the middle of the ganglion are the Retzius cells, with smaller mechanosensory neurons on either side (Muller et al., 1981). Optical recordings with the voltage-sensitive VF2.1.Cl obtained from the ventral surface of a leech ganglion

(Fig. 3.1B) detected well resolved spikes from the soma of Retzius cells, mechanosensory neurons, AP, AE and Leydig neurons, and also from other interneurons tentatively identified as cells 153, 158, 216, 222 and 261. A typical raster plot of detected spikes is shown in Fig. 3.1C. In all preparations Retzius cells, AP neurons, and AE neurons fired almost continuously at a frequency varying from 0.5 to 7 Hz, as did two other neurons, identified as cells 153 and 158 based upon their location in the ganglion and size (Muller et al. 1981). While neurons 153 and 158 fired almost continuously, irregular spikes were observed in other neurons, tentatively identified as neurons 216, 222 and 261 (Muller et al 1981).

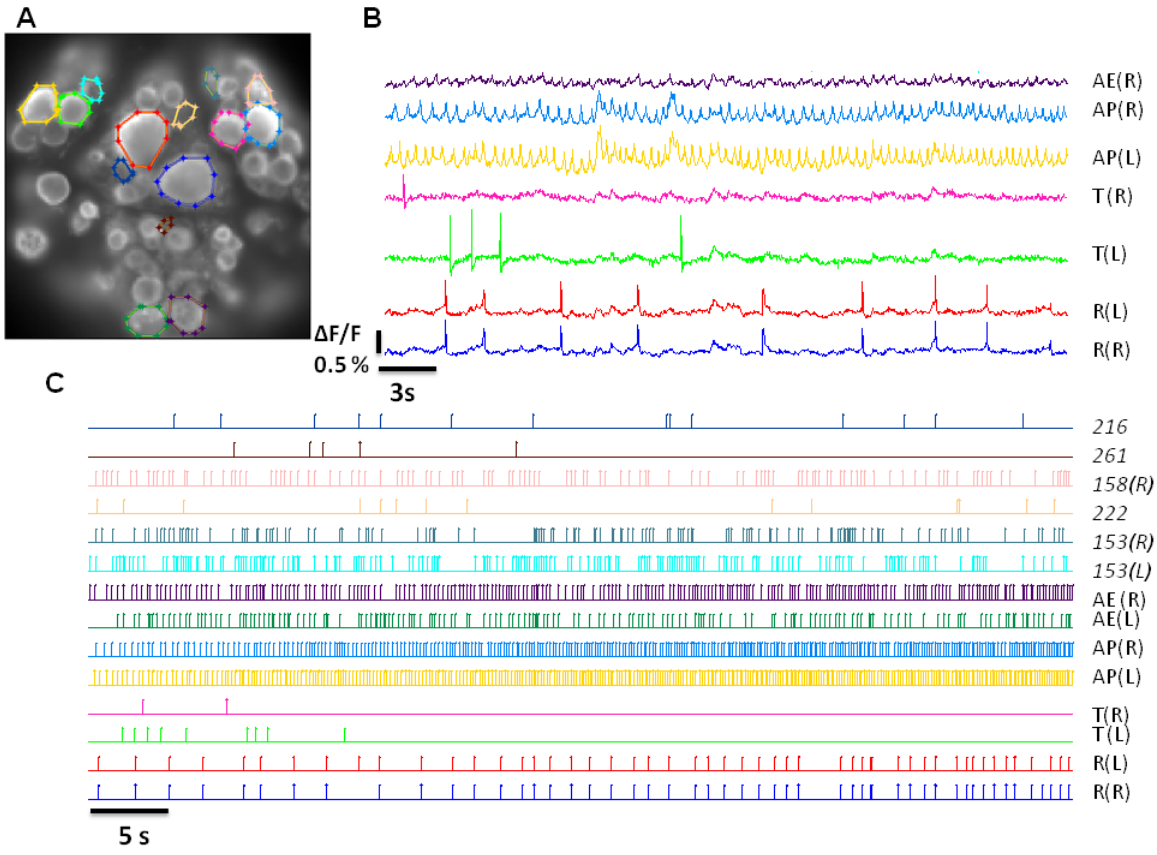


Figure 3.1. Optical recordings of the spontaneous electrical activity from the ventral surface of a leech ganglion. A: An image of a ventral surface of the

ganglion from which the optical recordings shown in B were obtained. The color of each line encircling a neuron in A corresponds to the optical recordings in B. B: ΔF/F recordings obtained during spontaneous activity of the ganglion. C: Raster plots of detected spikes from the obtained ΔF/F recordings. Neurons were identified by their location in the ganglion according to the classical atlas of leech neurons (Muller et al. 1981). The shape of recorded spikes provides and additional check for the identification of neurons, such as Retzius, APs, AEs and mechanosensory neurons. The identification of neurons indicated in *italics* is tentative. The letters R and L in parenthesis indicate whether the neuron was located on the right or left side of the ganglion.

The spontaneous activity of neurons imaged on the ventral surface of the leech ganglion had a rather stereotyped pattern: several neurons fired spikes almost continuously, such as the 2 Retzius cells, the 2 APs, the 2 AEs, interneurons 153 and 158. The remaining neurons either fired sparse, low-amplitude spikes or were non-spiking neurons. We never observed bursts of spikes in any neurons imaged on the ventral surface of a leech ganglion, with the exception of mechanosensory T cells. This pattern was consistently observed in 22 isolated leech ganglia stained with the VSD VF2.1.Cl.

Firing and correlation pattern of electrical signals in the ventral surface

We analyzed the firing pattern of neurons imaged on the ventral surface of the ganglion. We performed 30 experiments in which spikes could be clearly seen; in 7 experiments, we detected spikes in more than 12 neurons and computed the distribution of the firing frequency of these neurons (Fig. 3.2A). In each one of these 7 experiments we identified: a group of neurons with firing frequencies below 0.5 Hz such as neurons 216,222 and 261, plus other neurons with firing frequencies in the range of 1-5 Hz, such as Retzius, and neurons AP, AE, 50, and 153. We rarely observed a neuron firing below 0.5 Hz for some minutes and subsequently increasing its firing frequency above 2-3 Hz. Neurons visible in the ventral surface of the ganglion either fired almost rhythmically or fired hardly at all. The regularity of these two classes of spontaneous firing was measured as two peaks in the histogram of firing rates (Fig. 3.2A). Occasionally, mechanosensory neurons, primarily T cells, were seen firing bursts of spikes.

We analysed the degree of correlated firing of neurons present on the ventral surface of the ganglion, by computing the cross-correlations, $\sigma_{AP_{ij}}$, for all neuron pairs, using two different windows, 100 and 1000 msec (Fig. 3.2B). At the 100 msec window, only the firing of the two Retzius cells showed a large value close to 0.8, whereas all other pairs had cross-correlation values lower than 0.3. When the window was increased to 1000 msec, $\sigma_{AP_{ij}}$ became larger than 0.6 for several pairs of neurons, including the 2 AEs the 2 APs,

and the two 153 neurons.

We obtained optical recordings of the firing of the two Retzius cells, the two APs, the two AEs and the two 153 neurons in 6 ganglia and, in all these experiments, $\sigma_{AP_{ij}}$ increased significantly (1-way ANOVA $p<0.05$, followed by Tukey's post-hoc test) when the window increased from 100 to 1000 msec and entries of $\sigma_{AP_{ij}}$ for these pairs of cells were higher than 0.6. Neurons with a positively correlated firing at a window of 1000 msec also fired at a high frequency. To determine whether the observed positive value of $\sigma_{AP_{ij}}$ for the spikes was correlated with membrane potential changes, we analysed the degree of correlation of their slow changes of membrane potential. We filtered the spikes from the optical recordings by smoothing signals in a window of 50 msec (red traces of Fig. 3.2C), then we computed the cross-correlation matrix between all pairs of the smoothed optical signals. Although the pair of Retzius cells had a cross-correlation value close to 0.8, in agreement with their strong electrical coupling (Hagiwara and Morita, 1962) the pair of AP cells had a cross-correlation value close to 0 (Fig. 3.2D), indicating that their membrane potential oscillated in random phases. Also the pairs of AE and neurons 153 did not show any clear sign of correlation or anti-correlation among the graded signals recorded from their cell body.

