Variations in Interpulse Interval of Double Action Potentials During Propagation in Single Neurons

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ABSTRACT In this work, we analyzed the interpulse interval (IPI) of doublets and triplets in single neurons of three biological models. Pulse trains with two or three spikes originate from the process of sensory mechanotransduction in neurons of the locust femoral nerve, as well as through spontaneous activity both in the abdominal motor neurons and the caudal photoreceptor of the crayfish. We show that the IPI for successive low-frequency single action potentials, as recorded with two electrodes at two different points along a nerve axon, remains constant. On the other hand, IPI in doublets either remains constant, increases or decreases by up to about 3 ms as the pair propagates. When IPI increases, the succeeding pulse travels at a slower speed than the preceding one. When IPI is reduced, the succeeding pulse travels faster than the preceding one and may exceed the normal value for the specific neuron. In both cases, IPI increase and reduction, the speed of the preceding pulse differs slightly from the normal value, therefore the two pulses travel at different speeds in the same nerve axon. On the basis of our results, we may state that the effect of attraction or repulsion in doublets suggests a tendency of the spikes to reach a stable configuration. We strongly suggest that the change in IPI during spike propagation of doublets opens up a whole new realm of possibilities for neural coding and may have major implications for understanding information processing in nervous systems. **Synapse 67:68–78, 2013.**

INTRODUCTION

One central problem in *Neuroscience* is that of characterizing and understanding the neural code (Perkel and Bullock, 1968). The term "neural code" refers to the properties of a single sequence of action potentials (spike trains) or a spike train ensemble that may be used by postsynaptic neurons to be extracted or decoded (Abbott and Regehr, 2004; Eggermont, 1998; Grothe and Klump, 2000; Lestienne, 2001). Research in sound localization and electrosensory pathways has provided information about the basic understanding of neuronal mechanisms for detecting submillisecond differences in spike timing between neurons (which can be used by the nervous system in its purest sense), although many questions remain (Carlson, 2008; Grothe, 2003). However, much less is known about how information encoded into distinct patterns of activity over time (coding schemes based on sequences of interspike intervals within neurons rather than differences in spike timing between neurons) is decoded by postsynaptic neurons (Carlson, 2009).

On the other hand, it is usually accepted that, as in any good democracy, individual neurons count for little; it is the population activity that matters (Averbeck et al., 2006). However, in recent experiments, the behavioral relevance of single neuron activity is being demonstrated. For example, Carlson (2009)

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found that an all or none electrical potential is produced with variable timing or sequence of pulse intervals in single neurons. By varying the sequence of pulse intervals during signaling, he observed that mormyrids fishes are able to produce a rich repertoire of electric displays that play an important role in social behavior. Houweling and Brecht (2008) trained rats to respond to short trains of microstimulation pulses of single neurons in the somatosensory cortex. The 200-ms pulse train consisted of 14 spikes, each one of 0.4 ms in duration and a mean interpulse interval (IPI) of 12.2 ms, and these authors concluded that the stimulation of single neurons affects behavioral responses in a detection task.

Usually, the approaches to study the coding and decoding of temporal patterns are based on frequency and timing, but what would happen if the IPI of particular patterns varies while they propagate? What implications could have this effect? The aim of the present work is not to answer these questions but to show that IPI of short pulse trains varies while spikes propagate in neural axons. With this knowledge, we may interpret some basic results concerning the transmission of a neural code between presynaptic and postsynaptic neurons which would be a first step toward the understanding of information processing in nervous systems.

Recently, we discussed the possibility that coexisting pulse trains may exist in single neurons. That is, pulses with a separation less than the length of the nerve axon. For a particular case, we theoretically predicted, that they should possess minimum distances within the interval of 5–10 pulse widths (Villagran et al., 2011). In grasshopper nerves, we found pulse doublets displaying this minimum distance experimentally (Villagran et al., 2011). The appearance of short pulse trains like doublets has been reported by others, for instance Kirk and Glantz (1981) studying the impulse pattern generated in a crayfish abdominal postural motor neuron, obtained constant bursting in doublets in the largest tonic flexor motor neuron f6 induced by extrinsically injected current. The IPI histogram follows a Gaussian distribution and the mean was about 11 ms compared to a pulse width of 2.5 ms. Doublets with IPI greater than 37 ms did not appear. Harris and Stark (1973) carried out experiments to determining the fluctuations in the transmission delay caused by a synaptic link located in the subesophageal ganglion of the crayfish. The ganglion was stimulated with light evoking doublets in single light sensitive cells in some cases. On the other hand, Larimer and Moore (2003) investigated the effect of temperature on the basal activity of the caudal photoreceptor (CPR) of the crayfish and found variable temperature dependence for patterning, although the tendency is for single impulses at higher temperatures, and double and occasionally triple impulses at low temperatures. In addition to direct light sensitivity, the CPR responds trans-synaptically to the mechanoreceptive input (Larimer and Moore, 2003; Rodríguez-Sosa et al., 2012). Recently, Carlson (2009) investigating the temporal pattern recognition by single neurons of a sensory pathway in mormyrid fish, obtained but did not analyze doublets in a single neuron of the midbrain. Through whole-cell patch recordings from midbrain posterior exterolateral nucleus neurons, he found three patterns of IPI tuning: low-pass neurons tuned to long intervals, high-pass neurons tuned to short intervals and band-pass neurons tuned to intermediate intervals. The responses of band-pass postsynaptic neurons to an electrical stimulation programmed for a 30-ms IPI train turned out to be doublets and even triplets. In the above-mentioned studies, doublets were found in the discharge of single neurons when investigating other phenomena, and were not devoted to the analysis of doublets.

