รายงานวิจัยฉบับสมบูรณ์

โครงการ “วิธีการศึกษาวิธีใหม่ที่ใช้ในการคาดคะเนความสัมพันธ์บริเวณรอยต่อของเซลล์ประสาทชนิดที่มีระดับการกระตุ้นต่ำของเส้นประสาท common peroneal และเซลล์ประสาทยนต์ที่เกี่ยวกับการเคลื่อนไหวในมนุษย์”

โดย

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11 กรกฎาคม 2551

สัญญาเลขที่ MRG4980181

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คณะผู้วิจัย	สังกัด
1. ผศ.ดร. อรวรรณ ประศาสน์วุฒิ	**ภาควิชากายภาพบำบัด คณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่**
2. Binboga Erdal	**Department of Biophysics Ege University, Izmir, Turkey**
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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

บทคัดย่อ

การศึกษานี้ คณะผู้วิจัยต้องการผลการตอบสนองของรีเฟกซ์ (H-reflex) โดยทำการกระตุ้นเซลล์ประสาทยนต์ tibialis anterior motor units ในมนุษย์ และวิเคราะห์ผลการตอบสนอง ด้วยวิธีการวิเคราะห์ข้อมูล 2 วิธี วิธีที่ 1 เป็นวิธีแบบดั้งเดิมที่ใช้กันมานาน ที่เรียกว่า Peristimulus time histogram (PSTH) และวิธีที่ 2 เป็นวิธีการแบบใหม่ เรียกว่า Peristimulus frequencygram (PSF) คณะผู้วิจัยเชื่อว่าวิธีวิธีการวิเคราะห์ข้อมูลแบบ PSF เป็นวิธีที่มีความน่าเชื่อถือสูง เหมาะสมที่จะใช้ในการคาดคะเนความสัมพันธ์ที่บริเวณรอยต่อของเซลล์ประสาทยนต์ ดังนั้น คณะผู้วิจัย ต้องการเปรียบเทียบความแตกต่างระหว่างผลการวิเคราะห์ทั้งสองแบบ

อาสาสมัครสุขภาพดี ไม่มีความผิดปกติใดๆ ที่เกี่ยวข้องทางระบบประสาท จำนวน 11 คน เป็นชาย 7 คน หญิง 4 คน โดยอาสาสมัครนั่งบนเก้าอี้ที่มีพนักพิงรองรับ และได้รับการกระตุ้นโดยกระแสไฟฟ้าที่บริเวณเส้นประสาท common peroneal ในแต่ละครั้งของการศึกษา กระแสไฟฟ้าจะถูกปล่อยออกมากระตุ้นประมาณ 600 ครั้ง โดยปล่อยออกมาในลักษณะการสุ่ม ทุกๆ 1-2 วินาที บันทึกการเปลี่ยนแปลงทั้งคลื่นสัญญาณไฟฟ้าของกล้ามเนื้อ และบันทึกศักย์ไฟฟ้าของเซลล์ประสาทยนต์

ผลการศึกษาพบว่า การวิเคราะห์ข้อมูลแบบ PSF ให้ผลที่แตกต่างจากการวิเคราะห์แบบ PSTH โดยทันทีหลังการตอบสนองของรีเฟกซ์ การวิเคราะห์ข้อมูลแบบ PSTH พบช่วงเวลาที่มีการลดลงของการตอบสนองที่เรียกว่า silent period ซึ่งมีความหมายเป็นนัยว่าความสัมพันธ์บริเวณรอยต่อนั้นมีลักษณะของการยับยั้งเกิดขึ้น ในขณะที่การวิเคราะห์ข้อมูลแบบ PSF จะพบความต่อเนื่องของการเร้าหรือการกระตุ้น ดังแสดงจากการพบว่ายังมีศักย์ไฟฟ้าเกิดขึ้นอยู่ และความถี่ในการส่งกระแสประสาทสูงกว่าระดับก่อนการกระตุ้น นอกจากนี้ จากการวิเคราะห์ข้อมูลแบบ PSTH ในช่วงเวลาต่อมาพบว่าเกิดการเร้าหรือการกระตุ้นขึ้นอีก ที่เรียกว่า late excitation โดยสังเกตจากการเพิ่มขึ้นของการตอบสนอง ตรงข้ามกับการวิเคราะห์ข้อมูลแบบ PSF พบว่ามีผลการยับยั้ง โดยวัดได้จากการลดลงของความถี่ในการส่งกระแสประสาทเมื่อเทียบกับก่อนการกระตุ้น

เมื่อพิจารณา ผลการวิเคราะห์ที่แตกต่างกันของทั้งสองวิธี ร่วมกับผลการศึกษา Brain slice ในสัตว์ทดลอง สรุปได้ว่า วิธีการวิเคราะห์ข้อมูลแบบ PSF เป็นวิธีที่เหมาะสมในการนำมาใช้เพื่อคาดคะเนความสัมพันธ์แบบสุทธิระหว่างตัวรับความรู้สึกและเซลล์ประสาทยนต์ในระบบประสาทของมนุษย์

Abstract

Project Code : MRG4980181

Project Title : A study of synaptic connection between low threshold afferent fibres in common peroneal nerve and motoneurones in human tibialis anterior

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Project Period : 2 years (From July 1, 2006-June 30, 2008)

We have induced H-reflex responses in human tibialis anterior motor units and analysed the results using the classical technique, peristimulus time histogram (PSTH), and a new technique, peristimulus frequencygram (PSF). The PSF has recently been shown to be more reliable than the PSTH for indicating the synaptic connections on motoneurons, and therefore we wished to examine the differences between the two analysis methods.

Experiments were conducted on eleven healthy subjects (7 males and 4 females) that do not have any known neurological disorder. The subjects sat comfortably on a dental chair and the common peroneal nerve was stimulated. In each experiment, about 600 stimuli were applied randomly every 1-2 s. The recordings were taken with both by surface electromyogram (sEMG) and as single motor unit potentials.

Our findings suggest that PSF gives significantly different results compared with the PSTH in determining the synaptic connection of the low threshold nerve fibres to the motoneurons. While PSTH indicated that there was a silent period immediately after the

H-reflex, the PSF demonstrated that the silent period was actually a continuation of the net excitatory effect and not a genuine inhibition since the small number of action potentials occured during this period displayed higher discharge rates than the prestimulus level. Furthermore, the late excitation, as it was noted in the PSTH; was actually a net inhibitory effect since the large number of spikes that occured during that period had lower discharge rates than the prestimulus average.

In the lights of the recent brain slice findings and completely different results obtained using the two analysis techniques, we suggest that the PSF analysis should be used to illustrate the net synaptic connection between peripheral receptors and motoneurons in the human nervous system.

Keywords: peristimulus time histogram, peristimulus frequencygram, synaptic connection, excitatory postsynaptic potentials, inhibitory postsynaptic potentials

เนื้อหางานวิจัย

PROBLEM:

Via injecting current pulses into regularly discharging motoneurons in rat brain slice preparations, we have recently illustrated that all currently used methods for estimating pathways from peripheral receptors to the central nervous system contain errors (Türker and Powers 1999, 2001, 2002, 2003; reviewed in Türker and Powers, 2005). Therefore, in the human nervous system, the 'established' pathways are likely to be erroneous and need to be corrected urgently since the properties of these pathways are commonly used for diagnosis and determining the progress of treatment for many neurological disorders including lesions in the supra-segmental areas of the brain (Nadler et al., 2002, 2004).

SIGNIFICANCE OF THE STUDY:

This study could correct apparent errors in the most commonly studied and firmly 'established' pathway in the central nervous system. This correction needs to be done urgently since the existence, stability, strength and sign of pathways in the brain and spinal cord are used for diagnosis, treatment and assessment of the success of treatment of many neurological disorders (Cruccu, 1989; Chen et al, 1998; Nadler, 2004) especially in the elderly (Nadler et al, 2002).

Errors in the currently used techniques arise from the fact that they are indirect and rely upon the probability of spike occurrence in the surface or intramuscular EMG records (Fig. 1) to estimate the sign of connections (excitation/inhibition) between receptors and neurones in the central nervous system. In brain slice experiments, we have clearly shown that instantaneous discharge rate analysis (PSF) corrects these errors that are inherent in the probability-based methods and hence offers a new approach to reinvestigate 'previously-established' neuronal connections (Türker and Powers 1999, 2001, 2002, 2003).

AIMS

In this study, we aimed to test three hypotheses; firstly that the low threshold muscle afferents in the common peroneal nerve are connected to the homonymous motoneuones by only one pathway that is excitatory. Secondly, that the silent period that has been claimed to exist after the H-reflex response, is a net excitatory response. Thirdly, that the long-latency excitatory reflex response is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

BACKGROUND

Estimating the strength of synaptic connections between receptors and neurons in the human central nervous system has been a great challenge since direct recordings are impossible. Consequently, the networks between neurons are often expressed as a ‘black box’. Attempts to crack the black box utilize indirect methods in which a particular receptor system is stimulated and the responses of neurons that are affected by the stimulus are recorded to estimate the properties of the circuit. These experiments, although indirect and open to various interpretations, have claimed to discover the pathways that are activated when certain parts of the nervous system are stimulated. Such claims have been subject to a close examination by many investigators using similar stimulating and recording methods, and the connections of a number of receptors to the central nervous system have been ‘established’.

The classical studies in which the connection between receptor systems and the central nervous system are investigated involve electrical or mechanical stimulation of a selected receptor system and recording of the resultant changes in the muscle electromyogram (EMG) using surface and/or intramuscular electrodes. There are several techniques for quantifying these responses. The two most common are full-wave rectification and averaging of the EMG record around the time of stimulation (Jenner and Stephens 1982) and compiling peristimulus time histograms (PSTHs) from single motor unit (SMU) records (Stephens et al. 1976). These techniques are based on the principle that a significant increase in the occurrence of discharge (spike) probability (hence the term: probability-based analyses) at a time after the stimulus represents an excitatory postsynaptic potential (EPSP) and a significant decrease indicates an inhibitory postsynaptic potential (IPSP). This assumption has led to serious errors to the description of the patterns of connections between various elements of the central nervous system. These arise from the fact that the peaks and troughs in averaged EMGs and PSTHs which represent increases and decreases in spike probability, can reflect not only direct synaptic effects, but also secondary effects arising from the discharge statistics of the pre- and postsynaptic cells (Moore *et al.* 1970). Despite this early warning about the problems in the probabilistic analysis method, the peaks and troughs in an averaged response to a stimulus are still defined as synaptic excitation and inhibition, respectively (e.g., Brooke et al. 1999; Okdeh et al. 1999; Sonnenborg et al. 2000). This has lead to major misunderstandings about the nature of circuits in the human nervous system, which need to be corrected.

To show conclusively that the probability based analyses contain inherent errors and to put forward an error free method for the estimation of synaptic potential, and hence the wiring diagram, we have recently conducted a series of experiments on regularly discharging motoneurons in rat brain slices.