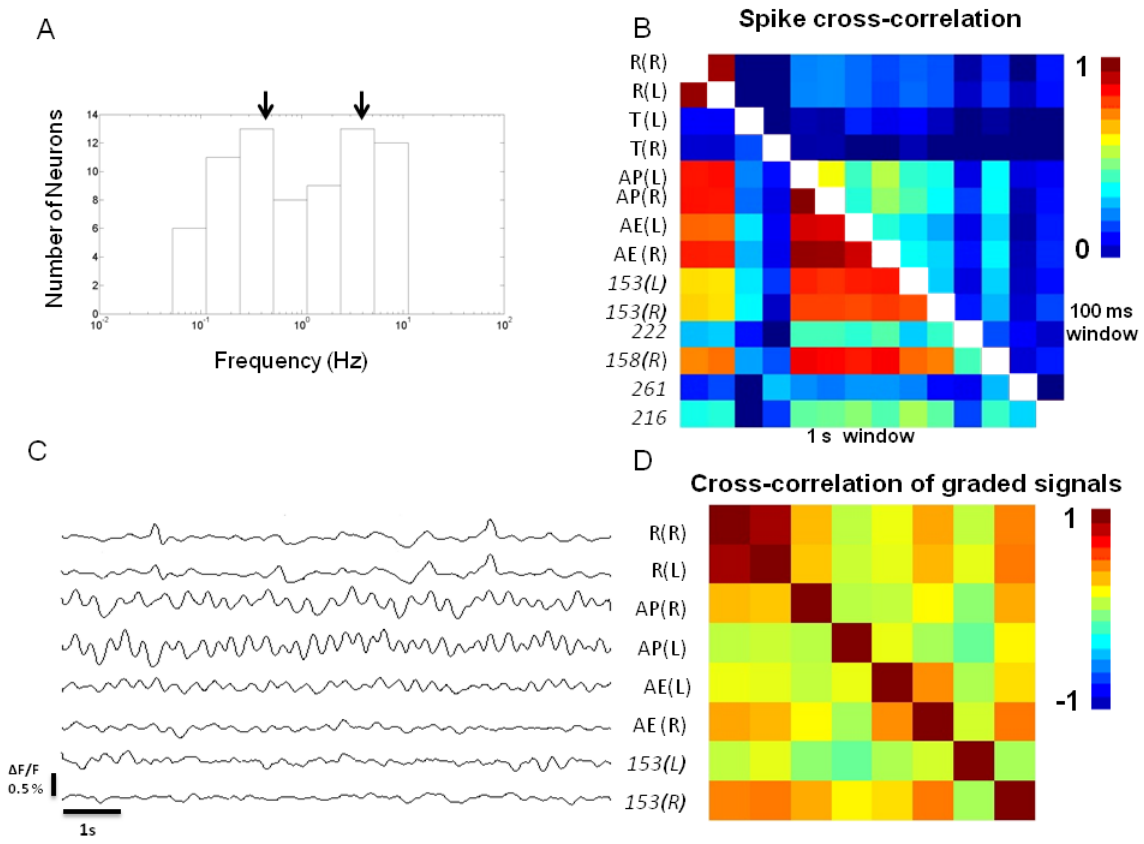


Figure 3.2. Correlation structure of electrical activity of neurons visible in the ventral surface of the ganglion. A: Distribution of firing frequencies of neurons visible in the ventral surface. The two peaks in the histogram indicate the existence of a population of neurons with an irregular firing rate below 0.3 Hz and of several neurons firing at a frequency above 1 Hz. These neurons were: Retzius, Ap, Ae, 251, T, 153, Leydig cell. Data collected from 12 experiments, each of them providing optical recordings for at least 30 seconds for a cumulative recording time of 8 minutes. The windows of the histogram varied logarithmically. B: The cross-correlation $\sigma_{AP_{ij}}$ of the firing of neuron i and neuron j computed at a 100 and 1000 msec window. Values of $\sigma_{AP_{ij}}$ vary between 0 and 1 according to the color coded scale shown at the right of the panel. Within the window of 100 msec only the two Retzius cells have a significant positive

value of $\sigma_{AP_{ij}}$. C: Smoothed optical recordings from the pairs of Retzius, Anterior Pagoda neurons, Annulus Erector neurons and neurons 153. Smoothing was obtained by averaging optical traces on a time window of 100 msec. Smoothing was performed to eliminate spikes and to isolate the graded and slow component of the signal. D: The cross-correlation $\sigma_{slow_{ij}}$ of slow signals obtained from the smoothed optical recordings in C. Values of $\sigma_{slow_{ij}}$ varies between -1 and 1 according to the color-coded scale shown at the right of the panel. Only the two Retzius cells have a positive value of $\sigma_{slow_{ij}}$. The almost oscillatory signals from the pairs of Anterior Pagoda neurons, Annulus Erector neurons and neurons 153 are not in-phase.

A large fraction of leech neurons were either non-spiking neurons or had spikes in the axons of the neurons and their amplitude in the soma did not exceed 2-5 mV (Ort et al 1974; Kristan& Calabrese 1976; Brodfuehrer & Friesen (1986a &1986b); Brodfuehrer et al 1995; Kristan etal. 2005). Because VF2.1.Cl can reliably detect voltage changes of 4 mV, we decided to analyze signals originating from all visible cell bodies of neurons in the ventral surface (Fig. 3.1A). Changes of optical signals could not only be caused by changes of membrane potentials but also by two kinds of artifacts: firstly sudden fluctuations in the light emitted by the illuminating lamp and secondly by small movements of the preparation. The first kind of artifact is present in the optical recordings from all imaged neurons and therefore it can be easily identified. The second kind of artifact produces optical signals obtained from two halves of the cell body to be in antiphase. Therefore, we

considered slow changes of optical signals as reporting slow changes of voltage those that were restricted to specific neurons – not caused by lamp glitches – and those that had the same time course when obtained from non overlapping regions of the cell body. With these criteria, we obtained 105 optical recordings from the ventral surfaces of ganglia in 11 experiments. The value of $\sigma_{slow_{ij}}$ for pairs of neurons visible in the ventral surface varied between -0.3 and 0.3 and only for the two Retzius cells $\sigma_{slow_{ij}}$ was higher than 0.7 and typically close to 0.85 (see Fig. 3.2D). The value of $\sigma_{slow_{ij}}$ for the pair of Retzius cells was significantly higher than the value for all the other neuron pairs (1-way ANOVA $p<0.05$, followed by Tukey's post-hoc test).

The results of Figs. 3.1 and 3.2 show that the great majority of neurons imaged on the ventral side of the leech are silent at rest and do not have any significant spontaneous activity. Some other neurons fire spikes vary either rarely or almost periodically but do not have bursts of spikes, as often observed during extracellular recordings from the nerve.

Optical recordings from the dorsal surface

We imaged also the dorsal surface of the ganglion, where the cell body of most leech motoneurons are visible (Fig. 3.3A).

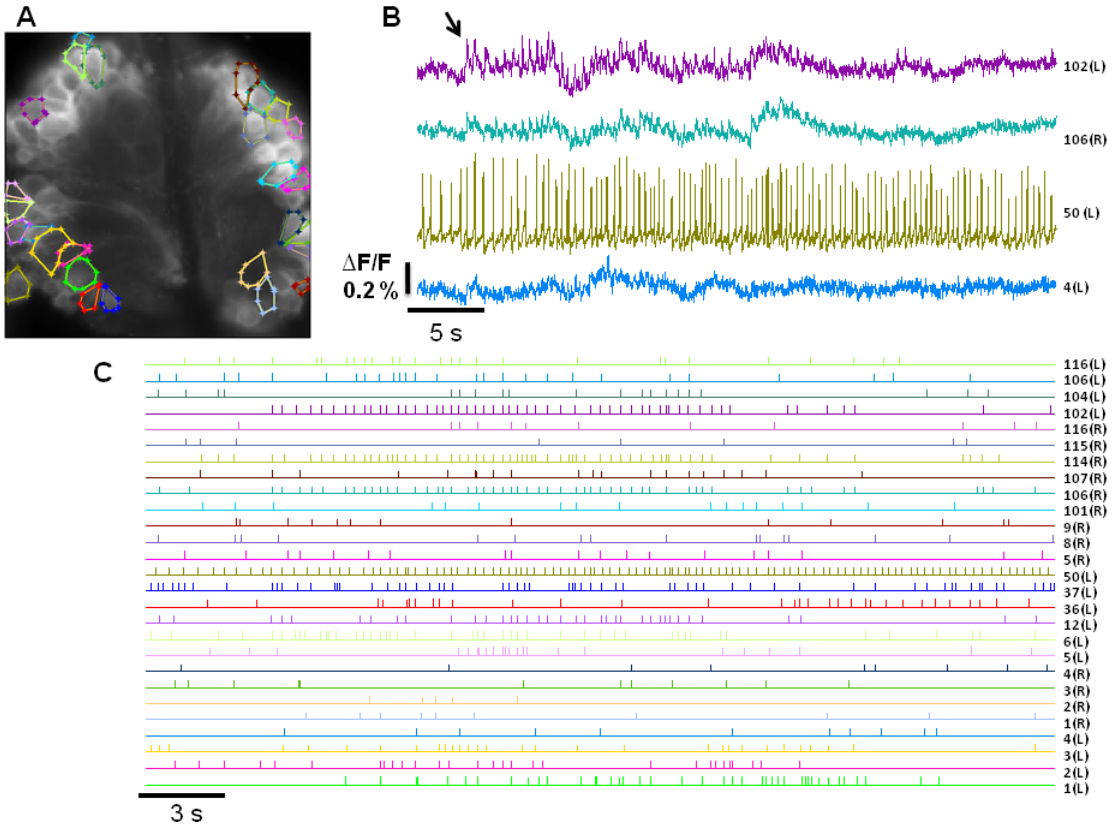


Figure 3.3. Optical recordings (ΔF/F) of the spontaneous electrical activity from the dorsal surface of a leech ganglion. A: An image of a dorsal surface of a leech ganglion from which the optical recordings shown in B were obtained. The colour of each line encircling a neuron in A corresponds to the optical recordings in B. B: ΔF/F recordings obtained during the spontaneous activity of the ganglion. Optical recordings were processed as described in the Methods section. C: Raster plots of detected spikes from the obtained ΔF/F recordings. Neurons were identified according to their location in the ganglion following the atlas of leech neurons (Muller et al 1981). The letters R and L in parenthesis indicate whether the neuron was located on the right or left side of the ganglion. Neurons were identified on the basis of their location following the leech neuron atlas (Muller et al 1981). As neurons in desheathed ganglia can be displaced from their original location, the identification of some neurons could be erroneous.