The present work focuses on the analysis of the internal dynamics of double and triple pulses, the shape, conduction velocity and interspike interval as they propagate as a single pattern along the neuron, to elucidate whether they influence each other. The structure of this article is as follows: Material and Methods section, experimental procedure, focuses on the method to obtain the double pulses and experimental conditions for three biological models: Lateral nerve in *Locusta migratoria*, the CPR in crayfish, and abdominal motoneurons also in crayfish. Results section is devoted to the analysis of single pulses. In addition, we analyze the dynamics of doublets and triplets. Discussion section is devoted to the discussion of results and conclusions.

MATERIAL AND METHODS

Lateral nerve in Locusta migratoria

Adult male and female *Locusta migratoria* were reared in a laboratory colony at about 30°C and 12:12 h dark–light cycle. Experiments were performed using amputated metathoracic legs which were mounted on a platform and fixed on their external lateral face with Plasticine™ (nontoxic modeling clay KR 25, Becher, Göttingen). The axis of rotation of the femoro-tibial joint was aligned with the origin of a semicircular angular plane. Inside a Faraday cage and with the aid of a stereo microscope, a small window of the posterior-lateral cuticle was removed to expose the peripheral lateral nerve properly called posterior tegumental femoral nerve n5B2c (Hustert et al., 1999; Mücke, 1991) and the tibia was cut to a stump to allow oxygenation inside the leg via the trachea. To record the electrical activity from the single multipolar stretch receptors, the anterior-lateral RDAL (nomenclature according to Burns, 1974; Coillot and Boistel, 1969) and the two

posterior-lateral receptors (RDPL; RVPL, which have synaptic connections onto the motor neurons involved in the control of the locust jump (Heitler and Burrows, 1977), monopolar platinum wire electrodes (diameter, 50 μm) were hooked on the nerve n5b2c at defined distances. With the aid of a pipette, a small drop of locust saline (NaCl 140 mM, KCl 10 mM, Na H_2PO_4 5 mM, $CaCl_2$ 2 mM, Saccharose 90 mM; pH 6.8) was deposited on the wounds to avoid the coagulation of the hemolymph and also used as a reference. A small amount of Vaseline (DAB 10, Roth GmbH, Karlsruhe, Germany) was added to isolate the system nerve-electrode as well as to avoid desiccation of the preparation.

In all cases, three electrodes were hooked on the nerve n5b2c. The distance from the rotation axis of the joint to the electrodes was 10, 12, and 15 mm, respectively. We did not put the first electrode closer to the origin of the system to avoid damaging or stimulating artificially the receptors. In all experiments, the tibia was extended from 0 to 80° where typically no activity could be recorded. Afterward, at each angle from 80 to 150° with steps of 10°, the tibia was fixed firmly with an entomological pin. Although primitive, the use of a pin to fix the angle is the most precise method, because the tibia always tends to flex and the pin, as a stopper, allows a perfect static equilibrium at the desired angle of extension. Sessions of 3–5 min were recorded for each angle. We found that 150° is the limit of the extension since beyond this angle the end of the tibia exerts pressure against the distal dorsal edge of the femur and therefore the conditions are no longer physiological (Coillot and Boistel, 1969). All experiments were performed at room temperature of 22–24°C.

The action potentials were recorded externally by platinum wire hook electrodes. A preamplifier (four channels, 1000× amplification, without any band-pass filter, 9 V battery source, workshop of the Institute for Zoology, Georg-August-University Göttingen) was used for primary signal amplification within the Faraday cage, a secondary amplifier (four channels, 10× amplification, 100 Hz high-pass filter, 30 kHz low-pass filter) was utilized to pre-filter the signals. The amplified action potentials were digitized via PC based data acquisition hardware (Packard Bell desktop computer, eight channels RUN Technologies acquisition port, PCI-DAS1200Jr board, Microsoft Windows XP Professional SP2, 2.66 GHz Pentium(R) 4 CPU, 512 MB RAM) and recorded and analyzed via the DATAPAC 2K2 software (RUN Technologies, Mission Viejo, CA).

Analysis of the recordings

The smallest sample period for digital recording with the system was 49.6 μs, though a period of 89.6 and 148.8 μm was chosen for longer recordings. No software-based secondary filtering was performed on the recorded channels. Pulse height was defined by the voltage distance between maximum depolarization and maximum hyperpolarization, to account for a potential signal baseline slope during the recording. Pulse duration was defined by the time difference between maximum depolarization and maximum hyperpolarization events during a pulse. In all pulse trains, the onset of the individual signals was defined by the maximum depolarization within the pulse, the offset by the maximum hyperpolarization. Signal velocity along the nerve was determined by subtracting the onset times of time-linked signals between the channels and multiplying the inverse with the measured spatial distance of the corresponding hook electrodes. The instantaneous pulse period was defined as the time difference between the current onset and the onset of the next signal within a pulse train.

For spike detection and sorting from a specific receptor, these were first identified according to their typical amplitude, and their spike-intervals were determined via a simple threshold pass analysis. The threshold was set at roughly 80% of the maximum depolarization amplitude of the unit of interest, and then adjusted manually in such a way as to maximize the sensitivity and specificity in regard to AP localization. The "onset" of the marked pulses was then moved to the first peak depolarization, the "offset" to the first peak hyperpolarization. If closely neurons fire in synchrony or with a small delay, less than 2 ms from peak to peak, then it is possible to get overlapping spikes; i.e., a spike shape generated by the sum of the spikes of the two neurons. Without a delay, overlapping spikes look like the firing of a third large neuron which was considered as a superimposed noise spike which was ruled out from the analysis.

To determine the correspondence of the spike waveforms from one channel to another several steps were followed: (1) since both electrodes were hooked onto the same nerve, only three classes of spikes are present in each recording. (2) The above mentioned amplitude discriminator was used. (3) For each recording, the patterns corresponding to each electrode were compared taking into account the tonic activity of the neurons. (4) The shift caused by the time delay was determined. This was done by setting the onset (vertical lines) at the peak of the spike in the first channel and the offset (vertical line) at the peak of the spike on the second channel.