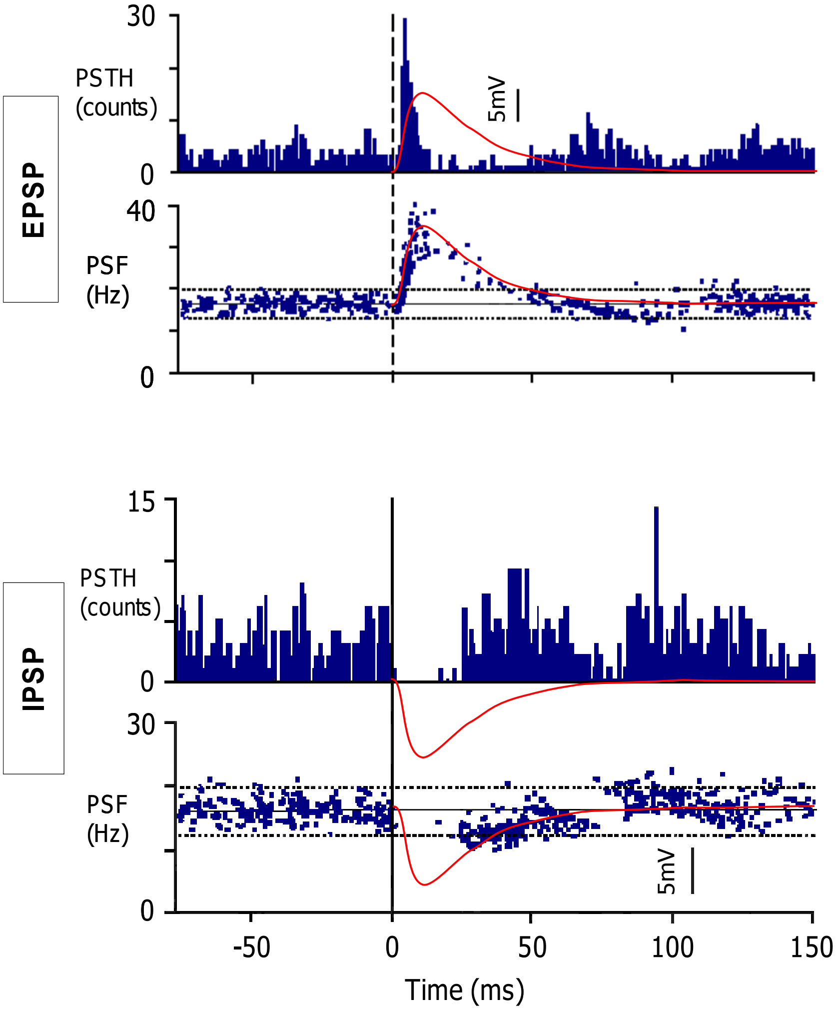

Figure 1: PSTH versus the new method (peristimulus frequencygram, PSF) in response to a known synaptic potential: Top diagram is produced by an EPSP (continuous line) with a rise time of 5ms that is injected into a regularly discharging motoneuron in a rat brain slice. The output of the cell is plotted using the classical method that uses probabilistic analysis (PSTH) and the PSF. PSTH users would have claimed five significant events due to peaks and troughs in the record. Hence five different neural pathways would have been claimed even though the injected synaptic potential contained only a simple EPSP (i.e., only an excitatory pathway between the stimulated nerve and the motoneuron. However, the PSF record shows that the discharge rate of the cell closely follows the time course of the injected EPSP.

Similarly an IPSP also induced several troughs and peaks in the record ‘indicating’ that many pathways exist between the stimulated nerve and the motoneurone. Again, the PSF record shows that the discharge rate of the cell closely follows the time course of the injected IPSP other than the peak of the current where no discharge is possible (redrawn from Türker and Powers, 2005).

In these experiments, we injected simple and complex current transients into regularly discharging hypoglossal motoneurones *in vitro* (Türker and Powers 1999). We examined the discharge output of these cells using both methods of analyses to identify which of the two more closely represented the profile of the postsynaptic potential (Fig. 1). We found that in a regularly discharging motoneuron, the rising phase of an EPSP moves the occurrence of spikes forward and hence induces a substantial peak in all probability-based records. This peak is followed immediately by a period of reduced activity ('silent period') during the falling phase of the EPSP due to the phase advance of spikes that were to occur in this period (count-related errors). The usual probability-based analyses would have led to the conclusion that the stimulus activated an excitatory pathway that was then followed by activation in an inhibitory pathway, i.e., that the EPSP was followed by an IPSP. Furthermore, secondary peaks and troughs would have led to the conclusion that the same stimulus also activated long latency excitatory and inhibitory pathways that may have been transcortical (due to the long time delay from the stimulus onset and the response). As this figure clearly illustrates, other than the sign of the very first pathway (excitatory), our claim regarding all other pathways would have been wrong since the changes in neural discharge are all the result of the initial phase advance of the action potentials by the beginning of the EPSP and their autocorrelation function: this was predicted by Moore *et al* as early as 1970.

Similarly, a stimulus that evoked a compound inhibitory post synaptic potential (IPSP) would also have led to erroneous conclusions since the falling phase of the IPSP delayed spikes which then occurred during the rising phase of the IPSP. During the delay, the probability-based analysis displayed a gap that would have correctly been interpreted as indicating an inhibitory pathway. However, since a substantial peak occurred in the PSTH due to the occurrence of the delayed spikes, this peak would have appeared to indicate an excitatory pathway. The PSTH records also induced secondary and tertiary peaks and troughs due to synchronization of the spikes in relation to the stimulus (synchronization-related errors). Again wrongly, these would have been labelled as long latency inhibitory and excitatory pathways. The brain slice experiments also clearly illustrated that the frequency-based analysis gave a better indication of the underlying synaptic potentials than the probability-based analyses and that it avoided the errors that are inherent in probability based analysis.

APPROACH:

We tested the most well known and claimed to be ‘established’ neural pathway using both classical probability based and the frequency based analyses.

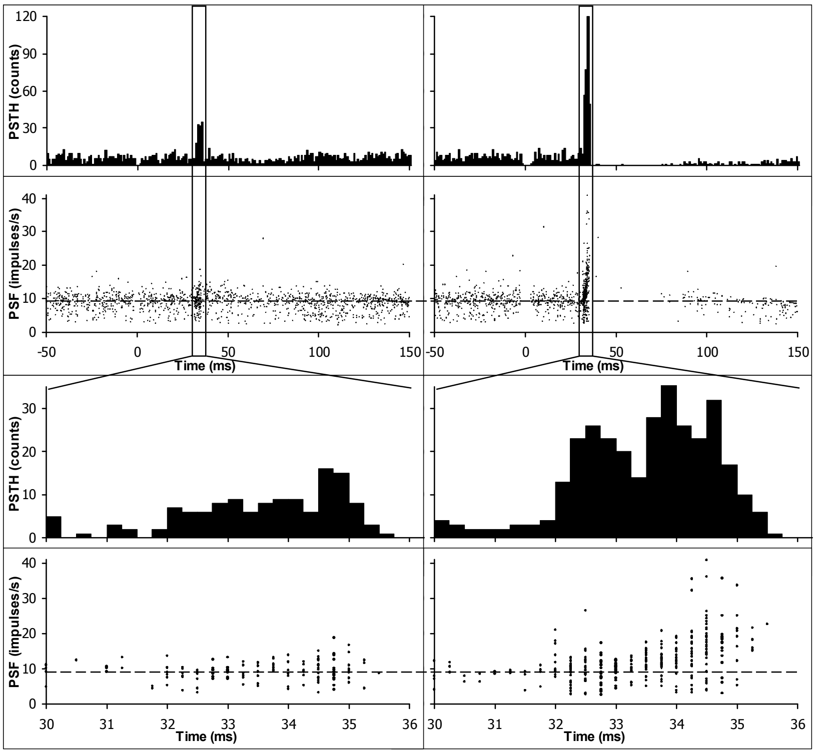

Figure 2: PSTH and PSF for common peroneal nerve stimulation: PSTH and PSF analysis of the same motor unit from the tibialis anterior during weak (left) and strong (right) electrical stimulation of the common peroneal nerve. Dashed horizontal lines show the mean firing frequency. Both trial durations were 30 minutes (approximately 880 stimuli). Frequency analysis shows that the reduction in unit firing probability after the initial excitation is not associated with a reduced firing rate, hence is an artefact not a true inhibition. A close-up view of the H-reflex (bottom section) shows a steady increase in both the occurrence rate and instantaneous frequencies in response to the weak stimulation. Strong stimulus, however, showed a dip approximately 1ms after the commencement of the reflex in both the PSF and PSTH. It is possible that this trough corresponds to activation of Ib fibres.

Wiring of the low threshold afferent fibres in the common peroneal nerve to the motoneurones that innervate the tibialis anterior muscle: We examined this circuitry that has been most intensely examined in human motoneurons. It is well ‘established’ that low intensity stimulation of the common peroneal nerve generates large compound EPSPs in the human tibialis anterior motoneurons, which is the basis for the H-reflex (the electrical equivalent of the stretch reflex; Táboȑková and Sax 1968; Katz et al. 1988). However, it has been claimed that the same low-intensity stimulus also activates the Ib (Golgi tendon organ) fibres that evoke strong IPSPs in motoneurons and hence cut short the expression of the EPSP generated by the Ia activation (Burke et al. 1983; Burke et al. 1984). From this concept, the gap in spike probability immediately after the initial peak (silent period) has been claimed to illustrate the existence of the Ib IPSP. Hence, it has been claimed and widely used that the Ia activation alone can be studied only during the initial peak (Pierrot-Deseilligny 1996). However, our brain slice work (Fig.1) has already established that a single compound EPSP will generate a reduced probability of spike occurrence during its falling phase. Therefore, it is possible that the so-called Ib IPSP may actually be an artefact of the probability-based analyses. On the other hand, it is also possible that the gap may be generated by a genuine IPSP. Our slice studies have shown that a single EPSP and an EPSP followed by an IPSP generates very similar probability-based records (Türker and Powers 1999; Türker and Powers, 2003). However, when it is used in combination with the PSF, we were able to distinguish between the excitatory and inhibitory PSPs. Therefore we plan to test whether or not a genuine IPSP follows the Ia EPSP by using the PSF along with the classical probability based methods. Preliminary experiments at low stimulus intensities have shown that, while only a few spikes occur during the silent period, their discharge rates are higher than the background rate, indicating that there is no inhibition at low stimulus intensities. However, we have also noticed that when the stimulus intensity is high, the number of occurrences at the H-reflex latency becomes less than the spikes that could be counted as phase-advanced. Close inspection of the PSTH and PSF around the H-reflex latency shows a ‘dip’ in the increase of both occurrence and frequency about 1ms after the start of the reflex response to a large stimulus, a phenomenon that is not present when a small stimulus is used (expanded scale, bottom of Fig. 2). The reduction in the expected spike numbers and frequency 1ms after the commencement of the H-reflex may be caused by the activation of Ib axons injecting IPSPs into the same motoneuron. Therefore it is necessary to study the response of the tibialis anterior motoneurons at various levels of stimulus intensities to pinpoint whether, and at what stage, Ib axons are activated. There is a limit to the increase in the stimulus intensity that can be used since very large stimuli will recruit some previously silent units at the H-reflex latency and make the recognition of spikes difficult. However, we will use selective recording methods so that superimposition is limited (see Methodology below).

METHODOLOGY:

Subjects: For the study, eleven healthy subjects who provided written informed consent were recruited. The protocol of these experiments was approved by the Human Ethics Committee of the University of Adelaide, Chiangmai University and conformed to the Declaration of Helsinki. The subject sat on the comfortable chair. A footrest was designed to support the right foot, positioned at 0^O plantar flexion, and provided the resistance required to produce large levels of voluntary dorsiflexion.

Recording

General protocol: All data were recorded on computer sampling at 15,000 samples/second using a CED system. Zero-phase band pass filtering was performed on the recorded data as the first step during off-line analysis. The stimuli were delivered randomly between 1 and 2 seconds in all studies. These triggers for the stimuli were seen in Figure 3 at the top panel.

Surface EMG: Two SEMG recording electrodes were used. They were placed 2 cm apart lengthwise on the belly of the muscles. The SEMG was recorded from the right tibialis anterior muscle. The bipolar surface EMG outputs were amplified (1,000 to 10,000 times) and high pass filtered (5Hz) to reduce movement artefacts. The impedance of the surface electrodes was set below 10kΩ; and subjects were grounded by lip clip electrodes (Türker et al. 1988). An example of sEMG recordings was showed in Figure 3 at the bottom panel (TA-sEMG)

Single unit EMG: Activity of single motor units was recorded from the tibialis anterior muscle using bipolar wire electrodes. To achieve this, a pair of Teflon® insulated silver wires (100µm diameter with insulation; 70µm core diameter) was inserted into the muscle with a 25G needle. The needle was then withdrawn, leaving the wires in the belly of the muscle. To insure that the wires did not move from the original recording site, the ends of the wires were "fish hooked" and the subject was asked to contract the muscle a number of times so that the electrodes are firmly embedded in it. However, to ensure that the activity of only a few units was recorded from each electrode, the insulation was not removed except at the cut end of the wire. The SMU recordings were amplified (1,000 to 10,000 times) and high pass filtered (5Hz). An example of SMU recordings was seen in Figure 3 at the third panel (TA-SMU).