Although spikes recorded from the soma of these motoneurons do not exceed 5-7 mV (Ort et al 1974; Kristan & Calabrese 1976; Brodfuehrer & Friesen 1986a,b); Brodfuehrer etal. 1995; Kristan et al. 2005), we could clearly detect spikes from our optical recordings (Fig. 3.3B). In contrast with what we observed on the ventral surface of the ganglion, motoneurons fired spikes in bursts, and a slow upward optical signal was often seen to initiate a burst of spikes (see arrow in Fig. 3.3B). For instance, motoneurons 102, 114 and 116, after a quiet period lasting some tens of seconds, started to fire spikes, following an upward deflection of optical traces, caused by a depolarization of these motoneurons. While the burst of spikes in motoneurons lasted for 10-20 seconds, neuron 50 fired spikes regularly at a rate of 2-4 Hz. From these optical recordings the raster plot of spikes (Fig. 3.3C), the concomitant firing of 27 motoneurons was observed. In other experiments, motoneurons 1, 2, 3, 4, 5 and 7 produced bursts of spikes. Only occasionally we observed simultaneous burst in several motoneurons, suggesting that bursts of motoneuron firing were segregated. Raster plots from the ventral (Fig. 3.1C) and dorsal surfaces (Fig. 3.3C) of a ganglion indicate some basic properties of the spontaneous firing of neurons in the ventral and dorsal surface: firstly, motoneurons – visible in the dorsal surface – do not fire spontaneously spikes at the same high rate as the Retzius cells and the AP neurons and AE motoneurons – visible on the ventral surface; secondly, diffuse and frequent bursts of spikes characterize the electrical activity of motoneurons visible on the dorsal surface. Therefore, the dynamics of the spontaneous electrical activity appears to be segregated with distinct properties in the ventral and dorsal surfaces of the

ganglion.

Correlation pattern of slow signals from motoneurons

We analyzed the degree of correlated firing among pairs of neurons visible in the dorsal surface of leech ganglia (Fig. 3.4A). The value of $\sigma_{AP_{ij}}$ computed within the window of 100 msec was consistently low and rarely exceeded 0.5. Also within the larger window of 1000 msec, the firing was very poorly correlated and we did not find any pair of neurons showing an appreciable degree of correlated or synchronous firing. The degree of simultaneous firing was significantly lower than what observed among pairs of neurons visible on the ventral surface.

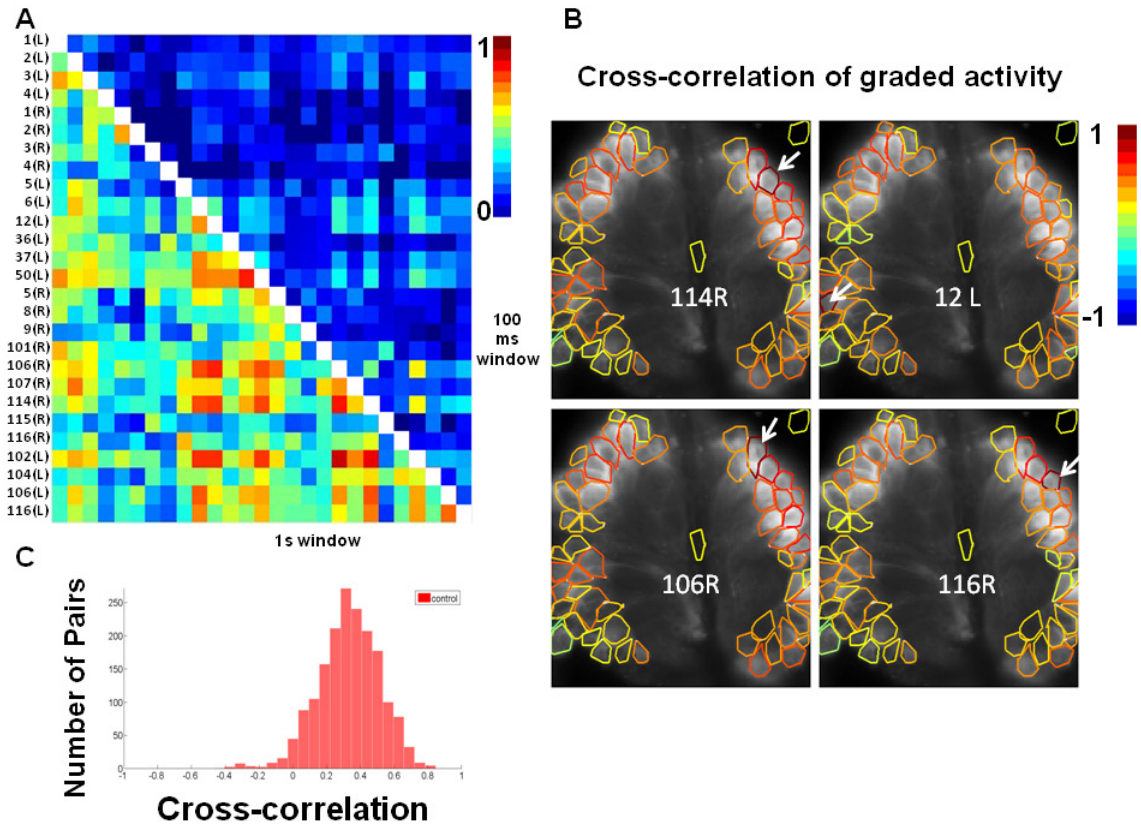


Figure 3.4. Correlation structure of electrical activity of neurons visible in the dorsal surface of the ganglion. A: The cross-correlation $\sigma_{AP_{ij}}$ of the firing of neuron i and neuron j computed at a 100 and 1000 msec window size. Entries of $\sigma_{AP_{ij}}$ vary between 0 and 1 according to the color-coded scale shown on the right of the panel, with a window of 100 msec $\sigma_{AP_{ij}}$ was rarely larger than 0.5 (2/351) and the window of 1 s was occasionally larger than 0.5 (97/351). B: Spatial profile of $\sigma_{slow_{ij}}$ among motoneurons visible in the dorsal surface. In each panel the motoneuron indicated at the centre was taken as the reference neuron i and $\sigma_{slow_{ij}}$ was computed for all the other visible neurons j. The profile of the reference neuron i is indicated in dark red, all the other neurons are circled with a colored line indicating the degree of cross-correlation of the graded activity measured by $\sigma_{slow_{ij}}$ using the color coded scale at the

right of the panel. C: distribution of values of $\sigma_{slow_{ij}}$ measured from neurons visible in the dorsal surface of the ganglion. Data from 3 experiments.

The analysis of the cross-correlation of slow optical signals shows a moderate degree of cross-correlation among neurons of the dorsal surface (Fig. 3.4C). Data from more than 1000 pairs of optical signals from the cell body of neurons on the dorsal surface of the ganglion indicate a mean value of the cross-correlation of $\sigma_{slow_{ij}}$ equal to 0.3 with only occasional negative entries.

Optical recordings of the spontaneous activity with fast transients identified as spikes from at least 12 different neurons were obtained in more than 8 experiments from the ventral surface and 9 experiments from the dorsal surface of the ganglion. In these experiments, we always detected spikes from Retzius, Annulus Erector and Anterior Pagoda neurons, as their amplitude is relatively large. Smaller spikes originating from neurons 216, 222, and 261 were detected in 5 experiments when neurons were imaged from the ventral surface. In all experiments (9) imaging neurons on the dorsal surface, the fraction of entries of $\sigma_{AP_{ij}}$ larger than 0.5 within the window of 100 and 1000 msec was smaller than 5% and 30% respectively.

Simultaneous electrical recordings from motoneurons and neurons in the ventral surface

The optical recordings shown in Fig. 3.2 and 3.3 show that Retzius cells, AP, and AE neurons fire spikes almost periodically, while motoneurons located on the dorsal surface do not fire periodically, but alternate bursts of elevated firing with quiet periods during which no spikes are detected. These observations suggest that the spontaneous dynamics of leech neurons are segregated and that there are groups of neurons firing almost repetitively and neurons in the dorsal surface firing in bursts. In order to establish this observation it is desirable to have simultaneous recordings from neurons of the ventral and dorsal surfaces of the ganglion. For technical reasons it is not easy to obtain simultaneous optical recordings from the ventral and dorsal surfaces of a leech ganglion, but it is possible to record simultaneously the electrical activity of motoneurons with their cell body in the dorsal surface of the ganglion – with suction pipettes - and of Retzius, AE and other neurons visible in the ventral surface with intracellular electrodes.