The spike detection and sorting for double pulses was done in a similar way as for the single spikes but with a more detailed analysis consisting in measurements of duration from minimum to maximum, amplitude ratio from first to second pulse, conduction velocity of each spike, and IPI recorded with both electrodes. Care was taken to analyze only isolated double pulses from a specific neuron, this means that no spikes belonging to another neuron should be

present near or between the double pulse trains since it could cause overlapping spikes thus difficulty the determination of the maxima of the pulses.

The CPR in crayfish

We used adult crayfish, *Cherax quadricarinatus*, in intermolt and with random gender. Animals were commercially acquired in Mexico. The crayfish were kept in the laboratory under a 12:12 light–dark cycle with free access to food for 2 weeks.

Electrophysiology

Electrophysiological experiments were made according to the method described previously (Rodríguez-Sosa et al., 2011). Briefly, the abdominal ganglion chain was removed from the crayfish under the ice-cold modified saline solution of van Harreveld (1936) (VH), (205 NaCl, 5.4 KCl, 2.6, $MgCl_2$, 13.5 $CaCl_2$, and 10 HEPES, all concentrations in mM) at pH 7.4. After the nerve cords were dissected, some nerve bundles between the fifth and the sixth abdominal ganglion were isolated under a stereoscopic microscope. In addition, the corresponding dissection for exposing the nerve bundle between the second and first abdominal ganglion were carried out. The preparation was transferred to a Petri dish containing the modified VH solution, and then kept in the recording chamber mounted on a microscope (SMZ800, Nikon) and perfused with saline solution (about 1 mL/min) during about 30 min in the dark at room temperature (20–22°C), before beginning the recording. The extracellular recordings were made with suction electrodes, filled with the same saline VH. The suction electrodes were usually positioned on the proximal and distal nerves of the connective previously described. The reference electrode (Ag-AgCl) was in the bath solution. Signals were recorded with AC amplifiers (EX1, Dagan), filtered at 30 Hz to 10 kHz by a band-pass filter system, and displayed on an oscilloscope (Gould 1604). Four-second duration light pulses at 4-min interval were delivered with a photo stimulator (Grass PS33), which produced white incandescent light (1000 lux). The light intensity was attenuated with neutral density filters (Kodak, series no. 96). The calibration of intensity was done with a photographic light meter (Goossen, model Luna-Pro, Germany). Mostly, in this present study, the biological preparation was stimulated with a white light at an intensity of 100 lux. The electrophysiological signals originated from the CPR, in the abdominal ganglion chain, were captured at 20 kHz on a computer using the Spike2 software (Cambridge Electronic Designed, Cambridge, England), and then analyzed, in accordance with the waveform, amplitude, duration and their response to light pulses.

Motor neuron 3 in crayfish

We used specimens of the Australian red claw crayfish (*Cherax quadricarinatus*) with random gender in the experiments described in this section. The crayfish were kept in the laboratory under a 12:12 light–dark cycle with free access to food for 2 weeks.

Electrophysiological measurements

Electrophysiological experiments were made according to the method described in (Pedersen, 2010) where details of the anatomy may be found. A homemade specimen tray of dimensions 15 × 10 was used to place the crayfish tail in. The tail was fixed to the bottom of the tray using (an imitation of) ordinary Blue-Tack as well as a two-component self-curing rubber replica (Reprorubber, reorder no. 16135, from Flexbar, NY) and ordinary pins used to place the crayfish tail.

Subsequently, the tray was filled with a saline solution, also functioning as a buffer, until the solution covered the nerve. This was done in part to emulate the natural extracellular salt ion concentrations in the animal and in part to keep the nerve and tissue separated. The voltage was picked up by the five electrodes; two were used for recording at the nerve, two were used as their references, and one for connecting the saline to ground reference (led to a PowerLab 26T amplifier from AD Instruments, using the cable (BioAmp cable, model Tronomed D-1540) that came with the amplifier). The amplifier was connected to a computer, with which data was collected using the "Chart" software from AD Instruments. Since it was necessary to resolve the individual peaks, which have duration on the order of milliseconds, we employed the amplifiers at the maximum frequency of data collection, 100 kHz. The data recorded with the "Chart" software was read into the "Igor Pro" software from WaveMetrics, for data processing. Basic peak detection was made as follows: The two data channels from "Chart", one for each recording electrode, were assigned to be the primary channel and secondary channel for the data processing. The primary channel is the one used for the initial detection of peaks; therefore it is chosen to be the one with the highest signal-to-noise ratio, since the detection is more reliable with a high signal-to-noise. See further details in Pedersen (2010).

RESULTS

Femoral nerve in *Locusta migratoria*

The posterior tegumental nerve n5B2c in the femur or lateral internal nerve is a bundle of several axons but under flexion only three neurons of the knee stretch-receptors are active. Extension of the femorotibial angle α excites three mechanosensory neurons whose activity can be recorded from this nerve. From

complete flexion of the joint, i.e., 0 to $\sim 80^\circ$ extension, the three neurons have no activity. For nearly 80 to 90°, a single neuron (RDPL) appears. From 90 to 150°, a second unit (RVPL) of slightly larger amplitude appears together with the first one. These two units are tonically active. From 120 to about 150° the largest unit of the three (RDAL) appears showing also a tonic activity. This is in good agreement with results obtained by Kuster and French (1983) and Coillot and Boistel (1969). For simplicity, we will label as unit A the neuron with the smallest amplitude pulses (RDPL), unit B, the one with the medium-sized amplitude spikes (RVPL) and unit C the largest of the three (RDAL).