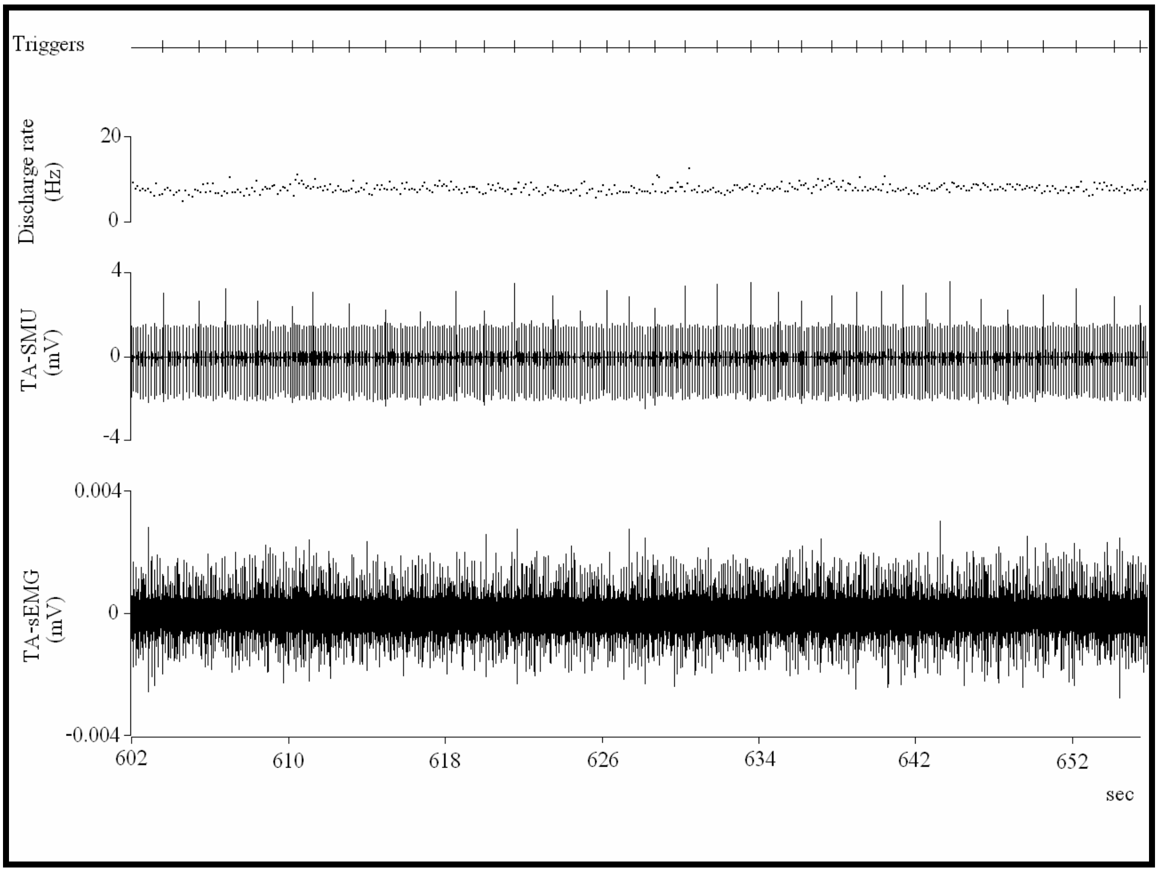

Figure 3 illustrates a short section of a recording session. The top panel (triggers) shows the timing of the electrical stimulation to common peroneal nerve. The second panel shows the discharge rate of an identified single motor unit; the third panel shows the single motor unit potentials recorded from right tibialis anterior. The bottom panel shows the sEMG of the right tibialis anterior.

Feedback of voluntary contraction level: During experiments SMU frequency data were used for feedback. Single motor unit potentials were discriminated on-line using microprocessor-based waveform analyses method, which matched the shapes of the unit potentials to pre-established templates (8701-Signal Processing Systems; Türker et al. 1989). Acceptance pulses from the discriminator were converted to discharge frequency and were displayed in front of the subject. The subject was asked to keep the discharge rate at around 10Hz. Most subjects can achieve this with little training.

Nerve stimulation: Up to six hundred stimuli were delivered. Two levels of intensities were used for all experiments. Therefore the experiments were last on average for two hours. The Ia (and possibly Ib) fibres were activated in each subject via electrical stimulation of the common peroneal nerve of the ipsilateral leg (Táborìková and Sax 1968; Katz et al. 1988; Miles et al. 1989). The common peroneal nerve was stimulated using a cathode that is positioned against the skin slightly below the head of the fibula with an anode directly opposite. Square pulse stimuli of 0.5 ms duration were triggered from a computer and delivered by a Grass S44 stimulator. The stimulus artefact was removed using an artefact-suppressing amplifier. The stimulus intensity was increased until it advances the occurrence of the SMU action potential to the H-reflex latency in 1-2 out of 10 trials (weak stimulus) and 3-4 out of 10 trials (strong stimulus). This would indicate a compound EPSP size of 1-2mV for the weak stimulus and 3-4mV for the strong stimulus (Miles et al, 1989). The stimuli need to be strong enough to study the Ia input to motoneurons but weak enough not to recruit units that are otherwise silent. If silent units are recruited at the H-reflex latency, this would obscure the unit of interest and hence correct estimation of the H-reflex size would be impossible. This adjustment of the stimulus level is essential otherwise the shape of the single unit action potential will not be recognised by the template-matching program. Since the success of the analysis programs depends upon the correct recognition of the single unit spikes, especially at the reflex latency, it is essential to record from at most a few (preferably one) units in each electrode so that superimposition of spikes from different units did not occur during the entire period of the reflex activity. Low stimulus intensity also preferred since the direct motor response (M response) did not generate and the circuit was not further complicated by the involvement of the Renshaw cell pool that is activated by the antidromic pulses in the thick motor axons.

Analyses

Surface EMG: After filtering, the SEMG record was full-wave rectified, extracted around the time of the stimuli and averaged. The cumulative sum (CUSUM**;** Ellaway, 1978) of the averaged EMG record was then constructed. From the prestimulus period of the CUSUM records, maximal positive and negative deflections obtained. The larger of the two values was then used to make a symmetrical ‘error box’ (Türker et al, 1997; Brinkworth and Türker, 2003). The existence of a reflex response was determined by comparing the size of the error box with the deflections in the poststimulus CUSUM before the earliest possible voluntary response (reaction time). CUSUM values that were larger than the limits of the error box, and that occur before the reaction time, indicate a significant reflex response.

Single motor unit: Single motor unit potentials from each of the electrodes were discriminated off-line using a microprocessor-based waveform analysis method, which matched the shapes of the unit potentials to pre-established templates (Spike 2, CED). We were usually able to discriminate reliably the activity of 1-2 single motor units. We use this data to build PSTHs, PSF and their CUSUMs.

Statistics: Approximately, eleven subjects were included in each experiment. This number was based on our previous experience of similar experiments in which we demonstrated the existence of new reflex pathways. It is often the case that the new reflex/pathway does not exist in each subject or it can be demonstrated only under certain combinations of background muscle activity, stimulus intensity, fatigue (both central and peripheral) and stimulus rate, all of which can affect the outcome. The variables that affect the success of the appearance of the neural pathway were scrutinised using two intensities and several hundred stimuli to bring out the profile of the response (Fig. 1). Repeated measures analysis was performed. The level for significance was set to 0.05 and power to 80%.

RESULTS

In 11 subjects, 22 motor units were analysed. Out of these motor units, 9 were recorded during experiments where the stimulus only generated an H-reflex response; remaining 13 units were recorded in experiments where the stimulus generated both the M response and the H-reflex (see summaries in the Table 1 and 2).

H-reflex only responses

When the stimulation induced only an H-reflex in the sEMG, it generated three distinct responses in the PSTH; the H-reflex, the silent period and the late excitation. When number of counts in the silent period was compared with the number of counts in the prestimulus period, it was found that all 9 units illustrated a significant decrease in the number of counts during the silent period (see Fig 4, PSTH panel). In addition, during the late excitation, 5 out of 8 units showed a significant increase in the number of counts and 3 units showed no change compared to the pre-stimuli. When the discharge rate of the spikes that occurred during the silent period was examined however we found that 6 out of 9 units illustrated a significant increase in the discharge rate during the silent period (see Fig 4, PSF-CUSUM and PSF). Furthermore, all 9 units displayed significant reduction in the discharge rates during the ‘late excitation’ phase (details in Table 1).

Table 1 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced only the H-reflex response.

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decreased (units)	No change (units)	Increased (units)	Decreased (units)	No change (units)
PSF (Hz)	6/9*	2/9*	1/9	-	8/8*	-
PSTH (counts)	0	9/9*	0	5/8*	-	3/8