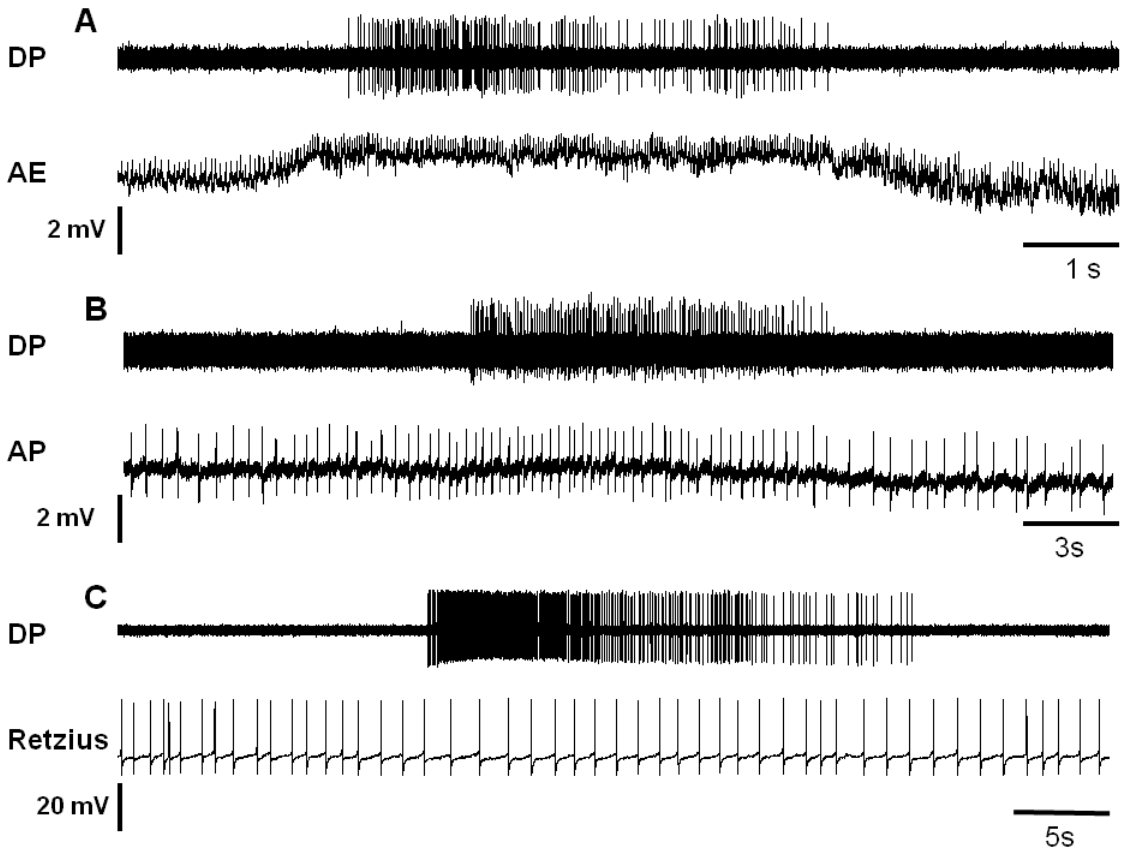


Figure 3.5. Simultaneous electrical recordings during bursts of spikes. A: Simultaneous intracellular recording from an Annulus Erector neuron (lower trace) and extracellular recording with a suction pipette from a DP nerve (upper trace). During the burst observed in the extracellular recording the AE neuron depolarized by 2-4 mV and increased its firing rate. B: Simultaneous intracellular recording from neuron AP (lower trace) and extracellular recording from a DP nerve (upper trace). During the burst of spikes observed in the extracellular recording the neuron AP increased its firing rate. C: Simultaneous intracellular recording from a Retzius cell (lower trace) and extracellular recording from a DP nerve (upper trace). During the burst of spikes observed in the extracellular recording the Retzius cell did not appreciably change either its firing rate or its membrane potential.

Fig. 3.5 illustrates simultaneous extracellular recordings from a DP nerve and intracellular recordings from neurons visible in the ventral surface of the ganglion. The AE neuron fires spikes at a high rate (around 10 Hz; Fig. 3.5A), similar to what is seen with optical recordings (Fig. 3.1). When a burst of spikes was recorded from the DP nerve, the membrane potential of the AE neuron depolarized by 2-4 mV and slightly increased its firing rate. Changes of the membrane potential of AE neurons usually preceded by 200-600 msec the onset of bursts recorded from DP nerves but not always: in some occasions (2/15 bursts) the burst in the DP nerve initiated approximately 300 msec before the occurrence of a clear change of the membrane potential in AE neurons. When the AP neuron was impaled and a burst of spikes was observed on the DP nerve, its membrane potential depolarized by 1-2 mV and its firing rate increased (Fig. 3.5B). We impaled several Retzius cells under similar conditions and when a burst of spikes was observed in the DP nerve (Fig. 3.5C), its spontaneous firing rate did not significantly change. We also impaled another spontaneously firing neuron, tentatively identified as neuron 152 and when a burst of APs was observed on the DP root, its membrane potential hyperpolarized by 2-4 mV and its firing rate substantially decreased. These electrical recordings confirm the observation that some leech neurons fire spikes almost periodically and other neurons fire spikes primarily in bursts.

Changes of the resting membrane potential of impaled neurons could precede (see Fig. 3.5A) or follow (see Fig. 3.5B) the initiation of APs bursts measured on the DP nerve. This variability was observed during continuous recordings from AEs, APs and other neurons and indicates that the initiation

of bursts is not a deterministic process but it is more likely a random event that could be described by self organized criticality (Mazzoni et al 2007).

As shown in Figs. 3.3- 3.5, in contrast to what observed in the ventral side, neurons imaged on the dorsal side have a significant spontaneous activity and often fire spikes in bursts, in agreement with extracellular recordings from cut ends of nerves.

Optical recordings in the presence of serotonin

We have also analyzed the spontaneous activity of leech neurons in the presence of 20 µM of serotonin while imaging both the ventral and dorsal surfaces of the ganglion.

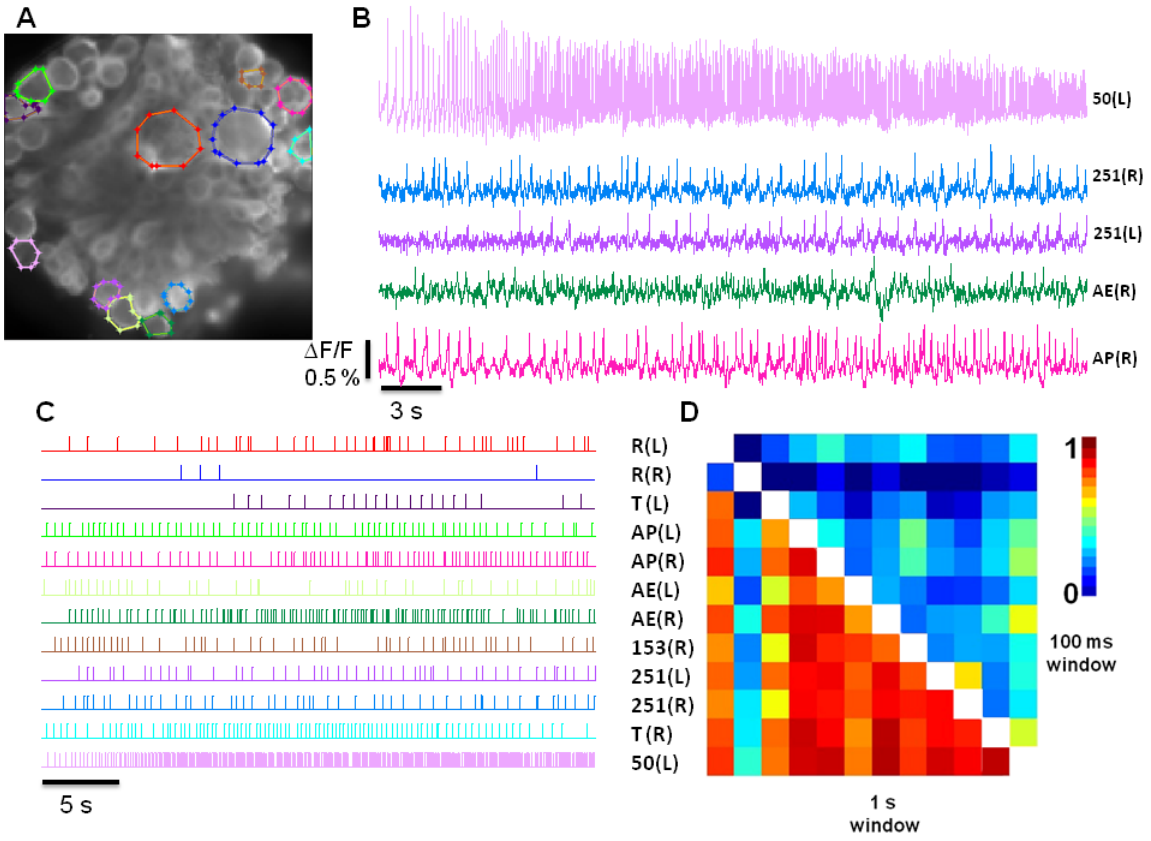


Figure 3.6: Optical recordings of the spontaneous electrical activity from the ventral surface of a leech ganglion in the presence of 20 µM serotonin. A: An image of a ventral surface of a leech ganglion from which the optical recordings shown in B were obtained. The colour of each line encircling a neuron in A corresponds to the optical recordings in B. B: ΔF/F recordings obtained during the spontaneous activity of the ganglion. C: Raster plots of spikes detected in the ΔF/F

recordings. D: The cross-correlation $\sigma_{AP_{ij}}$ of the firing of neuron i and neuron j computed at a 100 and 1000 msec window size. Entries of $\sigma_{AP_{ij}}$ vary between 0 and 1 according to the colour-coded scale shown on the right of the panel. In the presence of serotonin, Retzius cells hyperpolarize and stop firing, but the great majority of the other neurons fire more robustly and their firing appears to be more correlated at the larger window size of 1000 msec as indicated by the measured larger entries of $\sigma_{AP_{ij}}$. Some neurons, such as Retzius cells stop firing spikes in the presence of serotonin while other neurons, which were silent in control conditions, start firing in the presence of serotonin and therefore it is difficult to determine precisely how the matrix $\sigma_{AP_{ij}}$ changes from control conditions and when serotonin is added.

Two minutes after adding serotonin to the extracellular medium bathing the ganglion, we started optical recordings. When the ventral surface was imaged (Fig. 3.6B) optically recorded spikes from the Leydig neuron (or neuron 50) progressively decreased in amplitude and decreased their interspike interval, in agreement with what has already been observed when the same neuron is impaled with an intracellular electrode and serotonin is added to the extracellular medium (Dierkes et al. 2005). In contrast with their activity in control conditions, Retzius cells decreased their firing rate and eventually became silent, as already observed during intracellular recordings (Nusbaum et al. 1986; Dierkes et al. 2005).

within the window of 100 msec, entries of $\sigma_{AP_{ij}}$ were rarely large and in fact only less than 8% of them were larger than 0.5 (Fig. 3.6D). However, at the window of 1000 msec, most of the entries of $\sigma_{AP_{ij}}$ were larger than 0.5 with the exception of entries related to Retzius cells: indeed the pair of Retzius cells, in the presence of serotonin, reduce their firing rate and eventually become silent often losing their mutual electrical coupling and entries of $\sigma_{AP_{ij}}$ corresponding to these cells became small. In all 8 experiments in which more than 8 neurons were observed firing spikes in the presence of serotonin, entries of $\sigma_{AP_{ij}}$ for pairs of neurons visible in the ventral surface were larger than those in control conditions (1-way ANOVA $p<0.05$, followed by Tukey's post-hoc test).