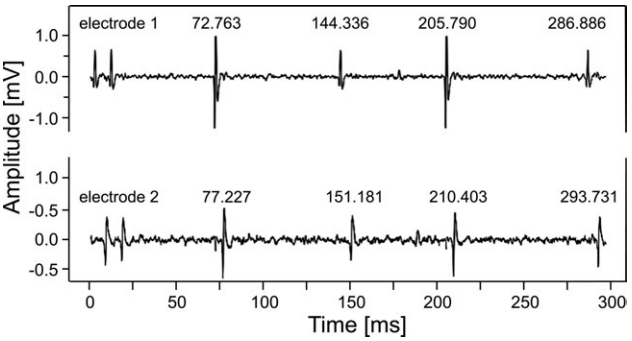

Fig. 1. The tonic activity of neurons A (large spikes) and B (small spikes) of the locust nerve n5B2c at a fixed femoro-tibial angle of 90° recorded with two electrodes spaced 5-mm apart. At the top of the spikes, the time at which they were recorded is indicated. IPI difference in singlets is zero within the sampling resolution.

Firing rate

All three units increase their mean rate of firing with increasing the femoro-tibial angle of extension α. The rate of firing of neurons B and C are almost linear with respect to the extension for angles between 90 and 140°. Within this interval the mean number of spikes per second of unit C is slightly greater than that of unit B. This is in good agreement with results obtained by Kuster and French (1983). The slight difference we observed appears at 150°, where the impulse rate of neuron B becomes lower than the rate of neuron C. The impulse rates are regular and their interval distribution can be well approximated by a Gaussian function with a small standard deviation compared to the mean interval.

Singlets: Conduction velocity and diameter of the axons

We have calculated the conduction velocity of pulses belonging to the three active neurons after recording the action potentials with two electrodes separated by 5 mm at two positions of the posterior tegumental nerve at 90, 120, and 150°. The obtained values at each angle for unit A were: 0.73 ± 0.01 m/s, 0.73 ± 0.02 m/s, and 0.73 ± 0.01 m/s, respectively. The value of unit B was 0.79 ± 0.01 m/s in the three cases while for unit C at 120 and 150° the velocity was 1.02 ± 0.01 m/s. Therefore the velocities are independent of the angle of extension.

Although conduction velocity specifically for these units is not reported in the literature, the values that we obtained fall within the interval of velocities of 0.3–3 m/s obtained for other locust motor nerve fibers (Gwilliam and Burrows, 1980; Pearson et al., 1970) and our values agree with those obtained by Hughes et al. (2005) who measured propagation velocities in the interval 0.29–1.44 m/s recorded in the nerve trunk supplying the metathoracic leg of the locust.

On the other hand, Hoyle (1955) found that some nervous axons in the extensor motor nerve in the midfemur of the locust leg have the same value for both the long and short axis. This implies that such neurons are geometrically regular. In addition, taking into account the logarithmic relationship between neuron diameter and conduction velocity derived by Pearson et al. (1970) for the locust neurons, the conduction velocity of 1 m/s corresponds to a neuron having a diameter of 4 μm. Thus we are dealing with geometrically regular nervous axons with the axis of around 4 μm. For axons with 5 μm, the maximum relative refractory period is about 15 ms and the absolute refractory period is about 3 ms (Hoyle, 1955).

The length of the nerve is about 2 cm and the whole duration of a spike is about 3 ms. Therefore, two pulses separated by more than 30 ms do not coexist in the same nervous axon, for this reason, we call them single pulses or singlets in this system.

Constant IPI in singlets

If we analyze the dynamics of the singlets of both neurons A and B separately by using two electrodes, we find that the IPI difference between two successive spikes traveling along the same nerve axon is zero within the sampling resolution (see Fig. 1). In the upper pattern the first electrode recorded the regular activity of neurons A and B. Spikes 1, 2 (doublet), 4, and 6 from left to right, belong to the neuron A whereas spikes 3 and 5 belong to neuron B. Electrode 2 records the same pattern but with some delay since it is placed 5 mm away from the first one. The IPI between singlets (4 and 6) of neuron A is 142.5504 ms for both electrodes, therefore the *interpulse interval difference is zero*. In the same way, for the spikes belonging to neuron B, the IPI is 133.0272 and 133.1312 ms recorded by electrodes 1 and 2, respectively, therefore the IPI difference is 0.1488 ms, which is exactly one time resolution unit which is considered to be inside the error. This example is perfectly illustrative for the general statistics of this analysis since

it follows the same behavior, i.e., IPI difference between successive singlets of the same neuron is zero.

Short pulse trains: Doublets

By analyzing carefully the regular firing of the three units separately, we found the presence of a particular pattern consisting of a pair of pulses of nearly the same amplitude and duration. These pairs of pulses appear in all the three units, but it is easier to distinguish them at angles of extension α less than 120° where only neurons A and B are active. In Figure 2A, we observe the recording of the activity for these two neurons. Unit B fires at a frequency of 10 spikes per second resulting in a mean IPI of 103 ms while unit A fires at a frequency of 12 spikes per second resulting in a mean IPI of 82 ms. At the center of the recording, in neuron B, a pair of spikes appears in the same shape while, due to the prevailing tonic discharge frequency of this unit, there should be a single one. The IPI in this pair is 9.8 ms which, compared with the mean IPI of singlets in the same neuron, is one order of magnitude shorter: that phenomenon we call a doublet. It must be stressed that this pattern appears with a probability of 10–20% in each sample.

In Figure 2B, we observe the complete histogram for an IPI of neuron B at an angle of extension of 100°. The Gaussian distributions centered at 11 and 150 ms correspond to doublets and singlets respectively. The peak of the singlets distribution is dependent on the angle of extension whereas the peak of doublets distribution does not (Villagran et al., 2011). We have measured the IPI of 400 doublets taken from 10 different legs from different animals and found that it follows a Gaussian distribution with a mean of 11 ms over all the samples with a SD of about 2 ms (see Fig. 2C). Along the length of the femoral nerve (about 2 cm) and with 3 ms duration of spikes and an IPI of about 11 ms, the two pulses of any doublet coexist in the axon while they propagate along the axon. For this reason we call this pattern a short pulse train.