* significant difference $p < 0.05$

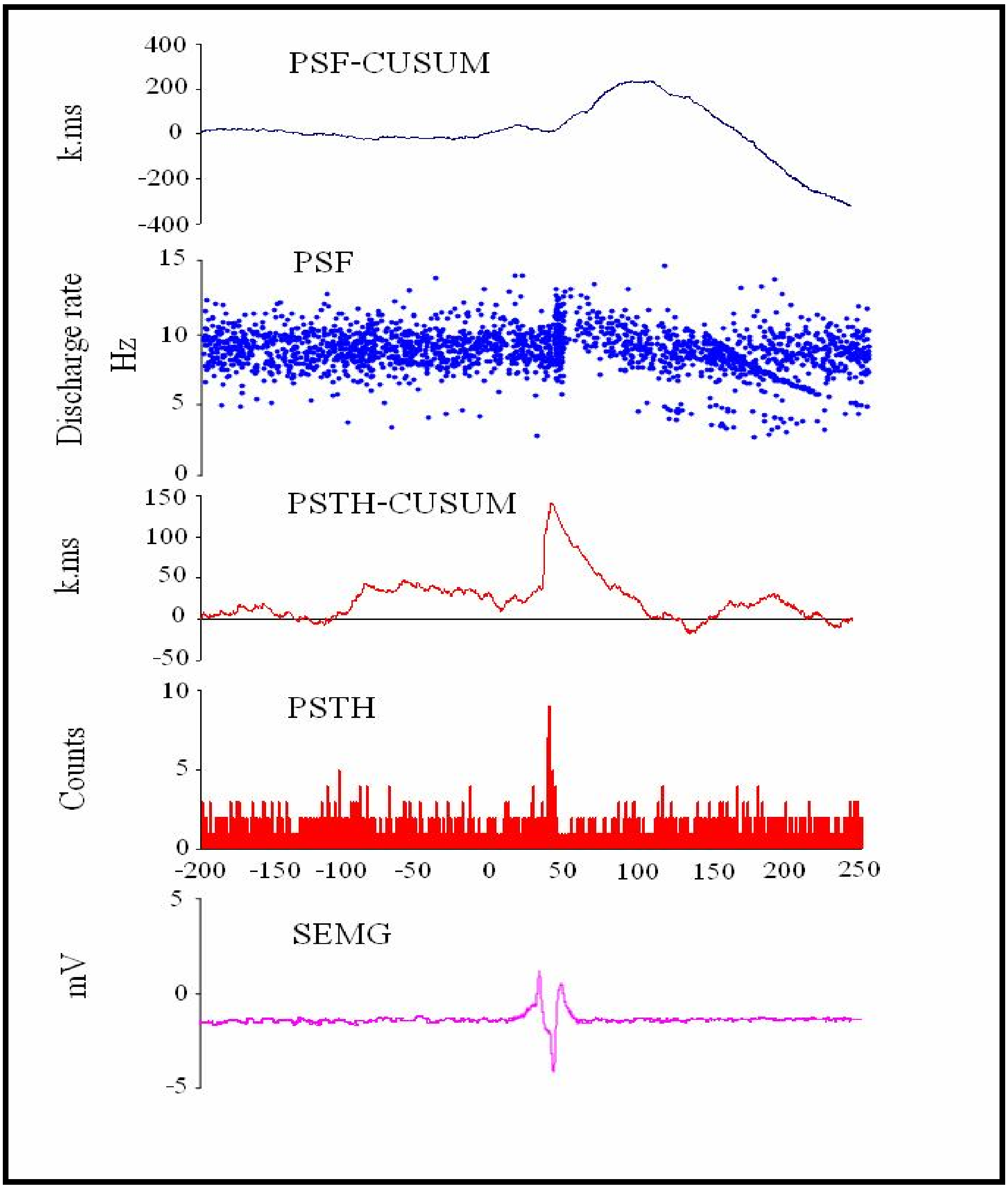

Figure 4 illustrates an example of the H-reflex only response results as can be seen in the last panel for surface electromyogram (SEMG). Top panel is the cumulative sum of peristimulus frequencygram (PSF-CUSUM) which indicates a long-lasting excitatory postsynaptic potential (EPSP) effect. The second panel shows the peristimulus frequencygram (PSF) which clearly shows the existence of increased discharge rates after H reflex response. In contrast, the third panel (CUSUM of peristimulus time histogram, PSTH-CUSUM) and the fourth panel (PSTH) show a reduction in number of spikes immediately after the H-reflex response and a large increase later which is termed the late excitation.

Comparison between PSF and PSTH analysis for H-reflex responses

Using PSTH and PSF analysis, either during the silent period or during the late excitation, the results illustrated the opposite findings. After the H-reflex response the PSTH demonstrates statistically significant decreases in the number of counts represented as the "silent period" whereas the majority of units which analyzed by PSF increased their discharge rates during the same period. For the late excitation, PSTH analysis indicated that most units increased the number of counts during that period whereas the discharge rates of all units during the same period decreased significantly.

M + H-reflex responses

Thirteen units were studied in experiments where the stimulus generated both M and H responses in the sEMG. All 13 units demonstrated significant decreases in the number of counts during the 'silent period'. During the late excitation, 10 out of 12 units showed significant increases in the number of spike counts (see Fig 5, PSTH and PSTH-CUSUM). In one unit, there was no noticeable increase in the CUSUM to be counted as the late excitatory response. While PSF analysis showed three patterns of the discharge rates during the silent period: no change, found in 6 out of 13 units, an increase in discharge rate found in 5 out of 13 units and a decrease in 2 out of 13 units. In addition, three patterns of the discharge rates response were also found during the late excitation: 9 out of 12 units decreased their discharge rates (see Fig 5, PSF), 2 out of 12 showed the no change in discharge rates and only 1 unit displayed an increase in the discharge rate (see Table 2).

Table 2 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced M + H responses

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decreased (units)	No change (units)	Increased (units)	Decreased (units)	No change (units)
PSF (Hz)	5/13*	2/13*	6/13	1/12*	9/12*	2/12
PSTH (counts)	-	13/13*	-	10/12*	-	2/12

* significant difference $p < 0.05$

Comparison between PSF and PSTH analysis for M and H responses

Again for our M+H responses, the opposite results were found in both periods for both analysis methods. While PSTH analysis found that all units decreased the number of counts in responses during the silent period, the PSF analysis indicated that nearly half of the units illustrated discharge rate increases and the other half no change in their discharge rates. Similarly, during the late excitation, analysis using PSTH showed an increase in the number of counts whereas PSF demonstrated a decrease in the discharge rate.

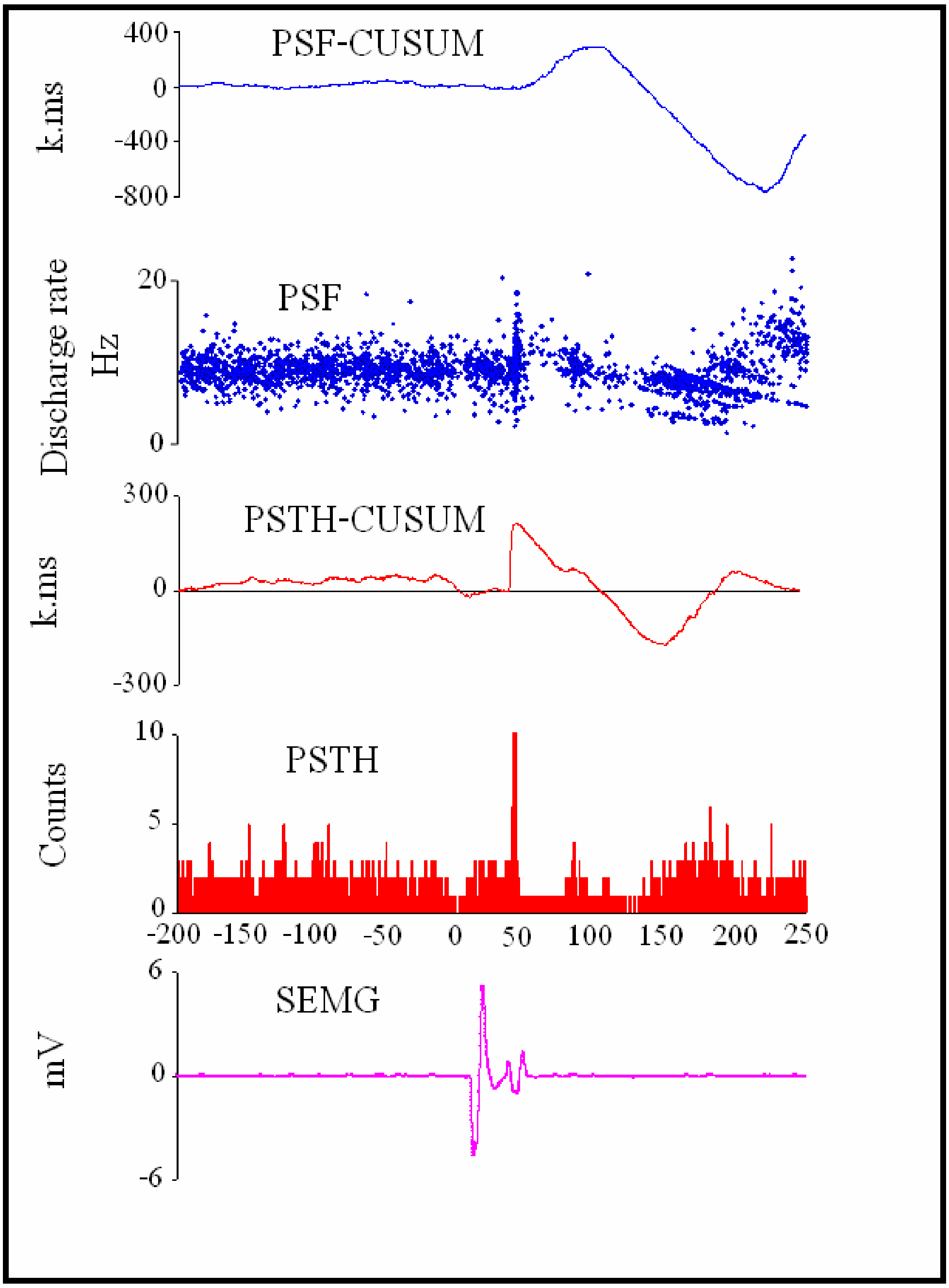

Figure 5 illustrates an example of the M + H response results as can be seen in the last panel for surface electromyogram (sEMG). Top panel is the PSF-CUSUM which indicates a long-lasting excitatory effect. The second panel shows the PSF which clearly shows the existence of discharge rates after the H-reflex response. In contrast, the third panel (PSTH-CUSUM) and the fourth panel (PSTH) indicate a clear reduction in spike counts immediately following the H-reflex (during the silent period) and a significant increase in spike counts later which induces a peak in the PSTH and an upswing in the PSTH-CUSUM (Fig 3).

DISCUSSION

Current results can be interpreted in two different ways depending on the analyses used. With classical PSTH analysis, an increase followed by a decrease could imply that following the stimulus there was an excitatory postsynaptic potential followed by inhibitory postsynaptic potential (EPSP+IPSP) (Ashby and Zilm 1978; Miles et al. 1989). On the other hand, with the PSF analysis, during the same period, there is only an increase in the discharge rate, which should be interpreted as a single long EPSP.

However, we prefer that the PSF interpreted as illustrating the net effect of the stimulation on the motoneurone pool rather than a single distinct pathway. This means that the pool had received several post synaptic potentials but the end product (the net effect) was a single long lasting excitation. If the EPSP and IPSP occur around the same time, depending on the dominant PSP, one could have seen a net excitation, a net inhibition, or no change at all as the end product. We therefore cannot rule out the occurrence of IPSP as a result of the stimulation on the common peroneal nerve in this study especially when the stimulus intensity induces both the M and H responses.

When the stimulus induced both the M and H responses, the PSF analyses were not as clear cut as the ones obtained when the stimulus induced only the H-reflex response (Tables 1 and 2). In deed, when a stimulus induces an M response, one would expect it to generate recurrent inhibition on the motoneurone pool (Bussel and Pierrot-Deseilligny 1977) and hence the net effect may reflect the sum of the Ia EPSP and the Renshaw cell IPSP. Hence, in 6 out of 13 units the discharge rate was not significantly different than the prestimulus. Therefore, involvement of the IPSPs cannot be ruled out in the PSF work as it represents the net effect.

Our conclusion as it stands today is that the activity in the low threshold fibres in the common peroneal nerve generates a single long lasting excitation in the motor pool of the tibialis anterior muscle if the stimulus intensity only generates the H-reflex response on the sEMG. However, even this single long excitation may be due to simultaneous stimulation of Ia and Ib fibres in the nerve. However, until we can distinctly suppress one of these pathways and determine the synaptic potential, we cannot conclude that a single long lasting excitation that we have observed means a single distinct excitatory pathway.

Therefore, we cannot confirm or reject our first hypothesis as we are observing the net effect of the stimulus but not a pure PSP from one source.

We can however confirm our second hypothesis, since, during silent period, discharge rate of most motor units were significantly higher than the prestimulus average indicating that during this ‘silent’ period, the net excitation on the motoneurone pool continued. However, this conclusion may only apply to the units obtained when the stimulus only induced the H-reflex response. With the other 13 units that are studied with the stimulus that induced both M and H responses, we may have a combination of excitation and inhibition occurring around the same time. We can also confirm our third hypothesis since we found that the discharge rate of motor units actually significantly declined during the “long-latency excitatory reflex response” illustrating that this period is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

Why do the classical methods contain errors: The classical techniques are based on the principle that a significant increase in the occurrence of discharge (spike) probability at a time after the stimulus represents an excitatory postsynaptic potential (**EPSP**) and a significant decrease indicates an inhibitory postsynaptic potential (**IPSP**). This assumption has led to serious errors in descriptions of connections between various elements of the central nervous system. These arise from the fact that the peaks and troughs in averaged surface EMG or PSTH can reflect not only direct synaptic effects, but also secondary effects arising from the discharge statistics of the pre- and postsynaptic cells (Moore et al. 1970). Despite this early warning about the problems in the probabilistic analysis method, the peaks and troughs in an averaged response to a stimulus are still defined as synaptic excitation and inhibition, respectively (e.g. Brooke et al. 1999; Okdeh et al. 1999; Sonnenborg et al. 2000).