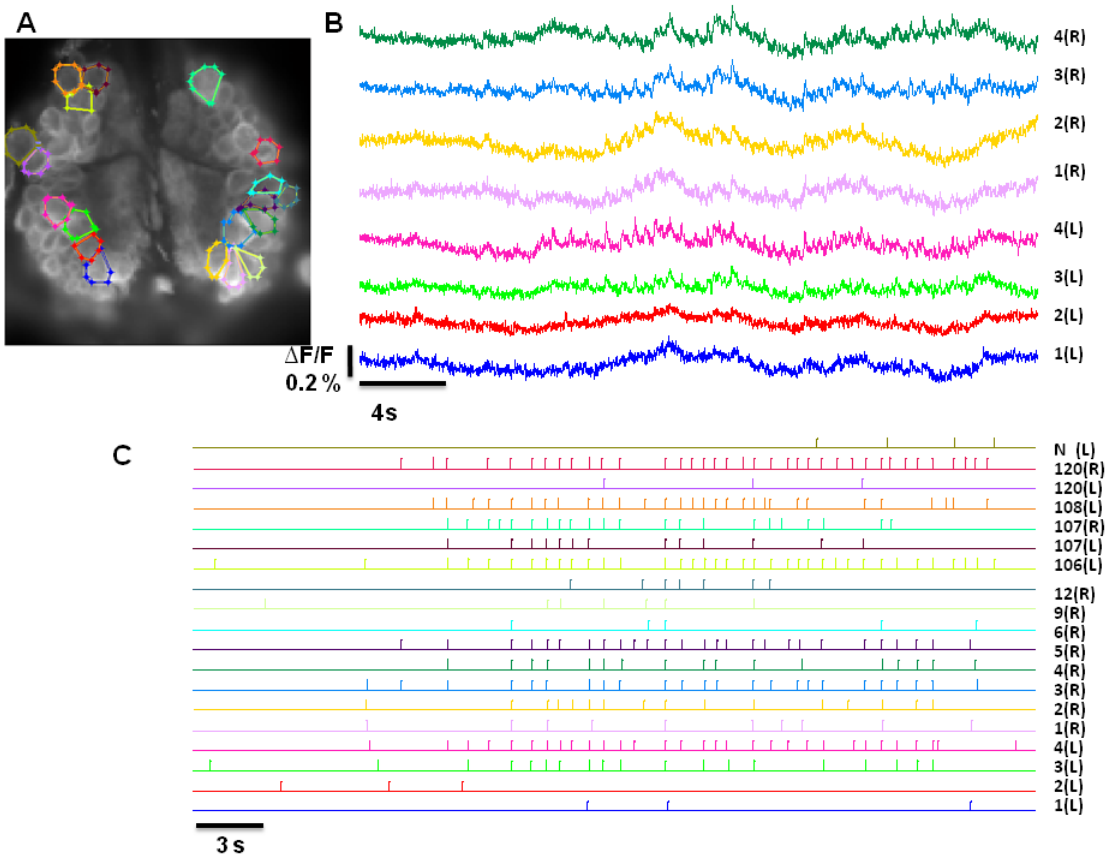


Figure 3.7: Optical recordings (ΔF/F) of the spontaneous electrical activity from the dorsal surface of a leech ganglion in the presence of 20 μM serotonin. Panels A,B and C as in Fig. 3.3. In the presence of serotonin, many more neurons produce simultaneous bursts of spikes.

We also recorded the optical activity in the presence of serotonin from the dorsal surface (Fig. 3.7) where slow changes of the optical signals were

more evident (Fig. 3.7B), indicating the occurrence of larger changes of the membrane voltage. In this case, the excitatory motoneurons 3 and 4 of both sides had a long burst of spikes (Fig. 3.7B) as did most of the neurons on the dorsal surface, as shown in the associated raster plot (Fig. 3.7C). In the presence of serotonin, spontaneous bursts of spikes in neurons visible on the dorsal surface lasted longer and activated more motoneurons than in control conditions.

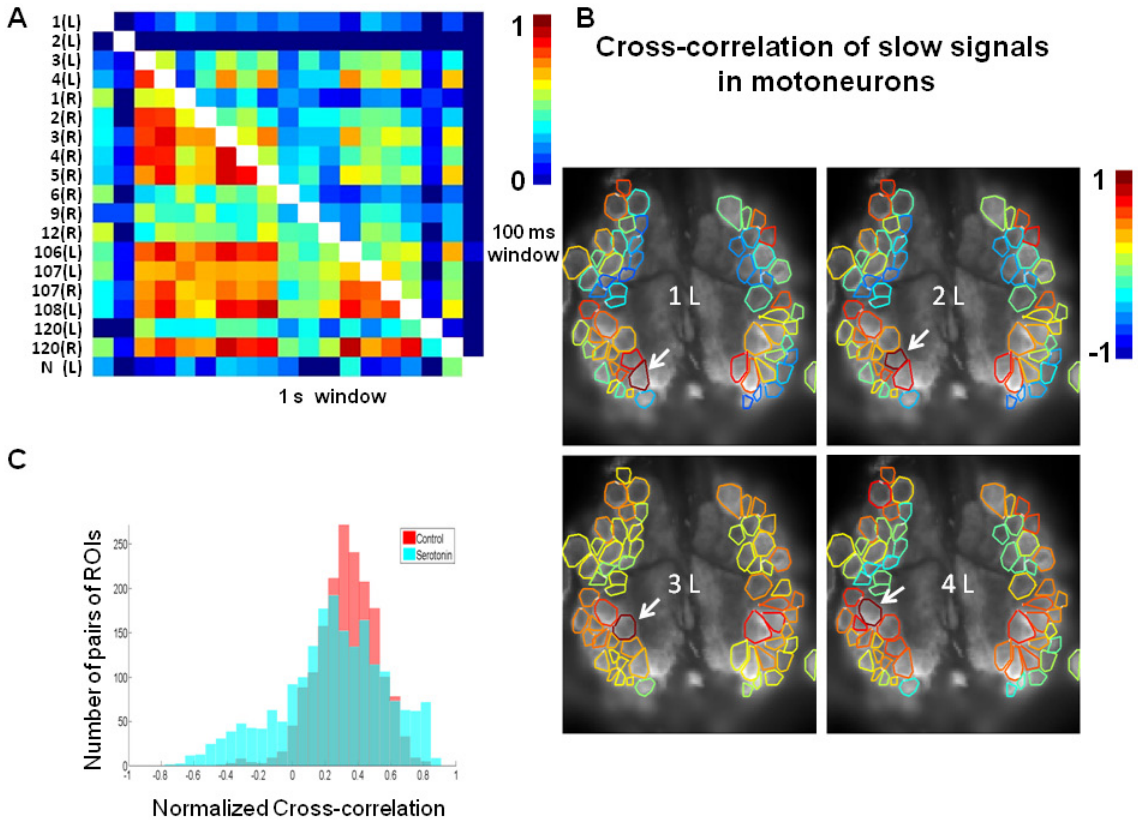


Figure 3.8: Correlation structure of electrical activity of neurons visible in the dorsal surface of the ganglion in the presence of 20 µM Serotonin. Panels A, B and C as in Fig. 3.4. In C data in presence of serotonin collected from 4

experiments. Observe that in the presence of Serotonin several pairs of motoneurons have higher entries of $\sigma_{AP_{ij}}$ (see panel A) and several pairs of motoneurons have entries of $\sigma_{slow_{ij}}$ above 0.6 or below -0.2.

The analysis of the cross-correlation of slow optical signals shows a significant fraction of pairs of neurons with a negative cross-correlation (see neurons with a blue circle in Fig. 3.8 B).

Neurons around the arrow in Fig. 3.8B are excitatory and inhibitory motoneurons of the dorsal and ventral muscles. The excitatory motoneurons 3 and 4 are highly correlated as well as the pair of inhibitory motoneurons 1 and 2. (Calabrese 1976; Willard 1981) but inhibitory and excitatory motoneurons are pairwise poorly correlated. Comparison of collected data of cross-correlation entries of pairs of neurons in control conditions (red histogram) and in the presence of serotonin (Blue histogram) in (Fig. 3.8 C) indicate that serotonin induces a more structured pattern of correlation of slow optical signals, associated to correlated or anticorrelated slow changes of membrane potential in leech neurons. Optical recordings of the spontaneous activity in the presence of serotonin were obtained in more than 8 experiments from the ventral surface and 6 experiments from the dorsal surface of the ganglion. In all these experiments Retzius cells were either silent or exhibited a significantly lower firing rate and spikes from the Leydig motoneuron decreased their amplitude during recording. In 4 experiments imaging the dorsal surface of the ganglion the fraction of entries of $\sigma_{AP_{ij}}$ larger than 0.5 at the window of 100 and 1000msec was larger than 5% and 30% respectively, therefore suggesting that serotonin increased the

degree of correlated firing in respect to control conditions (1-way ANOVA $p<0.05$, followed by Tukey's post-hoc test).

Chapter 4

Stimulus-evoked electrical activity

Stimulus-evoked electrical activity

The VSD allows the detection of spikes and also slow changes of the membrane voltage in population of neurons with small diameter (20-30 μm), which cannot be resolved not by calcium imaging (Grienberger & Konnerth (2012) , and not by previous voltage sensitive dye imaging (Briggman and Kristan 2006, Stein et al. 2011; Städele et al. 2012) . The new VSD permits efficient use of photons for excitation and emission, allowing lower light levels or dye concentration to be used (Miller et al. 2012). We had long term continuous optical recording exceeding several minutes which is a good progress in voltage sensitive dye imaging.