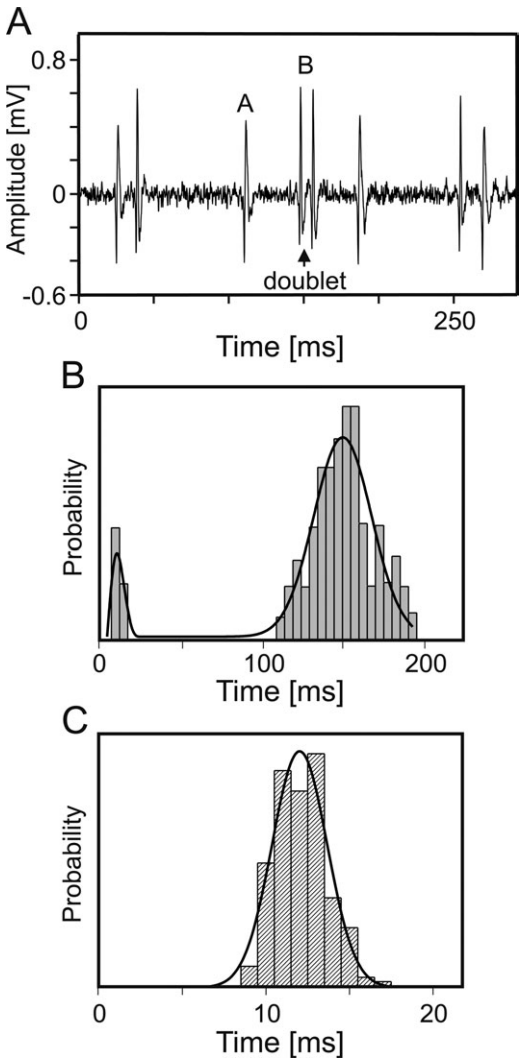

Fig. 2. **A**: Recording of the tonic activity of neurons A and B in the lateral external nerve n5B2c of the locust leg at a fixed femoro-tibial angle of 100°. At the center, a doublet in neuron B is observed. **B**: Histogram of IPI for neuron B. The peak to the left corresponds to doublets whereas the peak to the right centered at 150 ms corresponds to singlets. **C**: IPI distribution for doublets of unit B. The statistics corresponds to 400 doublets recorded from 10 different samples.

Constant IPI in doublets

We measured the IPI in doublets of neuron B at two different points separated by 5 mm along the nerve. In most of the cases, (about 90%, i.e., 360 doublets) we found that this IPI is constant within the sampling resolution which was taken as two-time resolution units (see Fig. 3). The conduction velocity for both the preceding and succeeding pulses is the same as for singlets of the corresponding neuron.

Increase of IPI in doublets

In a few cases (7%, i.e., 28 doublets), a particular phenomenon was observed: The IPI of two pulses of a doublet with nearly the same shape (same amplitude and duration from peak to trough) increases by about eight-time resolution units as pulses propagate, corresponding to 0.3 ms as shown in Figure 4 (top). The conduction velocity of the preceding pulses in neuron B is 0.78 ± 0.01 m/s, which is nearly the same for singlets of the same neuron, while the speed of the succeeding pulses is 0.74 ± 0.03 m/s, which is slower than that of singlets. Since it was determined through a detailed analysis (Hoyle, 1955) that the diameter of the axon is constant and we determined that IPI in singlets is constant (and even in some doublets), we cannot claim that this IPI variation is a function of the position along the axis of the nerve because this would imply that the axon is geometrically irregular which is not the case.

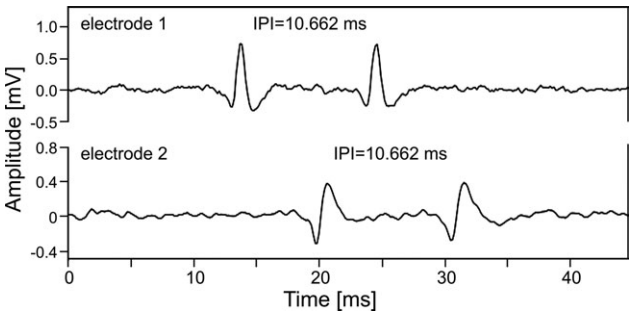

Fig. 3. Recording of a doublet of neuron B at a fixed femoro-tibial angle of 90°. Electrode 1 records: amplitude of both pulses 0.7301 V, duration from minimum to maximum D_{mM} (1) = 0.744 ms, D_{mM} (2) = 0.8928. (The difference is one resolution unit). IPI = 10.662 ms. Electrode 2 records: amplitude (1) = 0.3882 V, amplitude (2) = 0.3996 V; D_{mM} (1) = 0.744 ms, D_{mM} (2) = 0.10416 (the difference is of two resolution units). IPI = 10.662 ms.

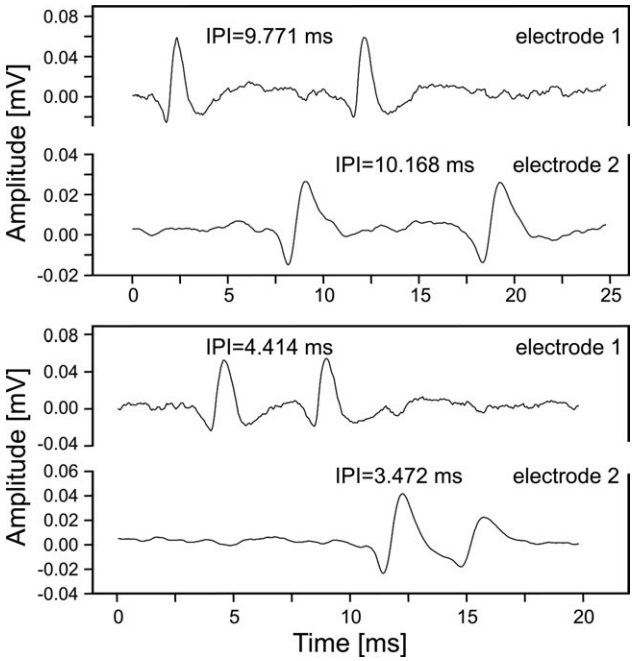

Fig. 4. Recording that shows IPI variation of doublets in neuron B at a fixed femoro-tibial angle of 100°. Top: IPI increase of 0.3968 ms. Bottom**:** IPI reduction of 0.9424 ms. Note the change in shape of the succeeding pulse.