To show directly that the probability based analyses contain inherent errors and to put forward an error free method for the estimation of synaptic potential, and hence the wiring diagram, Türker and Powers have conducted a series of experiments on regularly discharging motoneurons in rat brain slices. The brain slice experiments clearly illustrated that the frequency-based analysis gave a better indication of the underlying synaptic potentials than the probability-based analyses (Türker and Powers 1999; 2001; 2002; 2003;2005).

Significance: This study will correct apparent errors in the most commonly studied and firmly 'established' pathway in the central nervous system. This correction needs to be done urgently since the existence, stability, strength and sign of pathways in the brain and spinal cord are used for diagnosis, treatment and assessment of the success of treatment of many neurological disorders (Cruccu et al. 1989; Chen et al. 1998; Nadler et al. 2004).

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ภาคผนวก

A STUDY OF SYNAPTIC CONNECTION BETWEEN LOW THRESHOLD AFFERENT FIBRES IN COMMON PERONEAL NERVE AND MOTONEURONES IN HUMAN TIBIALIS ANTERIOR

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Key words: peristimulus time histogram, peristimulus frequencygram, synaptic connection, excitatory postsynaptic potentials, inhibitory postsynaptic potentials

ABSTRACT

We have induced H-reflex responses in human tibialis anterior motor units and analysed the results using the classical technique, peristimulus time histogram (PSTH), and a new technique, peristimulus frequencygram (PSF). The PSF has recently been shown to be more reliable than the PSTH for indicating the synaptic connections on motoneurons, and therefore we wished to examine the differences between the two analysis methods.

Experiments were conducted on eleven healthy subjects (7 males and 4 females) that do not have any known neurological disorder. The subjects sat comfortably on a dental chair and the common peroneal nerve was stimulated. In each experiment, about 600 stimuli were applied randomly every 1-2 s. The recordings were taken with both by surface electromyogram (sEMG) and as single motor unit potentials.

Our findings suggest that PSF gives significantly different results compared with the PSTH in determining the synaptic connection of the low threshold nerve fibres to the motoneurons. While PSTH indicated that there was a silent period immediately after the H-reflex, the PSF demonstrated that the silent period was actually a continuation of the net excitatory effect and not a genuine inhibition since the small number of action potentials occured during this period displayed higher discharge rates than the prestimulus level. Furthermore, the late excitation, as it was noted in the PSTH; was actually a net inhibitory effect since the large number of spikes that occured during that period had lower discharge rates than the prestimulus average.

In the lights of the recent brain slice findings and completely different results obtained using the two analysis techniques, we suggest that the PSF analysis should be used to illustrate the net synaptic connection between peripheral receptors and motoneurons in the human nervous system.

INTRODUCTION

Hoffmann (H) reflex is commonly used for investigation of synaptic connections between the low threshold muscle afferents and motoneurons (Knikou 2008). By using intramuscular electrodes, the discharge behaviour of single motor units in response to nerve stimulation can be recorded in human subjects and the recordings can be used to estimate the synaptic connections between the stimulated afferents and the motoneurones. Peristimulus time histogram (PSTH) is the classical method for illustrating such connections (Garnett and Stephens 1980). PSTH is constructed by counting the number of motor unit action potentials around periodic stimulation of afferents (Ashby and Zilm 1978).

In PSTH, an increase in the numbers of spikes following activation of an afferent system has been claimed to indicate an excitatory connection between the stimulated afferent system and the motoneuron. This increase in the spike probability following the stimulus appears as a peak in PSTHs (Garnett and Stephens 1980). If however the stimulated afferent system has an inhibitory connection to the motoneurone, the number of spikes following the activation of the afferent system would decrease which would appear as a trough in the PSTH (Garnett and Stephens 1981).

When a mixed nerve to a muscle is stimulated using low intensity electrical pulses, the resultant PSTH illustrates three significant peaks (Awiszus et al. 1991). A short latency large increase (the H-reflex response) followed by a decrease (also referred to as the silent period) and a long latency increase (long latency excitation) in the probability of motoneurone discharge. This finding has been interpreted to indicate a short latency possibly monosynaptic Ia excitatory postsynaptic potential (EPSP), an inhibitory postsynaptic potential (IPSP) or refractory period, and a long latency and possibly transcortical EPSP (Ashby and Zilm 1978; Lourenco et al. 2006). However, this conclusion can be criticized as these periods may have been due to count and/or synchronization related errors, as a novel analysis technique (PSF; Türker and Cheng 1994) did not indicate the existence of these periods.

In order to resolve this controversy and determine which of the two methods indicate the underlying postsynaptic potential (PSP) more reliably, we have used regularly discharging motoneurones in brain slices. In these experiments, we have inserted known PSPs into a regularly discharging motoneurones in brain slices and recorded the resultant action potentials to build PSTH and PSF (Türker and Powers 1999; 2003; 2005). These studies have indicated that the probability based classical methods, the PSTH and the averaged sEMG, had count and synchronization related errors and hence the synaptic potentials put forward using these techniques may not exist. On the other hand, the PSF techniques indicated the underlying PSP much more reliably. We therefore thought that the pathways that are activated as a result of low intensity electrical stimulation of a mixed muscle nerve should be re-investigated.

Other than our methodology paper where we have introduced the PSF technique for estimating PSPs in human motoneurones (Türker and Cheng 1994), only a few other papers exists where PSTH and PSF analyses are used simultaneously and compared (Deriu et al. 2005; Norton et al. 2008).

In this study, we aimed to test three hypotheses; firstly that the low threshold muscle afferents in the common peroneal nerve are connected to the homonymous motoneuones by only one pathway that is excitatory. Secondly, that the silent period that has been claimed to exist after the H-reflex response, is a net excitatory response. Thirdly, that the long-latency excitatory reflex response is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

MATERIALS AND METHODS

Eleven healthy volunteer subjects (7 men and 4 women) aged between 21-55 years old (mean ± SD = 33.4 ± 11.4, 27.8 ± 8.0 respectively) were recruited from students and staffs at University of Adelaide, Chiangmai University and Ege University. The protocol of the experiments was approved by the Human Ethics Committee of the University of Adelaide, Chiangmai University and Ege University and conforms to the Declaration of Helsinki. The subjects were informed about the study in a standardized way and signed an informed consent form. The subject sat comfortably on a dental chair with right foot positioned at 0º plantar flexion and the forefoot placed underneath a wood bar. This bar was set to provide the resistance during voluntary dorsiflexion and also measured the applied isometric force.

General protocol

All data were recorded on computer sampling at 15,000 samples/second using a CED® system. Subjects were instructed to activate selected single motor units at a predetermined rate using audio and visual feedback. While the subject fired the motor unit at a required level, stimuli were delivered randomly between 1 and 2 seconds in all studies. These triggers for the stimuli were seen in Figure 1 at the top panel.

Surface electromyography (sEMG)

Two sEMG recording electrodes were placed 2 cm apart lengthwise on the belly of the right tibialis anterior muscle. The outputs from the bipolar surface electrodes were amplified (1,000 to 10,000 times) and high pass filtered (5Hz). The skin resistance underlying the surface electrodes was reduced so that it was always below 10 kΩ; and subjects were grounded by lip clip ground electrodes (Türker et al. 1988). An example of sEMG recordings was showed in Figure 1 at the bottom panel (TA-sEMG)

Single motor unit (SMU) potentials

Teflon® insulated (except for their tips) silver bipolar wire electrodes (100µm diameter with insulation; 70µm core diameter) were inserted into the right tibialis anterior muscles using a 25G surgical needle. The needle was then withdrawn, leaving the fish hooked wires in the belly of the muscle. The subject was asked to fire a selected single motor unit tonically at a predetermined level using auditory and visual feedback of the unit's firing frequency. The SMU recordings were amplified (1,000 to 10,000 times) and high pass filtered (5Hz). An example of SMU recordings was seen in Figure 1 at the third panel (TA-SMU).

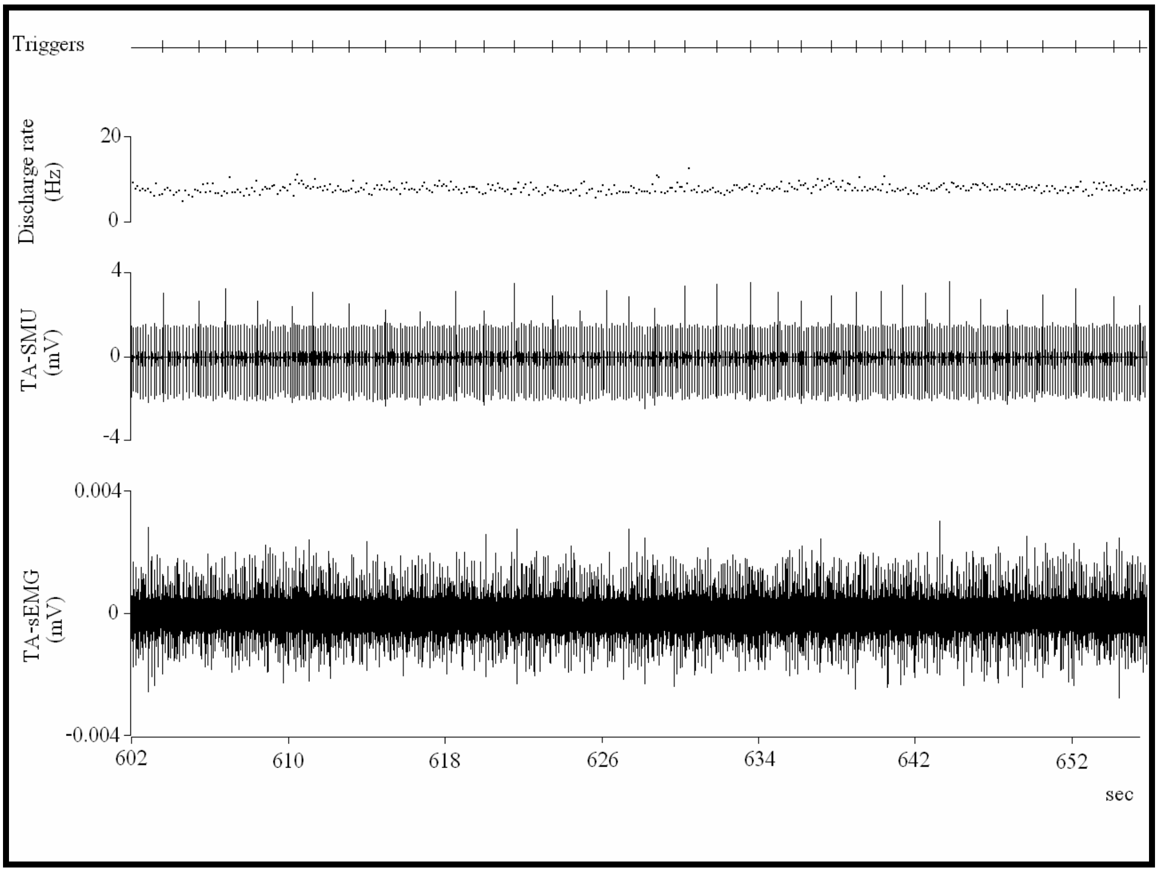

Figure 1 illustrates a short section of a recording session. The top panel (triggers) shows the timing of the electrical stimulation to common peroneal nerve. The second panel shows the discharge rate of an identified single motor unit; the third panel shows the single motor unit potentials recorded from right tibialis anterior. The bottom panel shows the sEMG of the right tibialis anterior.

Single motor unit potentials were discriminated on-line using microprocessor based waveform analyses method, which match the shapes of the units' potentials to pre-established templates (CED$^{®}$; Spike2 systems). Acceptance pulses from the discriminator were used to deliver auditory feedback and the discharge frequency of the unit was used as also used as visual feedback.