Also we studied the evoked response in leech ganglia after stimulating mechanosensory neurons. To study the stimulus-evoked electrical activity by new VSD, we stimulated nociceptive mechansensory neuron (N cell) and pressure mechanosensory neuron (P cell).

Long term recording

The VSD VF2.1.Cl allows satisfactory optical recordings within a few minutes continuously, 3-10 minutes depending on the intensity of the excitation light as shown in Fig. 4.1.

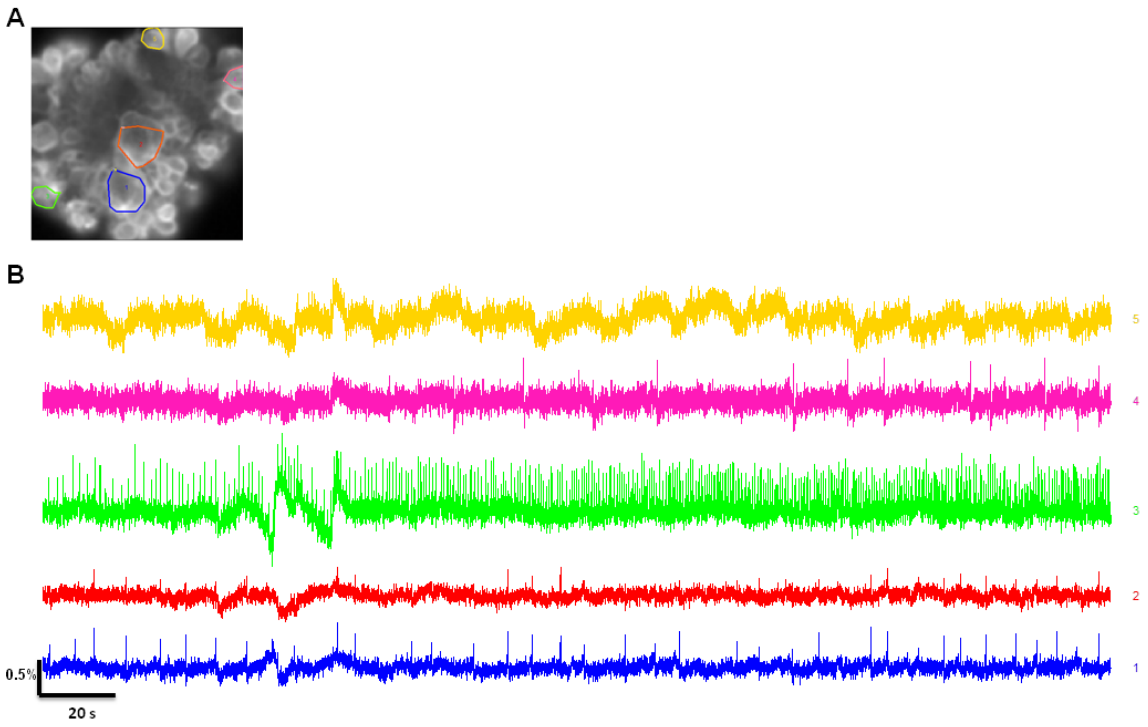


Fig 4.1. Long-term continuous optical recording. (A) Image of the population of neurons from ventral side of the ganglion; the neurons from which the activity recorded are encircled and numbered with the color correspondent to their trace in B (B) optical recording traces from neurons indicated in A; traces are average of the intensity of the pixels inside each neuron field.

We used a combination of neutral density filters to attenuate the intensity of the excitation and avoiding the dye bleaching. By using ND8 we were able to record up to 12 minutes in 4 minutes episodes recorded continuously with 94 frame/s, interspersed by 10 minutes darkness but only from neurons which were both bigger in size and had bigger action potentials. A four minutes recording is shown in Fig. 4.1. By using ND4 we were able to record from all of the neurons of the ventral or dorsal side up to 3 minutes continuous recording.

Stimulus-evoked electrical activity

To study the stimulus-evoked electrical activity by new VSD, we stimulated N cell and P cell. By stimulating P cell we expected to see signs of local bending reflex behavior in the leech.

This behavior has been well studied using electrophysiological recordings by Kristan and Lockery (Kristan 1982, Kristan et al. 1982, Lockery and Kristan 1990a&b, Lockery and Kristan 1991).We also stimulated N cell and recorded the response on ventral side of the ganglion (Fig. 4.2). We see response in several neurons encircled and numbered as region of interest (ROI). The stimuli response is visible in Roi's 3, 4, 6, 7, 9, 11, 12, 17, 18, 21, 22, 23, 24, 36, 37, 41, 51, 53, 55, 59, 73, 74. We see the biggest response in following neurons 9, 12, 17, 21, 22, 53, 73, 74; which are approximately identified according to their cell body location, respectively as Anterior Pagoda, Heart motoneuron, Anterior Pagoda, Retzius, Retzius, Leydig, cell 251 and cell 251 according to the map of the neurons in (Macagno 1980). We expect to see the evoked response in two Retzius neurons and Leydig neurons (Lockery and Kristan 1991).

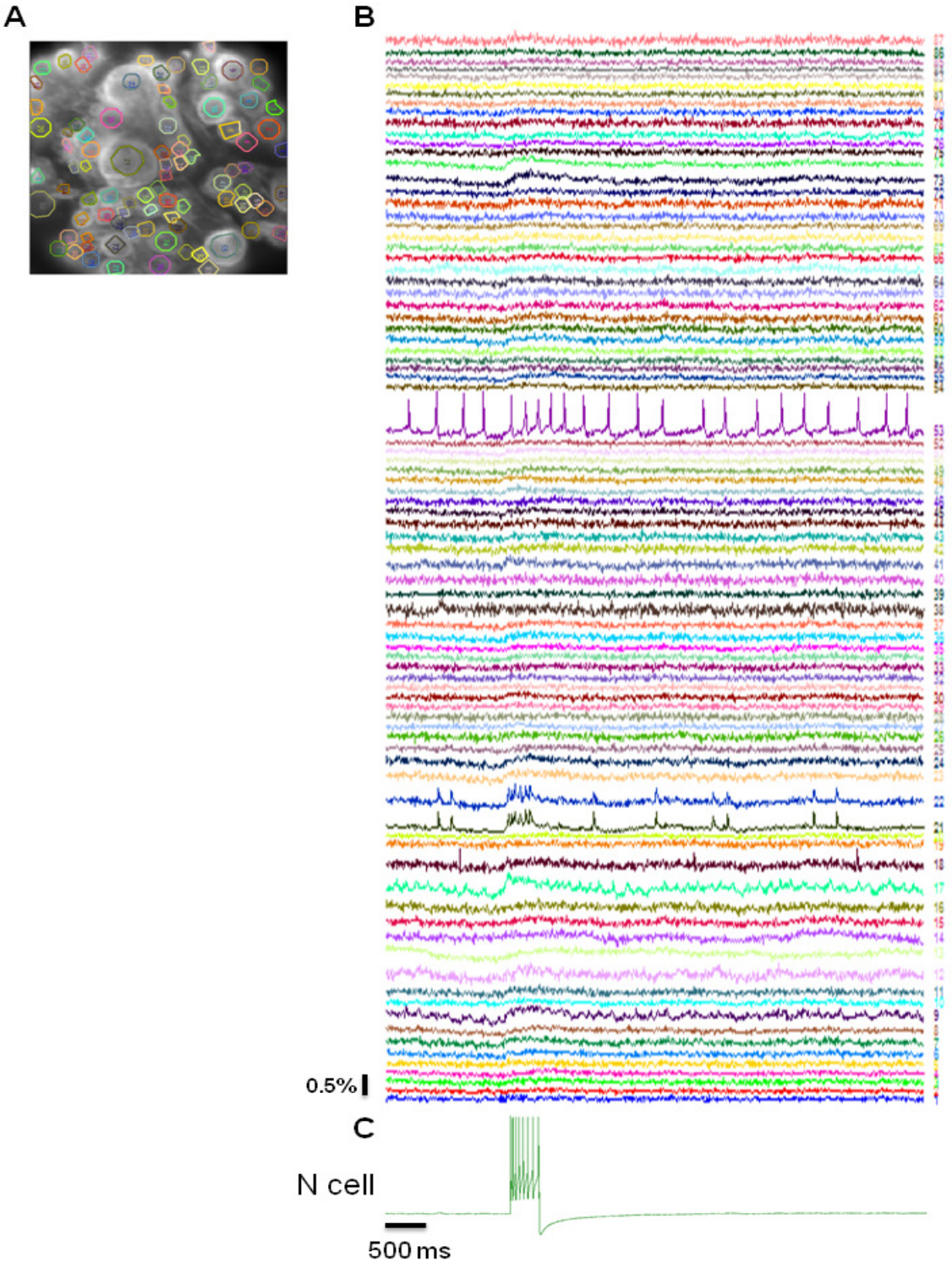


Fig 4.2. Nociceptive mechanosensory neuron (N cell) electrical stimulation for local bending behavior. (A) Image of the population of neurons from ventral side

of the ganglion; the neurons from which the activity recorded are encircled and numbered with the color correspondent to their trace in B (B) optical recording traces from neurons indicated in A; traces are average of the intensity of the pixels inside each neuron field (C) electrical recording of the N cell response to stimulation

Also we stimulated dorsal P cell and recorded the response on dorsal side of the ganglion which contains mostly motoneurons and produces the local bending response (Fig. 4.3). The stimuli response is visible in Roi's 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 18, 19, 20, 21, 22, 26, 27, 28, 38, 40, 45, 46, 48, 49, 54, 56, 60, 61, 62, 63, 64, 65, 66, 67. The response is inhibitory hyperpolarization in Neurons 18, 45, 48, 56. We observe the biggest excitory response in neurons 2, 8, 10, 13, 19, 21, 22, 27, 38, 46, 54, 62, 66. We approximately identified neurons 2 and 19 according to their cell body location as cell 3 which are excitors of dorsomedial longitudinal muscles on the right and left side of the ganglion as they have been recorded electrophysiologically in (Lockery and Kristan 1990a). We observe the biggest inhibitory response in neurons 45, 48, 56. Neuron 56 is approximately identified according to its cell body location as cell 102 the inhibitor of dorsal longitudinal muscles.