Reduction of IPI in doublets

In 3% (12 doublets) of the double pulses analyzed for neuron B, we found that the two pulses of a doublet with nearly the same amplitude and duration recorded with the first electrode reduce their IPI as they travel along the axon as recorded by the second electrode placed at a distance of 5 mm from the first one. Figure 4 (bottom) shows an IPI reduction of a doublet from 4.414 to 3.472 ms. The speed of the preceding pulse is about 0.78 m/s, which is slightly below the value for the singlets in the same neuron but that of the succeeding pulse is 0.82 m/s, which is above the normal value. In addition, a change in shape (amplitude and duration) is observed. This example, however, is an exception to the overall behavior of attraction since most of the approaching doublets appear with an IPI of 12–16 ms. As a general statistic, the conduction velocity of the preceding pulses in this case is $\nu = 0.78 \pm 0.01$ m/s while for the succeeding pulses is 0.82 ± 0.03 m/s. Unfortunately, the reduction in IPI in this biological model occasionally appears, and it becomes difficult to determine a correlation of velocities ratio as a function of the pulse separation.

The CPR in crayfish

Recordings from the sixth abdominal ganglion show spontaneous and multiunit activity in the dark. By a careful selection and examination it is possible to analyze the activity of the individual neuron identified as CPR which presents doublets with an average IPI of 11 ms as shown in Figure 5 (top).

Variation in IPI

We have carried out the analysis of IPI distribution of 253 doublets taken from a representative crayfish and recorded with two electrodes separated by 34 mm along the CPR. Figure 5 (middle) shows comparative histograms with 2 ms bins corresponding to IPI distributions recorded by the electrodes A and B. If both histograms were identical, it would imply that IPI remains constant, however, by simple inspection, one may observe slight differences, meaning that IPI changes in some cases by more than 2 ms. In an attempt to understand whether the IPI increases or decreases, the scatter plot of the change in IPI *versus* initial IPI is depicted in Figure 5 (bottom). All the points on the horizontal axis on level zero of the ordinate correspond to doublets whose IPI remains constant, i.e., IPI change is zero. On the other hand, the higher density of points is found at an initial IPI between 8 and 9 ms. Most of those points have a change in IPI of about 0.5 ms, however, there are points with an IPI change of about 3 ms and even more, with an IPI change of −2 ms which means IPI reduction. A similar behavior is shown by the doublets with initial IPI of 11 ms. Since doublets IPI between 8 and 11 ms may increase or decrease, we may state that changes in IPI are independent of the initial IPI.

The obtained conduction velocity for singlets (or double pulses separated by more than 25 ms) in the CPR was 4.25 ± 0.2 m/s. Speed of pulses in doublets, which maintain a constant IPI during propagation fall within this interval of values. We also measured the conduction velocity for both impulses of the doublets, which present an increase in IPI, obtaining a mean for the first impulses of 4.15 ± 0.9 m/s whereas the mean conduction velocity for the second impulses is of 4.04 ± 0.6 m/s. The conduction velocity of the

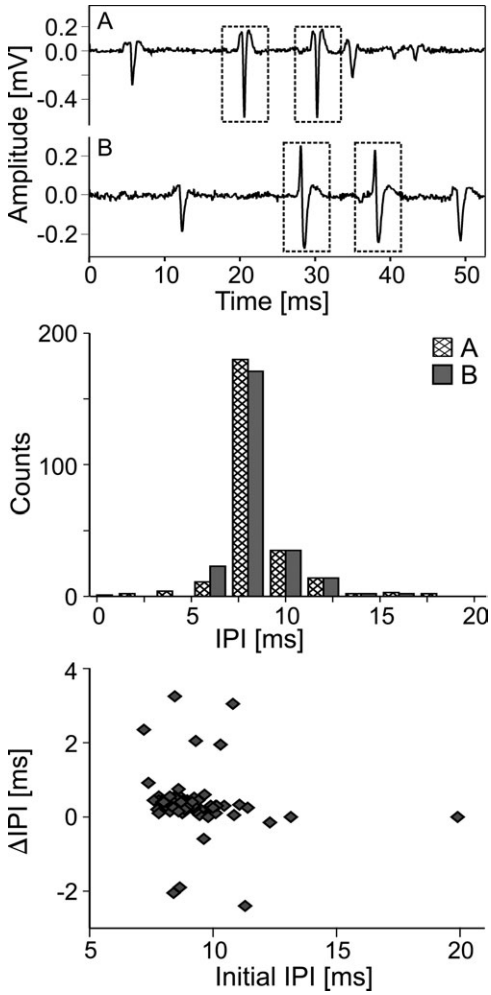

Fig. 5. Top: Electrophysiological recordings from the CPR that shows the spontaneous activity in the dark in the isolated chain of six abdominal ganglia of crayfish. Electrode A records the spontaneous multiunit activity in the proximal region of the sixth abdominal ganglion. Note the presence of the doublets in the neuron identified as CPR. Electrode B, placed at a distance of 34 mm from A, records the spontaneous multiunit activity in the anterior region of the chain abdominal ganglia. Also, the presence of the doublets in the firing of action potentials from CPR is shown. Middle: IPI distribution histograms of doublets recorded from CPR. **A**: Data analysis from the proximal region to the sixth abdominal ganglion, the electrode is in the zone "A." **B**: Data obtained from the second electrode, at the anterior region or place "B." The statistics corresponds to 253 doublets lumped from one representative animal. Bottom: Scatter plot of ΔIPI versus initial IPI recorded from the CPR of the crayfish. Positive values on the vertical axis indicate IPI increase, whereas a negative value IPI reduction (see text).

preceding pulses for doublets with IPI reduction is 4.15 ± 0.9 m/s, whereas that of the succeeding pulses is 4.3 ± 0.7 m/s.