Nerve stimulation

The common peroneal nerve was stimulated using a cathode that was positioned against the skin slightly below the head of the fibula with an anode directly opposite. Square pulse stimuli of 0.5 ms duration was triggered from a computer and delivered by Digitimer constant current stimulator. The stimulus artefact was removed using an artefact-suppressing amplifier. The stimulus intensity was increased until it advanced the occurrence of the single motor unit action potential to the H-reflex latency in 2-3 out of 10 trials (Miles et al. 1989). This low intensity stimulus was also preferred as it did not generate synchronous activity of many motor units at the H-reflex latency which makes the recognition of the selected unit impossible.

Analysis

For sEMG, after filtering, the record was full-wave rectified, extracted around the time of the stimuli and averaged. The cumulative sum (CUSUM; Ellaway 1978) of the averaged EMG record was then constructed. From the prestimulus period of the CUSUM records, maximal positive and negative deflections were obtained. The larger of the two values was then used to make a symmetrical 'error box' (Türker et al. 1997; Brinkworth and Türker 2003). The existence of a reflex response was determined by comparing the size of the error box with the deflections in the poststimulus CUSUM.

For SMU, the recordings were discriminated off-line using a microprocessor-based waveform analysis method, which matched the shapes of the unit potentials to pre-established templates (CED® Spike2 program). These discriminated data were then used to build PSTHs, PSFs and their CUSUMs. For CUSUMs, similar analyses to the one described for the sEMG was used as well as a statistical comparison of the number of action potentials in selected areas (for PSTH) and discharge rates for selected areas (for PSF).

The silent period was determined as follows: two cursors were lined up at the onset and the end of the negative slope on the PSTH-CUSUM immediately after the positive slope representing the H-reflex response. During this period, the number of occurrences in the

PSTH and the discharge rates in the PSF were compared with the values in the prestimulus period using Student's t-test for comparing independent samples (see also Garnett and Stephens 1980).

Similarly, two cursors were placed at the onset and the end of the long latency positive slope on PSTH-CUSUM (see PSTH-CUSUMs in Figs 2 and 3). Again, the number of occurrences in the PSTH and the discharge rates in the PSF during this selected period were compared with the values in the prestimulus period using the same test; Student's t-test for comparing independent samples (the level for significance was set to 0.05 for all tests).

RESULTS

In 11 subjects, 22 motor units were analysed. Out of these motor units, 9 were recorded during experiments where the stimulus only generated an H-reflex response; remaining 13 units were recorded in experiments where the stimulus generated both the M response and the H-reflex (see summaries in the Table 1 and 2).

H-reflex only responses

When the stimulation induced only an H-reflex in the sEMG, it generated three distinct responses in the PSTH; the H-reflex, the silent period and the late excitation. When number of counts in the silent period was compared with the number of counts in the prestimulus period, it was found that all 9 units illustrated a significant decrease in the number of counts during the silent period (see Fig 2, PSTH panel). In addition, during the late excitation, 5 out of 8 units showed a significant increase in the number of counts and 3 units showed no change compared to the pre-stimuli. When the discharge rate of the spikes that occurred during the silent period was examined however we found that 6 out of 9 units illustrated a significant increase in the discharge rate during the silent period (see Fig 2, PSF-CUSUM and PSF). Furthermore, all 9 units displayed significant reduction in the discharge rates during the ‘late excitation’ phase (details in Table 1).

Table 1 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced only the H-reflex response.

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decreased (units)	No change (units)	Increased (units)	Decreased (units)	No change (units)
PSF (Hz)	6/9*	2/9*	1/9	-	8/8*	-
PSTH (counts)	0	9/9*	0	5/8*	-	3/8

* significant difference $p < 0.05$

Comparison between PSF and PSTH analysis for H-reflex responses

Using PSTH and PSF analysis, either during the silent period or during the late excitation, the results illustrated the opposite findings. After the H-reflex response the PSTH demonstrates statistically significant decreases in the number of counts represented as the "silent period" whereas the majority of units which analyzed by PSF increased their discharge rates during the same period. For the late excitation, PSTH analysis indicated that most units increased the number of counts during that period whereas the discharge rates of all units during the same period decreased significantly.

M + H-reflex responses

Thirteen units were studied in experiments where the stimulus generated both M and H responses in the sEMG. All 13 units demonstrated significant decreases in the number of counts during the 'silent period'. During the late excitation, 10 out of 12 units showed significant increases in the number of spike counts (see Fig 3, PSTH and PSTH-CUSUM). In one unit, there was no noticeable increase in the CUSUM to be counted as the late excitatory response. While PSF analysis showed three patterns of the discharge rates during the silent period: no change, found in 6 out of 13 units, an increase in discharge rate found in 5 out of 13 units and a decrease in 2 out of 13 units. In addition, three patterns of the discharge rates response were also found during the late excitation: 9 out of 12 units decreased their discharge rates (see Fig 3, PSF), 2 out of 12 showed the no change in discharge rates and only 1 unit displayed an increase in the discharge rate (see Table 2).

Table 2 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced M + H responses

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decreased (units)	No change (units)	Increased (units)	Decreased (units)	No change (units)
PSF (Hz)	5/13*	2/13*	6/13	1/12*	9/12*	2/12
PSTH (counts)	-	13/13*	-	10/12*	-	2/12

* significant difference $p < 0.05$

Comparison between PSF and PSTH analysis for M and H responses

Again for our M+H responses, the opposite results were found in both periods for both analysis methods. While PSTH analysis found that all units decreased the number of counts in responses during the silent period, the PSF analysis indicated that nearly half of the units illustrated discharge rate increases and the other half no change in their discharge rates. Similarly, during the late excitation, analysis using PSTH showed an increase in the number of counts whereas PSF demonstrated a decrease in the discharge rate.

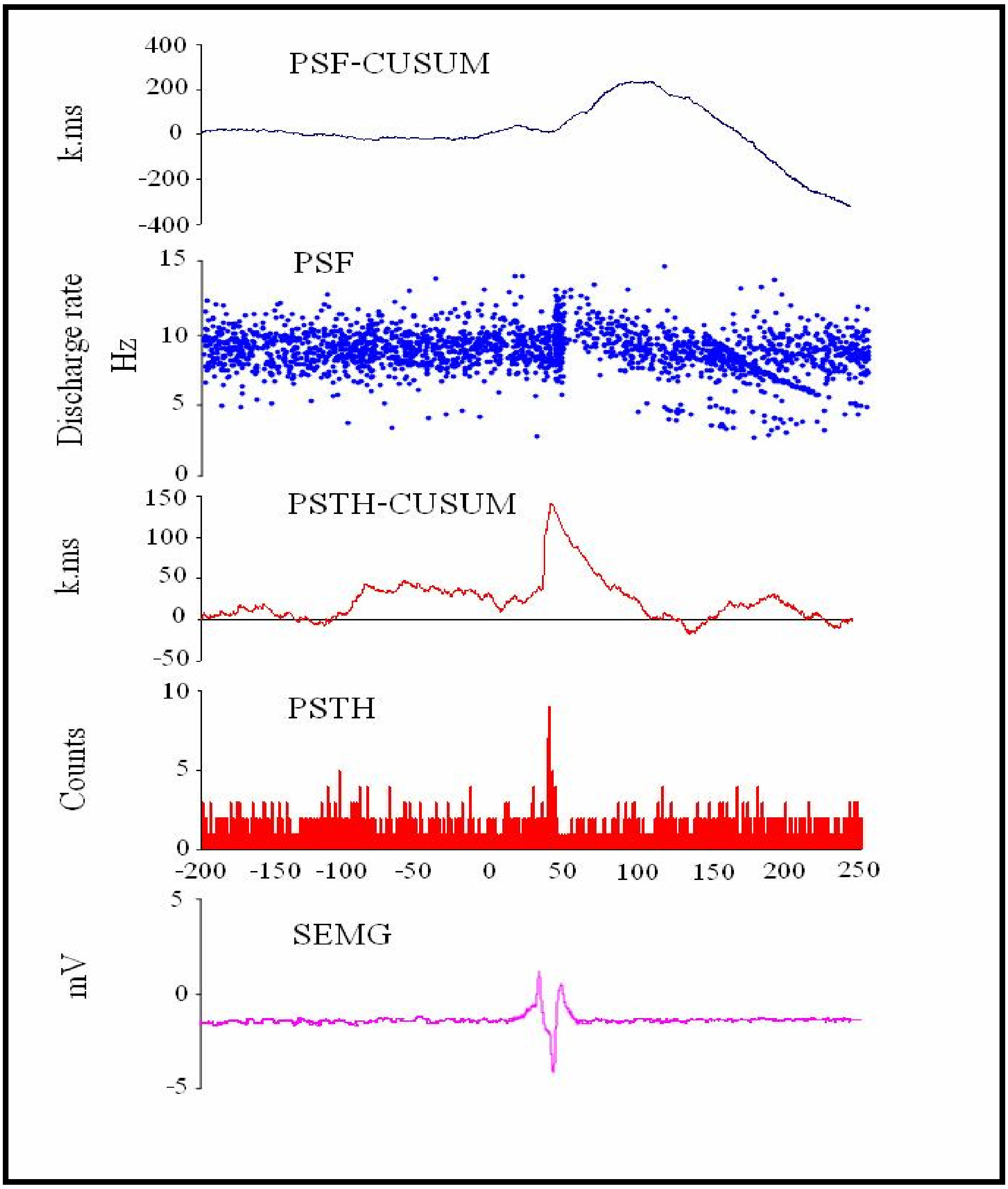

Figure 2 illustrates an example of the H-reflex only response results as can be seen in the last panel for surface electromyogram (SEMG). Top panel is the cumulative sum of peristimulus frequencygram (PSF-CUSUM) which indicates a long-lasting excitatory postsynaptic potential (EPSP) effect. The second panel shows the peristimulus frequencygram (PSF) which clearly shows the existence of increased discharge rates after H reflex response. In contrast, the third panel (CUSUM of peristimulus time histogram, PSTH-CUSUM) and the fourth panel (PSTH) show a reduction in number of spikes immediately after the H-reflex response and a large increase later which is termed the late excitation.