In Fig. 4.4. we superimposed traces of the evoked response of N cell stimulation and P cell stimulation. We can see the responses are reproducible in several trials.

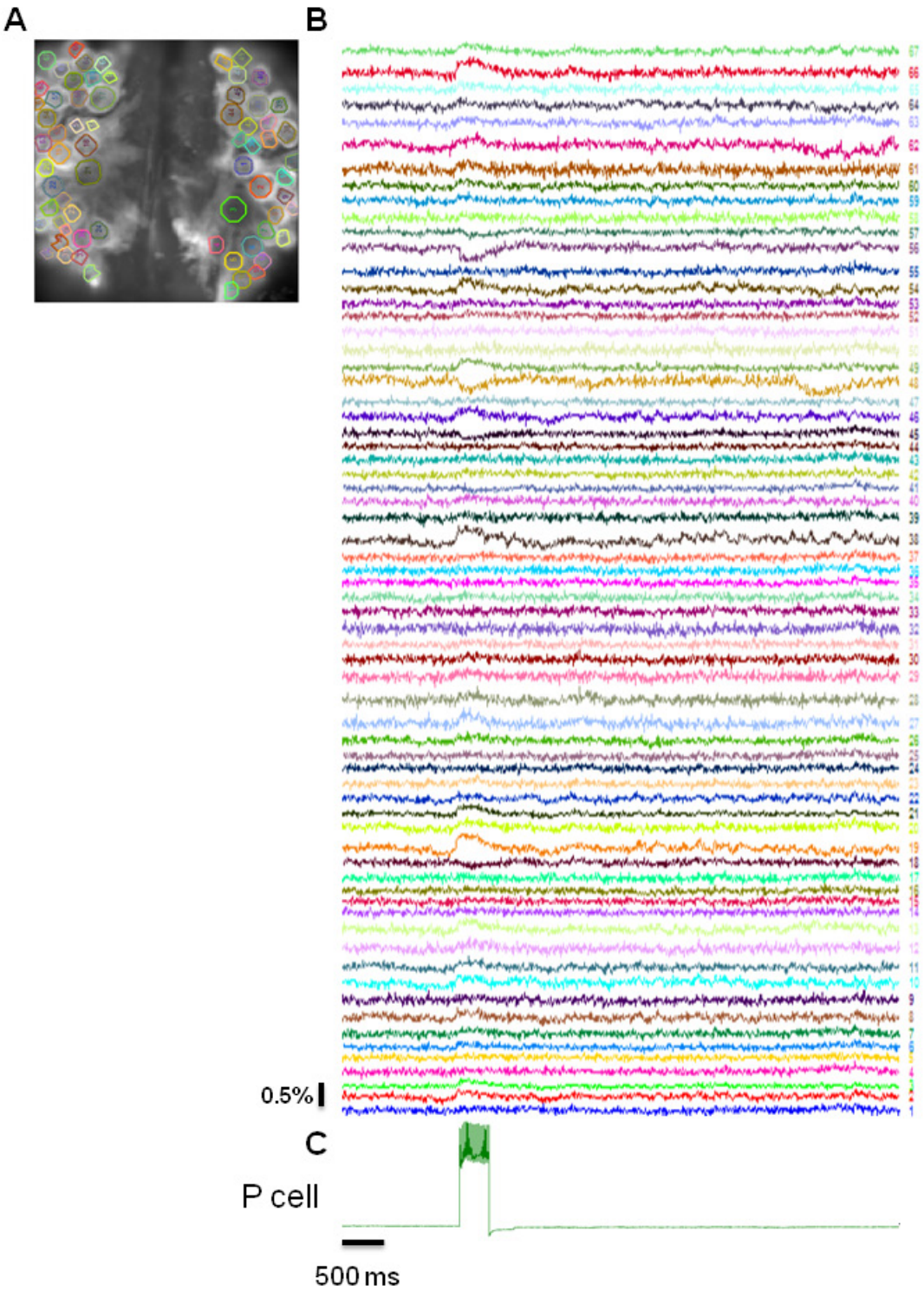


Fig 4.3. Pressure mechanosensory neuron (P cell) electrical stimulation (A) Image of the population of neurons from dorsal side of the ganglion; the neurons from

which the activity recorded are encircled and numbered with the color correspondent to their trace in A (B) optical recording traces from neurons indicated in B; traces are average of the intensity of the pixels inside each neuron field (C) electrical recording of the P cell response to stimulation

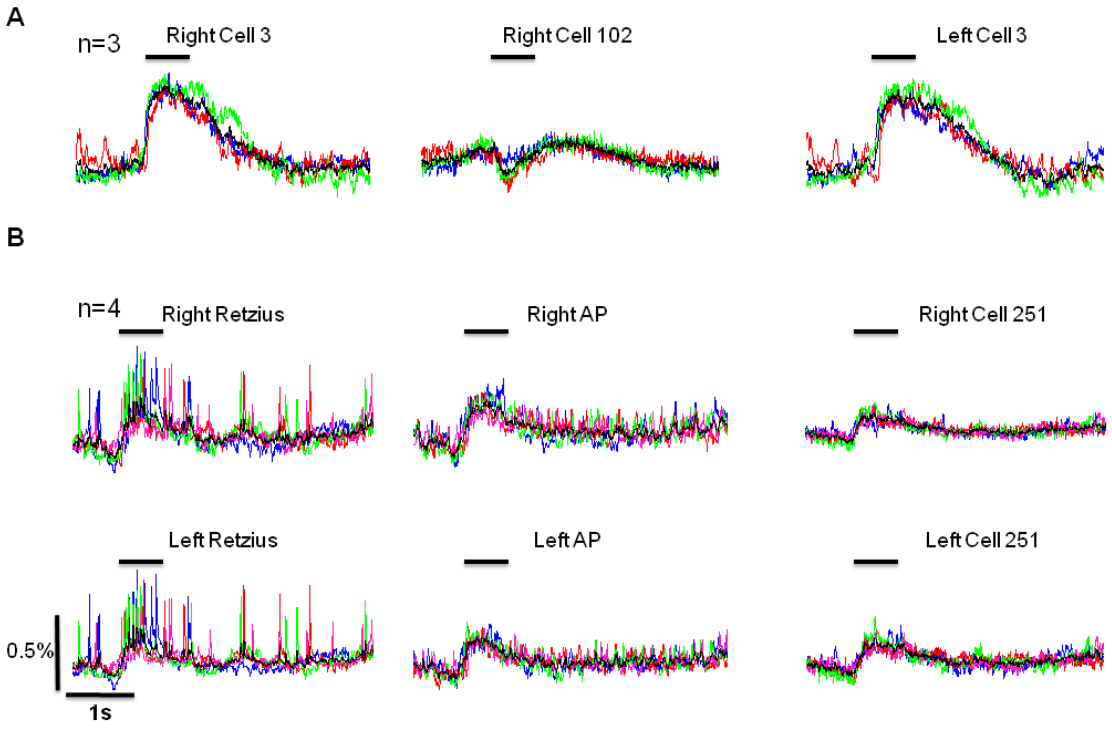


Fig. 4.4. Single sweeps and average (Black) of the optically recorded of stimuli response (A) superimposed optical traces of three trials of three different neurons responded to the electrical stimuli of pressure mechanosensory neuron (P cell) on the dorsal side (B) superimposed optical traces of four trials of six neurons on right and left side of the ventral face of the ganglion responded to the electrical stimuli of nociceptive mechanosensory neuron (N cell).

In Fig 4.5. we showed the average of the evoked response from four preparation for N cell stimulation and three preparation for P cell stimulation.

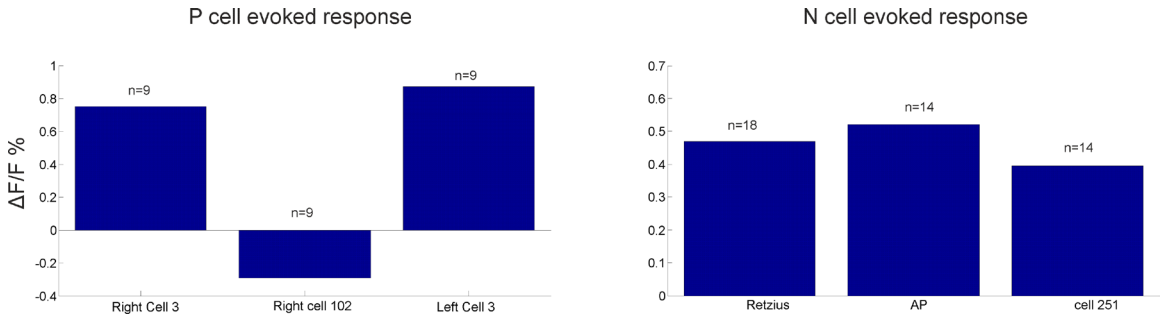


Fig. 4.5. Response of several neurons to single P cell and single N cell stimulation. (A) Average peak synaptic potential in response to stimulation of a single P cell. (B) Average peak synaptic potential in response to stimulation of a single N cell.

Discussion

In this section I summarize the main results and conclusions from my work.