Motor neuron 3 in crayfish

Nerve 3 exhibits a spontaneous activity of six neurons. However, it is possible to distinguish clearly the activity of all of them by amplitude. Figure 6 (left) shows the appearance of doublets and even triplets, whereas Figure 6 (right) shows the corresponding histograms for IPI distribution in three of these neurons labeled as class A1 (small amplitude), class A2 (medium-sized neuron), and class A3 (larger amplitude). By correlating the recording and the histograms, we may observe that the larger the amplitude the shorter the mean IPI in doublets for these three neurons.

To characterize the individual pulses of a doublet, we measured the time from negative to positive peak of each spike, D_{mM}. In Figure 7, we observe the D_{mM} for three neurons of the nerve 3. As shown, unit class A3 contains pulses with a mean D_{mM} of 0.87 and 0.90 ms for the preceding and succeeding pulses respectively; unit class A2, a mean D_{mM} of 0.96 and 1.1 ms for the first and second pulses respectively; and unit class A1, a mean D_{mM} of 1.05 and 1.11 ms for first and second pulses respectively. This result is of major importance since it implies that pulses in a doublet are not identical as it would be expected since both belong to exactly the same neuron, but they are slightly different in shape.

Variation of IPI in doublets

After analyzing 1000 doublets (taken from 10 animals) belonging to the neuron class A2, we found the following behavior: 14% of doublets maintain constant IPI, independently of the initial IPI at which pulses are generated; 2% of doublets reduces their IPI independent of the amplitude ratio, i.e., pulses may have the same or even slightly different amplitude. The remaining 84% of the doublets increase their IPI as they travel along the nerve axon.

The calculated conduction velocity for singlets in the neuron class A2 turned out to be 2.405 ± 0.008 m/s. The calculated conduction velocity of the preceding pulses in doublets of neuron class A2 with an increase in IPI is 2.407 ± 0.006 m/s whereas of the succeeding pulses the obtained value is 2.395 ± 0.008 m/s. In the case of IPI reduction, the speed of the succeeding pulses is 2.407 ± 0.006 m/s while that of the succeeding pulses is 2.415 ± 0.009 m/s. We have chosen neuron class A2 for this analysis because the number of doublets spontaneously generated is greater than that of the other neurons in nerve 3.

DISCUSSION

Doublets have been observed in several biological systems. This work represents the first attempt to analyze their dynamics during propagation in the nerve axon of a neuron. To better understand the origin of short pulse trains and compare their internal dynamics, we have studied three biological systems and obtained those patterns in a natural way, namely, physiological conditions and without electrical stimulation. This means that we have control neither over the pulse shape nor over the

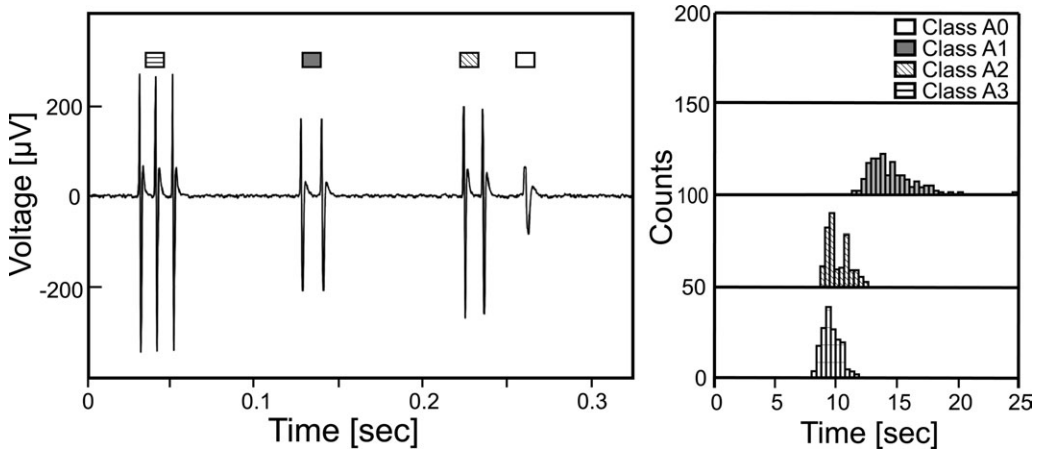

Fig. 6. Left: Spontaneous activity of four neurons in nerve 3 in the crayfish (*C. quadricarinatus*) thorax. Unit class A3 exhibits a triplet, whereas units class A1 and class A2 exhibit doublets. Right: Histograms of IPI distribution for units class A1, class A2, and class A3, as it is indicated, respectively.

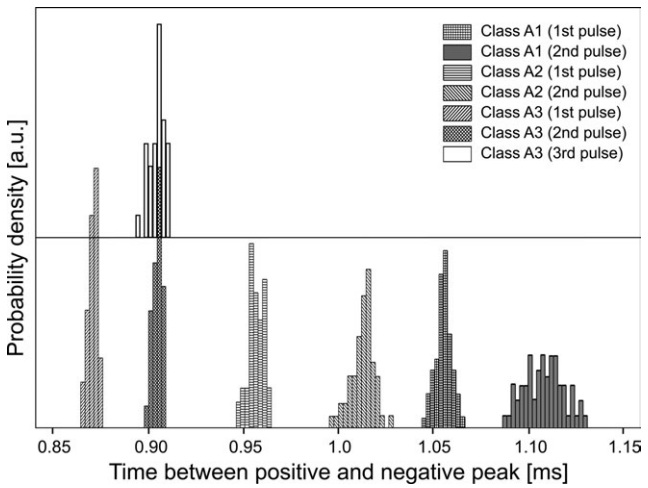

Fig. 7. Histograms of time interval distribution corresponding to duration from minimum to maximum of pulses in doublets and triplets for three neurons in the crayfish motor nerve 3. The histograms correspond to the preceding and succeeding pulses of doublets of the neuron class A1, neuron class A2, and triplets in neuron class A3, as it is indicated, respectively. Analysis corresponds to 1000 doublets from 10 animals.

separation between spikes. Since doublets and even triplets originate in single neurons from the process of sensory mechanotransduction in a system and spontaneous activity in the other, we may state that their appearance is independent of the external process by which they are triggered.