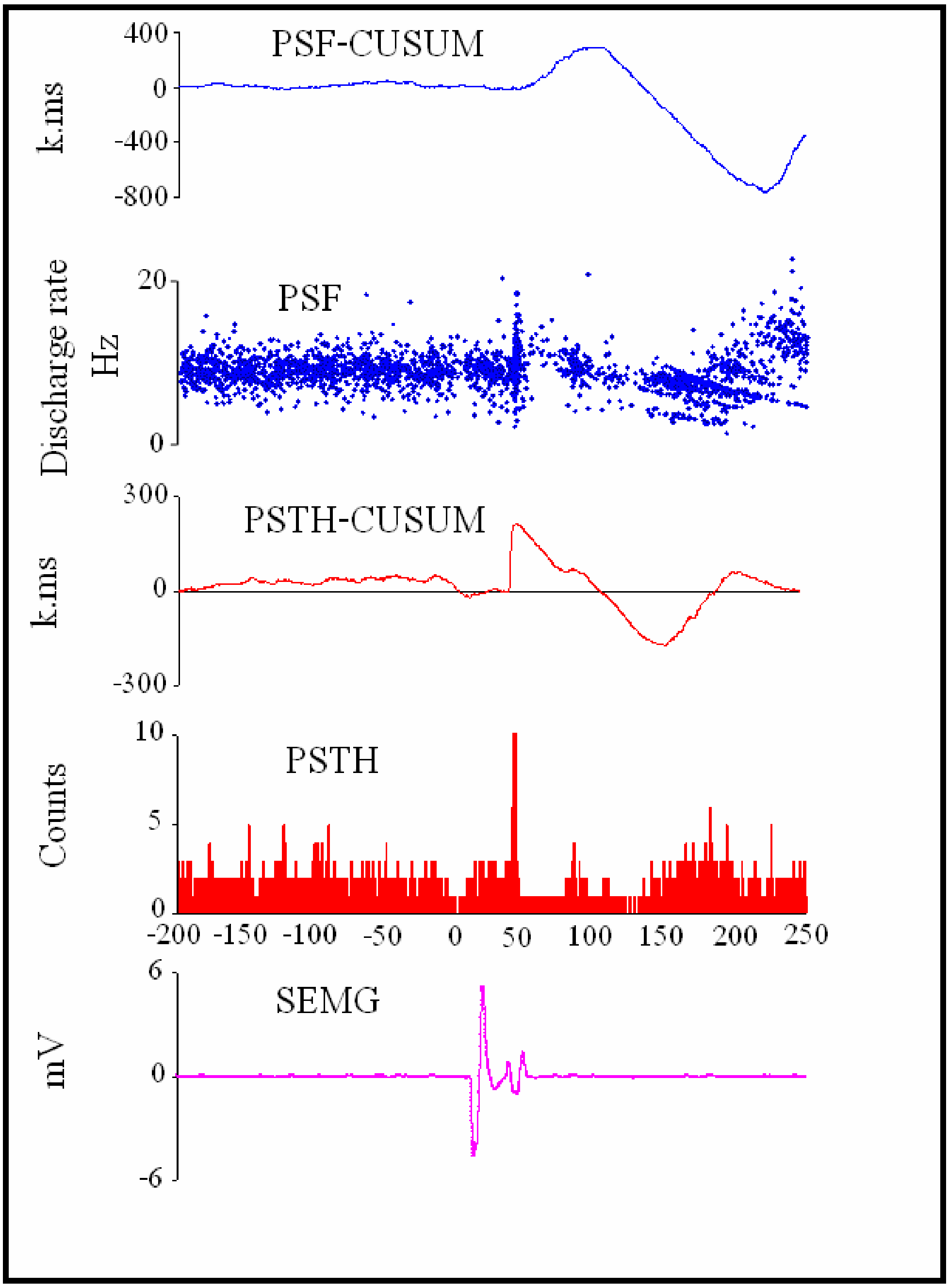

Figure 3 illustrates an example of the M + H response results as can be seen in the last panel for surface electromyogram (sEMG). Top panel is the PSF-CUSUM which indicates a long-lasting excitatory effect. The second panel shows the PSF which clearly shows the existence of discharge rates after the H-reflex response. In contrast, the third panel (PSTH-CUSUM) and the fourth panel (PSTH) indicate a clear reduction in spike counts immediately following the H-reflex (during the silent period) and a significant increase in spike counts later which induces a peak in the PSTH and an upswing in the PSTH-CUSUM (Fig 3).

DISCUSSION

Current results can be interpreted in two different ways depending on the analyses used. With classical PSTH analysis, an increase followed by a decrease could imply that following the stimulus there was an excitatory postsynaptic potential followed by inhibitory postsynaptic potential (EPSP+IPSP) (Ashby and Zilm 1978; Miles et al. 1989). On the other hand, with the PSF analysis, during the same period, there is only an increase in the discharge rate, which should be interpreted as a single long EPSP.

However, we prefer that the PSF interpreted as illustrating the net effect of the stimulation on the motoneurone pool rather than a single distinct pathway. This means that the pool had received several post synaptic potentials but the end product (the net effect) was a single long lasting excitation. If the EPSP and IPSP occur around the same time, depending on the dominant PSP, one could have seen a net excitation, a net inhibition, or no change at all as the end product. We therefore cannot rule out the occurrence of IPSP as a result of the stimulation on the common peroneal nerve in this study especially when the stimulus intensity induces both the M and H responses.

When the stimulus induced both the M and H responses, the PSF analyses were not as clear cut as the ones obtained when the stimulus induced only the H-reflex response (Tables 1 and 2). In deed, when a stimulus induces an M response, one would expect it to generate recurrent inhibition on the motoneurone pool (Bussel and Pierrot-Deseilligny 1977) and hence the net effect may reflect the sum of the Ia EPSP and the Renshaw cell IPSP. Hence, in 6 out of 13 units the discharge rate was not significantly different than the prestimulus. Therefore, involvement of the IPSPs cannot be ruled out in the PSF work as it represents the net effect.

Our conclusion as it stands today is that the activity in the low threshold fibres in the common peroneal nerve generates a single long lasting excitation in the motor pool of the tibialis anterior muscle if the stimulus intensity only generates the H-reflex response on the sEMG. However, even this single long excitation may be due to simultaneous stimulation of Ia and Ib fibres in the nerve. However, until we can distinctly suppress one of these pathways and determine the synaptic potential, we cannot conclude that a single long lasting excitation that we have observed means a single distinct excitatory pathway.

Therefore, we cannot confirm or reject our first hypothesis as we are observing the net effect of the stimulus but not a pure PSP from one source.

We can however confirm our second hypothesis, since, during silent period, discharge rate of most motor units were significantly higher than the prestimulus average indicating that during this ‘silent’ period, the net excitation on the motoneurone pool continued. However, this conclusion may only apply to the units obtained when the stimulus only induced the H-reflex response. With the other 13 units that are studied with the stimulus that induced both M and H responses, we may have a combination of excitation and inhibition occurring around the same time. We can also confirm our third hypothesis since we found that the discharge rate of motor units actually significantly declined during the “long-latency excitatory reflex response” illustrating that this period is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

Why do the classical methods contain errors: The classical techniques are based on the principle that a significant increase in the occurrence of discharge (spike) probability at a time after the stimulus represents an excitatory postsynaptic potential (**EPSP**) and a significant decrease indicates an inhibitory postsynaptic potential (**IPSP**). This assumption has led to serious errors in descriptions of connections between various elements of the central nervous system. These arise from the fact that the peaks and troughs in averaged surface EMG or PSTH can reflect not only direct synaptic effects, but also secondary effects arising from the discharge statistics of the pre- and postsynaptic cells (Moore et al. 1970). Despite this early warning about the problems in the probabilistic analysis method, the peaks and troughs in an averaged response to a stimulus are still defined as synaptic excitation and inhibition, respectively (e.g. Brooke et al. 1999; Okdeh et al. 1999; Sonnenborg et al. 2000).

To show directly that the probability based analyses contain inherent errors and to put forward an error free method for the estimation of synaptic potential, and hence the wiring diagram, Türker and Powers have conducted a series of experiments on regularly discharging motoneurons in rat brain slices. The brain slice experiments clearly illustrated that the frequency-based analysis gave a better indication of the underlying synaptic potentials than the probability-based analyses (Türker and Powers 1999; 2001; 2002; 2003;2005).

Significance: This study will correct apparent errors in the most commonly studied and firmly ‘established’ pathway in the central nervous system. This correction needs to be done urgently since the existence, stability, strength and sign of pathways in the brain and spinal cord are used for diagnosis, treatment and assessment of the success of treatment of many neurological disorders (Cruccu et al. 1989; Chen et al. 1998; Nadler et al. 2004).

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A STUDY OF SYNAPTIC CONNECTION BETWEEN LOW THRESHOLD AFFERENT FIBRES IN COMMON PERONEAL NERVE AND MOTONEURONES IN HUMAN TIBIALIS ANTERIOR

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A STUDY OF SYNAPTIC CONNECTION BETWEEN LOW THRESHOLD AFFERENT FIBRES IN COMMON PERONEAL NERVE AND MOTONEURONES IN HUMAN TIBIALIS ANTERIOR

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Key words: peristimulus time histogram, peristimulus frequencygram, synaptic connection, excitatory postsynaptic potentials, inhibitory postsynaptic potentials

ABSTRACT

We have induced H-reflex responses in human tibialis anterior motor units and analysed the results using the classical technique, peristimulus time histogram (PSTH), and a new technique, peristimulus frequencygram (PSF). The PSF has recently been shown to be more reliable than the PSTH for indicating the synaptic connections on motoneurons, and therefore we wished to examine the differences between the two analysis methods.

Experiments were conducted on eleven healthy subjects (7 males and 4 females) that do not have any known neurological disorder. The subjects sat comfortably on a dental chair and the common peroneal nerve was stimulated. In each experiment, about 600 stimuli were applied randomly every 1-2 s. The recordings were taken with both by surface electromyogram (sEMG) and as single motor unit potentials.

Our findings suggest that PSF gives significantly different results compared with the PSTH in determining the synaptic connection of the low threshold nerve fibres to the motoneurons. While PSTH indicated that there was a silent period immediately after the H-reflex, the PSF demonstrated that the silent period was actually a continuation of the net excitatory effect and not a genuine inhibition since the small number of action potentials occured during this period displayed higher discharge rates than the prestimulus level. Furthermore, the late excitation, as it was noted in the PSTH; was actually a net inhibitory effect since the large number of spikes that occured during that period had lower discharge rates than the prestimulus average.

In the lights of the recent brain slice findings and completely different results obtained using the two analysis techniques, we suggest that the PSF analysis should be used to illustrate the net synaptic connection between peripheral receptors and motoneurons in the human nervous system.

INTRODUCTION

Hoffmann (H) reflex is commonly used for investigation of synaptic connections between the low threshold muscle afferents and motoneurons (Knikou 2008). By using intramuscular electrodes, the discharge behaviour of single motor units in response to nerve stimulation can be recorded in human subjects and the recordings can be used to estimate the synaptic connections between the stimulated afferents and the motoneurones. Peristimulus time histogram (PSTH) is the classical method for illustrating such connections (Garnett and Stephens 1980). PSTH is constructed by counting the number of motor unit action potentials around periodic stimulation of afferents (Ashby and Zilm 1978).

In PSTH, an increase in the numbers of spikes following activation of an afferent system has been claimed to indicate an excitatory connection between the stimulated afferent system and the motoneuron. This increase in the spike probability following the stimulus appears as a peak in PSTHs (Garnett and Stephens 1980). If however the stimulated afferent system has an inhibitory connection to the motoneurone, the number of spikes following the activation of the afferent system would decrease which would appear as a trough in the PSTH (Garnett and Stephens 1981).

When a mixed nerve to a muscle is stimulated using low intensity electrical pulses, the resultant PSTH illustrates three significant peaks (Awiszus et al. 1991). A short latency large increase (the H-reflex response) followed by a decrease (also referred to as the silent period) and a long latency increase (long latency excitation) in the probability of motoneurone discharge. This finding has been interpreted to indicate, a short latency possibly monosynaptic Ia excitatory postsynaptic potential (EPSP), an inhibitory postsynaptic potential (IPSP) or refractory period, and a long latency and possibly transcortical EPSP (Ashby and Zilm 1978; Lourenco et al. 2006). However, this conclusion can be criticized as these periods may have been due to count and/or synchronization related errors, as a novel analysis technique (PSF; Türker and Cheng 1994) did not indicate the existence of these periods.

In order to resolve this controversy and determine which of the two methods indicate the underlying postsynaptic potential (PSP) more reliably, we have used regularly discharging motoneurones in brain slices. In these experiments, we have inserted known PSPs into a regularly discharging motoneurones in brain slices and recorded the resultant action potentials to build PSTH and PSF (Türker and Powers 1999; 2003; 2005). These studies have indicated that the probability based classical methods, the PSTH and the averaged sEMG, had count and synchronization related errors and hence the synaptic potentials put forward using these techniques may not exist. On the other hand, the PSF techniques indicated the underlying PSP much more reliably. We therefore thought that the pathways that are activated as a result of low intensity electrical stimulation of a mixed muscle nerve should be re-investigated.

Other than our methodology paper where we have introduced the PSF technique for estimating PSPs in human motoneurones (Türker and Cheng 1994), only a few other papers exists where PSTH and PSF analyses are used simultaneously and compared (Deriu et al. 2005; Norton et al. 2008).