In this dissertation, I imaged population of neurons of the leech ganglion with the technique that I developed, during spontaneous and evoked activity. In one aspect it is important that it is spontaneous, because in stimuli evoked behaviors like swimming and crawling (Kristan et al. 2005), we see central pattern generator (CPGs) activity. In the presence of CPGs, especially in Motoneurons there is a higher level of electrical activity because of burst of spikes while in spontaneous activity the level of electrical activity is lower and there are single spikes, this means that there is weaker optical signal than behavioral CPGs. Therefore this imaging method is very useful to study both behavioral CPGs and evoked response to identify unknown neuronal circuits and also spontaneous activity in which we have no stimulation and less neural activity. Especially one of the useful applications is to study how a behavior initiates or how behaviors switch to each other.

Simultaneous recording from a population of neurons in comparison to single cell electrophysiological recording gives us a much broader view of the network dynamics. I imaged dorsal and ventral side of the ganglion in separate preparations. On dorsal side I was able to monitor 50-60 neurons and on ventral side 80-90 neurons.

Long term recording

I showed here that with the help of the technique I presented and the new voltage sensitive dye, I was able to have continuous long-term optical recordings at a sampling rate of 94 to 170 frame/s with the duration of several minutes. In the previous studies the duration of the optical recording does not exceed one minute continuously (Cohen et al. 1989, Cacciatore et al. 1999, Briggman et al. 2005, Briggman and Kristan 2006, frost et al. 2007, stein et al. 2011, Hill et al. 2012, Städele et al. 2012). This will help to study the neural network for a longer time and enables us to understand better the dynamics of the system and to know what is happening between neurons in spontaneous activity and to understand better the mechanism of different kinds of behaviors and decision making.

Stimulus-evoked electrical activity

In previous work the input-output relations of the local bending reflex were studied. Kristan and his colleagues analysed the activation and inhibition of motoneurons by stimulating sensory neurons (Kristan 1982, Kristan et al. 1982, Lockery and Kristan 1990a&b, Lockery and Kristan 1991). But they had this limitation that they were able to study a few neurons response simultaneously, while by imaging, the response of a population of neurons including motoneurons, interneurons and sensory neurons can be studied simultaneously.

This feature of imaging enables us to distinguish a behavior from another one by imaging from the population of neurons of the nervous system.

The response has been seen in many neurons including cell 3 as an excitor motoneuron and cell 102 as an inhibitor motoneuron. Although this is not a complete comparison because the preparations are different by Comparing these results with the results of previous studies (Kristan 1982, Kristan et al. 1982, Lockery and Kristan 1990a&b, Lockery and Kristan 1991) that just used electrophysiological methods, it can be said that the activity reported here is not altered by the dye or the technique.

Spontaneous activity

The voltage sensitive dye VF2.1.Cl (Miller et al. 2012) allows the detection of spikes with small amplitude such as those present in the cell body of most leech neurons (Ort et al 1974; Kristan & Calabrese 1976; Brodfuehrer & Friesen 1986a & 1986b; Brodfuehrer et al 1995; Kristan et al 2005). This spontaneous electrical activity is segregated in three main groups: a group of neurons firing almost periodically but not in synchrony, a group of neurons firing sparsely and randomly, and a group of neurons firing bursts with varying duration and size. These three groups interact with each other only weakly and their dynamics appear segregated. This segregation – observed in all investigated ganglia – could not be revealed by extracellular recordings from the nerves or by the simultaneous intracellular electrical recordings generation of VSDs.

Segregation of the spontaneous activity

As shown in Fig. 3.1 and 3.6, when the ventral surface of the ganglion is imaged, it is possible to visualize the cell body of as many as 80 neurons. Although I was able to detect optical signals associated to spikes with amplitude of 4-6 mV, I detected changes of emitted fluorescence only in a limited number of visualized neurons. When the leech ganglion is involved in the generation of the electrical activity of a relevant behavior such as swimming or crawling, reliable optical signals are observed from a much larger portion of visualized neurons (Briggman et al., 2005; Briggman and Kristan, 2006), suggesting that a large portion of neurons present in the ventral side do not have a significant spontaneous electrical activity. In the experiments I observed an almost periodic and spontaneous firing of the two Retzius cells, the two APs and AEs neurons, two Leydigs and of some additional neurons such as neurons 153 and 158. I occasionally observed also the spontaneous firing of mechanosensory neurons such as T and N cells. When the cell body of other neurons in the ventral surface was imaged, I rarely detected optical signals indicating the occurrence of a spike. we were able to detect optically from the cell body of imaged neurons spikes with an amplitude of 4 mV. Therefore, the spontaneous electrical activity of the great majority of neurons visible in the ventral surface of leech ganglia is either very limited and almost absent or is segregated into their axons. A different picture emerges when neurons in the dorsal surface are imaged (Fig. 3.3) optical signals associated to the occurrence of spikes could be

recorded – in successful experiments – from the great majority of imaged neurons. These neurons do not usually fire in a repetitive or periodic mode in bursts of 3-10 spikes lasting for some seconds, as confirmed by extracellular recordings with suction pipettes from the nerves shown in Fig. 3.5. As shown in Figs. 3.1 and 3.5, the spontaneous firing of the Retzius, APs, AEs is almost periodic and does not occur in bursts, as in motoneurons visible in the dorsal surface. Therefore, under control conditions, the spontaneous activity of neurons in a leech ganglion has three different regimes: i – a repetitive and almost periodic firing around 1 Hz; ii – a sparse and random firing and iii – a firing in bursts of varying duration and size. These different regimes involve specific neurons and a given neuron does not change its firing regime.

Simultaneous electrical recordings from the nerves and from Retzius, AP and AE neurons (Fig. 3.5) show that when a burst occurs in motoneurons, the burst is not correlated with changes in the membrane potential of these neurons. As shown in Fig. 3.5, changes of the resting membrane potential of the impaled neurons visible on the ventral surface (AEs, APs, Retzius cells,….) could precede or follow the initiation of bursts, in agreement with the notion that bursts dynamics is not deterministic but reminiscent of self organized criticality (Mazzoni et al 2007). Therefore, the spontaneous electrical activity of leech neurons appears to be segregated in three distinct groups with their own regime.

Subthreshold interactions among leech neurons

The dye VF2.1.Cl is not only able to signal the occurrence of a spike with an amplitude larger than 4 mV, but can detect changes of the membrane potential larger than approximately 4-6 mV. Therefore imaging with this dye offers the possibility to evaluate and identify the extent of subthreshold interactions among leech neurons. The analysis of $\sigma_{slow_{ij}}$ among pairs of neurons visible in the ventral surface shows a strong positive correlation among the pair of Retzius cells and only weak and possibly negligible subthreshold coupling among other pairs of neurons. In contrast, neurons visible in the dorsal surface, exhibit some degree of subthreshold positive correlation and graded optical signals recorded from neighboring motoneurons have often a positive value of $\sigma_{slow_{ij}}$. In the presence of serotonin, neurons become more coupled, with the exception of Retzius cells (see Fig. 3.6). The comparison of $\sigma_{AP_{ij}}$ of neurons visible in the ventral surface indicates an increased correlated firing in the presence of serotonin (Fig. 3.6D) with respect to what is observed in control conditions (Fig. 3.2B). Neurons visible in the dorsal surface of the ganglion, which usually have positive entries of $\sigma_{slow_{ij}}$ at around 0.3 become either more strongly positively coupled or exhibit negative entries of $\sigma_{slow_{ij}}$. This effect is consistent with the swimming behavior induced in chains of ganglia by micromolar amounts of serotonin (Kristan et al. 2005).

The effect of Serotonin

My optical recordings of the perfusion of Serotonin (20μM) of a single isolated leech confirms several previous observations (Nusbaum et al.1986; Dierkeset al. 2005; Kristan et al 1993): indeed, the amplitude of Retzius cells response decreases and eventually these cells become silent and their electrical coupling is weakened (Fig. 3.6). Slow signals from neurons visible in the dorsal surface, which in control conditions did not have large positive or negative values (Fig. 3.4B and C), exhibited large positive and negative values, as shown in Fig. 3.8 B and C. It is well known that serotonin switches behaviors - such as swimming - on and off (Kristan & Calabrese 1976; Willard 1981). Therefore, the addition of serotonin to a single isolated leech ganglion creates groups of neurons with in-phase and antiphase slow changes of their membrane voltage, but the emergence of the swimming pattern – in which motoneurons fire with specific phase lags - requires the presence of a chain of ganglia.

Possible functional role of segregation of the spontaneous activity

The segregation of the spontaneous electrical activity here described can have an important functional role in information processing in the leech nervous system. Indeed, neurons on the ventral surface are sensory neurons and interneurons, while most of the motoneurons in the ganglion are on the dorsal surface. A highly irregular firing of spikes and/or the presence of

large bursts are certainly not beneficial to the processing of sensory information. From this point of view it is not surprising that neurons devoted to the analysis, processing and filtering of sensory and particularly mechanical inputs do not have a significant spontaneous activity: indeed, it is advantageous that interneurons involved in the first stage of sensory processing do not fire spontaneously and are relatively noise free. Neurons on the dorsal surface are not sensory neurons and are primarily effectors, i.e. are motoneurons.

My results relate to the current discussion of the role and properties of "stimulus driven" and "network driven" neuronal mechanisms (Benucci et al 2009; Churchland et al 2012). Neurons in the ventral surface of the ganglion seem to be primarily driven by sensory stimuli, while the electrical activity of neurons, and in particular motoneurons, in the dorsal surface is not only driven by sensory inputs but it is also influenced by network properties determining the occurrence of bursts. The observation of substantially higher entries of $\sigma_{slow_{ij}}$ in pairs of neurons visible in the dorsal surface than in the ventral surface supports the notion that neurons in the dorsal surface are more network driven than those in the ventral surface.

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