Interestingly, a common feature that doublets share in these systems is that the mean IPI is about 11–14 ms [see Figs. 2B, 5 (middle), and 6 (right)], which allowed us to study the various characteristics of these patterns by analogy, regardless of their origin.

Prior to analyzing the shape, speed and IPI variation of doublets, it was essential to carefully discard the sources of misinterpretations. Histological studies carried out for neurons of the locust femoral nerve (Hoyle, 1955) indicate that these units possess the same characteristic radius along the axon, in other words, they are geometrically regular. Thus, the differences observed in the shape and velocities of the pulses are due to intrinsic aspects of the neurons which will be studied in a future work.

As a starting point, we analyzed the singlets, i.e., pulses separated by more than 30 ms. As shown in Figure 1, the IPI between successive singlets of the same unit is constant within the sampling resolution as recorded by two electrodes. In other words, these individual pulses travel at a constant velocity.

Concerning the short pulse trains such as doublets and triplets, we observed three patterns as they travel along the nerve axon: (i) Constant IPI, (ii) increase in IPI, and (iii) reduction in IPI. The fraction of each of the three patterns varies in the three models studied. Regarding the mechanisms underlying those differences, we consider that it would be interesting to include this topic in future research protocols.

The results showed that the separation of doublets tends to a stable configuration, namely, the distance at which both pulses travel without changing IPI. This is illustrated in Figure 5 (bottom), where it is shown that the amount of doublets with initial IPI larger than 12 ms is quite low, and those with initial IPI between 12 and 20 ms have a change in IPI around zero which is a stable configuration. Moreover, the refractory period for the CPR is about 12 ms (Kennedy, 1963). Since about 95% of the doublets possess an initial IPI less than 12 ms, we propose that it could be possible to consider the refractory period only as a reference point for the mean IPI of doublets in the specific neuron.

The amount of doublets with IPI reduction is very low in the three models studied here, however, it is

worth mentioning that this behavior was predicted theoretically by several authors (Donati and Kunov, 1976; Evans et al., 1982; Feroe, 1982; Hastings, 1982; Yanagida, 1987, 1992) who suggested that the equation introduced by FitzHugh (1969) may have double pulse solutions which are close to the superposition of two *widely* separated single pulse solutions. This phenomenon of attraction has also been observed in the squid nerve axon by electrical stimulation (Scott, 1982; Scott and Vota-Pinardi, 1982), the velocity of the succeeding pulse is larger than the mean velocity for the axon, for this reason it was termed "supernormal speed."

As for the potential implications that the changes in interspike interval patterns during action potential propagation could have for understanding the information processing in nervous systems, we suggest that IPI changes could play an important role in the process of temporal pattern recognition by a postsynaptic neuron which may be considered as a decoding process. Harris and Stark (1973) investigated the synaptic delay and its effect on information transmission in the crayfish CPR system. As mentioned above, they obtained single and double pulse patterns for which they determined the probability distributions for the change in length of an IPI due to synaptic transmission. The synaptic delay was found to depend on the length of the interval preceding the pulse. The mean delay was found to vary from 2.9 ms (for pulses following intervals longer than 94 ms) to 4.4 ms (for pulses following intervals of <9.4 ms). Our findings explain those results in a direct way because, according to our analysis, the mean delay of 2.9 ms for pulses with intervals longer than 94 ms is due to the separation between electrodes which are placed at the presynaptic and postsynaptic neurons separated by a synaptic link, but the delay of 4.4 ms for pulses with intervals <9.4 ms, which we consider doublets here, is caused by both the separation of electrodes and the change in IPI that such pulses undergo, explicitly speaking, the larger delay is caused by the slower velocity of the succeeding spikes of the pair.

It is important to stress the fact that in their investigation, Harris and Stark (1973) observed a near one-to-one correspondence between the impulse trains at the two recording sites. According to Kumar et al. (2010) it is vitally important that spiking activity can propagate from one module (or neuron) to the next while preserving the information it carries.

Now, we come back to the previously mentioned experiments carried out by Houweling and Brecht (2008) in which they demonstrated that the stimulation of single neurons affects behavioral responses in a detection task. The neural code they obtained consisted of a 200-ms pulse train containing 14 spikes, and a mean IPI of about 12 ms, thus probably some doublets exist in that pattern. Interestingly, the authors suggested further studies to establish how the frequency and number of action potentials are related to the evoked sensations. At this point we would like to add: While spikes are propagating, how the change in IPI could also be related to the evoked sensations?

We are convinced that the change in temporal codes during propagation opens up a whole new realm of possibilities for neural coding including processes where the spike timing is involved, specifically in the novel concept of cellular learning, where the temporal order instead of frequency is emphasized, known as Spike Timing Dependent Plasticity.

To conclude, we summarize the following:

(1) Doublets exist in the six neurons of the three biological models analyzed here. These two pulse trains originate in the process of sensory mechanotransduction in neurons of the locust femoral nerve and spontaneous activity both in the abdominal motor neurons and the CPR of the crayfish. (2) Single unidirectional pulses, separated by more than 30 ms, in geometrically regular neurons, considering a uniform diameter along the axon, travel at a constant speed and they do not change their shape while propagating. (3) Two pulses making up a doublet with IPI variation travel at different speeds in the same nerve axon. Therefore the temporal codes change during propagation.

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