In this study, we aimed to test three hypotheses; firstly that the low threshold muscle afferents in the common peroneal nerve are connected to the homonymous motoneuones by only one pathway that is excitatory. Secondly, that the silent period that has been claimed to exist after the H-reflex response, is a net excitatory response. Thirdly, that the long-latency excitatory reflex response is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

MATERIALS AND METHODS

Eleven healthy volunteer subjects (7 men and 4 women) aged between 21-55 years old (mean ± SD = 33.4 ± 11.4, 27.8 ± 8.0 respectively) were recruited from students and staffs at University of Adelaide, Chiangmai University and Ege University. The protocol of the experiments was approved by the Human Ethics Committee of the University of Adelaide, Chiangmai University and Ege University and conforms to the Declaration of Helsinki. The subjects were informed about the study in a standardized way and signed an informed consent form. The subject sat comfortably on a dental chair with right foot positioned at 0° plantar flexion and the forefoot placed underneath a wood bar. This bar was set to provide the resistance during voluntary dorsiflexion and also measured the applied isometric force.

General protocol

All data were recorded on computer sampling at 15,000 samples/second using a CED® system. Subjects were instructed to activate selected single motor units at a predetermined rate using audio and visual feedback. While the subject fired the motor unit at a required level, stimuli were delivered randomly between 1 and 2 seconds in all studies. These triggers for the stimuli were seen in Figure 1 at the top panel.

Surface electromyography (sEMG)

Two sEMG recording electrodes were placed 2 cm apart lengthwise on the belly of the right tibialis anterior muscle. The outputs from the bipolar surface electrodes were amplified (1,000 to 10,000 times) and high pass filtered (5Hz). The skin resistance underlying the surface electrodes was reduced so that it was always below 10 kΩ; and subjects were grounded by lip clip ground electrodes (Türker et al. 1988). An example of sEMG recordings was showed in Figure 1 at the bottom panel (TA-sEMG)

Single motor unit (SMU) potentials

Teflon® insulated (except for their tips) silver bipolar wire electrodes (100μm diameter with insulation; 70μm core diameter) were inserted into the right tibialis anterior muscles using a 25G surgical needle. The needle was then withdrawn, leaving the fish hooked wires in the belly of the muscle. The subject was asked to fire a selected single motor unit tonically at a predetermined level using auditory and visual feedback of the unit's firing frequency. The SMU recordings were amplified (1,000 to 10,000 times) and high pass filtered (5Hz). An example of SMU recordings was seen in Figure 1 at the third panel (TA-SMU).

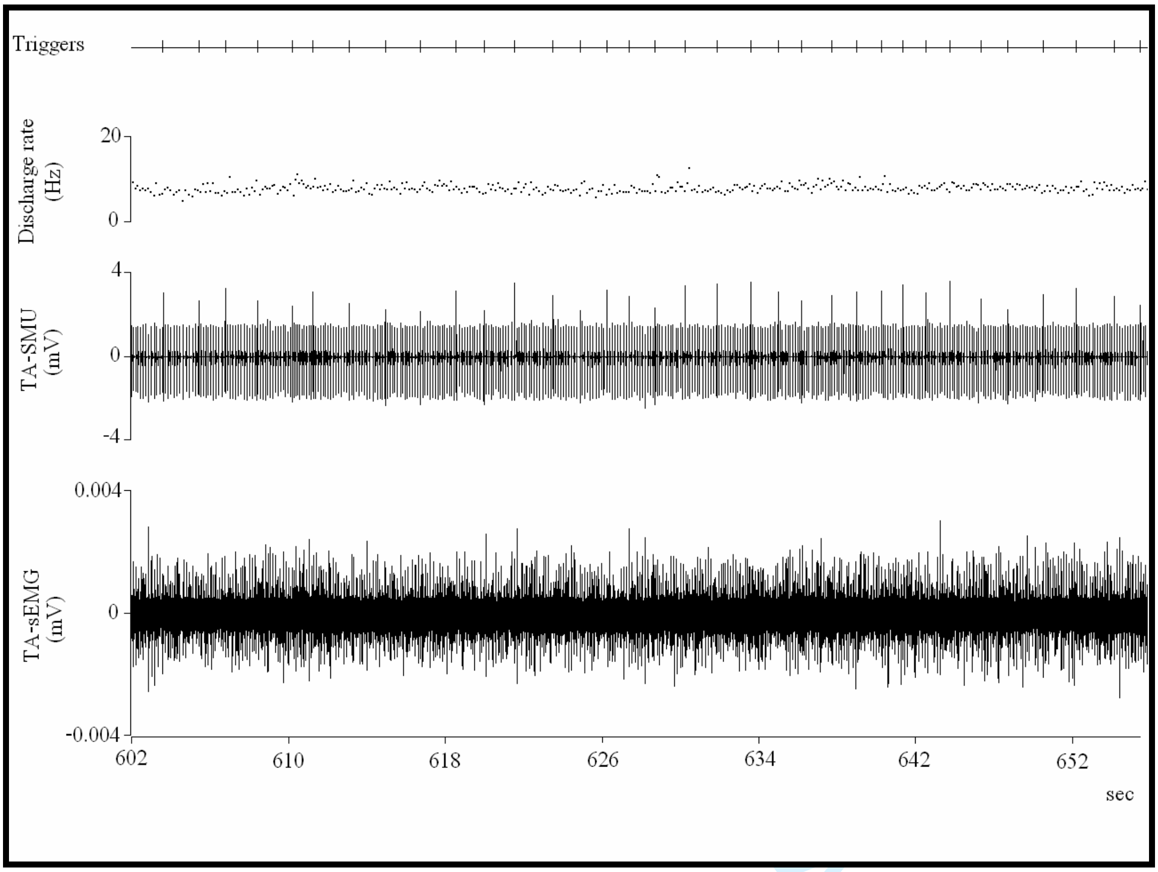

Figure 1 illustrates a short section of a recording session. The top panel (triggers) shows the timing of the electrical stimulation to common peroneal nerve. The second panel shows the discharge rate of an identified single motor unit; the third panel shows the single motor unit potentials recorded from right tibialis anterior. The bottom panel shows the sEMG of the right tibialis anterior.

Single motor unit potentials were discriminated on-line using microprocessor based waveform analyses method, which match the shapes of the units' potentials to pre-established templates (CED®; Spike2 systems). Acceptance pulses from the discriminator were used to deliver auditory feedback and the discharge frequency of the unit was used as also used as visual feedback.

Nerve stimulation

The common peroneal nerve was stimulated using a cathode that was positioned against the skin slightly below the head of the fibula with an anode directly opposite. Square pulse stimuli of 0.5 ms duration was triggered from a computer and delivered by Digitimer constant current stimulator. The stimulus artefact was removed using an artefact-suppressing amplifier. The stimulus intensity was increased until it advanced the occurrence of the single motor unit action potential to the H-reflex latency in 2-3 out of 10 trials (Miles et al. 1989). This low intensity stimulus was also preferred as it did not generate synchronous activity of many motor units at the H-reflex latency which makes the recognition of the selected unit impossible.

Analysis

For sEMG, after filtering, the record was full-wave rectified, extracted around the time of the stimuli and averaged. The cumulative sum (CUSUM; Ellaway 1978) of the averaged EMG record was then constructed. From the prestimulus period of the CUSUM records, maximal positive and negative deflections were obtained. The larger of the two values was then used to make a symmetrical ‘error box’ (Türker et al. 1997; Brinkworth and Türker 2003). The existence of a reflex response was determined by comparing the size of the error box with the deflections in the poststimulus CUSUM.

For SMU, the recordings were discriminated off-line using a microprocessor-based waveform analysis method, which matched the shapes of the unit potentials to pre-established templates (CED® Spike2 program). These discriminated data were then used to build PSTHs, PSFs and their CUSUMs. For CUSUMs, similar analyses to the one described for the sEMG was used as well as a statistical comparison of the number of action potentials in selected areas (for PSTH) and discharge rates for selected areas (for PSF).

The silent period was determined as follows: two cursors were lined up at the onset and the end of the negative slope on the PSTH-CUSUM immediately after the positive slope representing the H-reflex response. During this period, the number of occurrences in the PSTH and the discharge rates in the PSF were compared with the values in the prestimulus period using Student’s t-test for comparing independent samples (see also Garnett and Stephens 1980).

Similarly, two cursors were placed at the onset and the end of the long latency positive slope on PSTH-CUSUM (see PSTH-CUSUMs in Figs 2 and 3). Again, the number of occurrences in the PSTH and the discharge rates in the PSF during this selected period were compared with the values in the prestimulus period using the same test; Student’s t-test for comparing independent samples (the level for significance was set to 0.05 for all tests).

RESULTS

In 11 subjects, 22 motor units were analysed. Out of these motor units, 9 were recorded during experiments where the stimulus only generated an H-reflex response; remaining 13 units were recorded in experiments where the stimulus generated both the M response and the H-reflex (see summaries in the Table 1 and 2).

H-reflex only responses

When the stimulation induced only an H-reflex in the sEMG, it generated three distinct responses in the PSTH; the H-reflex, the silent period and the late excitation. When number of counts in the silent period was compared with the number of counts in the prestimulus period, it was found that all 9 units illustrated a significant decrease in the number of counts during the silent period (see Fig 2, PSTH panel). In addition, during the late excitation, 5 out of 8 units showed a significant increase in the number of counts and 3 units showed no change compared to the pre-stimuli. When the discharge rate of the spikes that occurred during the silent period was examined however we found that 6 out of 9 units illustrated a significant increase in the discharge rate during the silent period (see Fig 2, PSF-CUSUM and PSF). Furthermore, all 9 units displayed significant reduction in the discharge rates during the 'late excitation' phase (details in Table 1).

Table 1 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced only the H-reflex response.

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decrease (units)	No change (units)	Increased (units)	Decrease (units)	No change (units)
PSF (Hz)	6/9*	2/9*	1/9	-	8/8*	-
PSTH (counts)	0	9/9*	0	5/8*	-	3/8

* significant difference $p < 0.05$

Comparison between PSF and PSTH analysis for H-reflex responses

Using PSTH and PSF analysis, either during the silent period or during the late excitation, the results illustrated the opposite findings. After the H-reflex response the PSTH demonstrates statistically significant decreases in the number of counts represented as the "silent period" whereas the majority of units which analyzed by PSF increased their discharge rates during the same period. For the late excitation, PSTH analysis indicated that most units increased the number of counts during that period whereas the discharge rates of all units during the same period decreased significantly.

M + H-reflex responses

Thirteen units were studied in experiments where the stimulus generated both M and H responses in the sEMG. All 13 units demonstrated significant decreases in the number of counts during the 'silent period'. During the late excitation, 10 out of 12 units showed significant increases in the number of spike counts (see Fig 3, PSTH and PSTH-CUSUM). In one unit, there was no noticeable increase in the CUSUM to be counted as the late excitatory response. While PSF analysis showed three patterns of the discharge rates during the silent period: no change, found in 6 out of 13 units, an increase in discharge rate found in 5 out of 13 units and a decrease in 2 out of 13 units. In addition, three patterns of the discharge rates response were also found during the late excitation: 9 out of 12 units decreased their discharge rates (see Fig 3, PSF), 2 out of 12 showed the no change in discharge rates and only 1 unit displayed an increase in the discharge rate (see Table 2).

Table 2 illustrates the differences between PSF and PSTH analysis during the silent period and during the late excitation when stimulus intensity induced M + H responses

	During the silent period Number of units significantly changed			During the late excitation Number of units significantly changed		
	Increased (units)	Decreased (units)	No change (units)	Increased (units)	Decreased (units)	No change (units)
PSF (Hz)	5/13*	2/13*	6/13	1/12*	9/12*	2/12
PSTH (counts)	-	13/13*	-	10/12*	-	2/12

* significant difference $p < 0.05$

Comparison between PSF and PSTH analysis for M and H responses

Again for our M+H responses, the opposite results were found in both periods for both analysis methods. While PSTH analysis found that all units decreased the number of counts in responses during the silent period, the PSF analysis indicated that nearly half of the units illustrated discharge rate increases and the other half no change in their discharge rates. Similarly, during the late excitation, analysis using PSTH showed an increase in the number of counts whereas PSF demonstrated a decrease in the discharge rate.

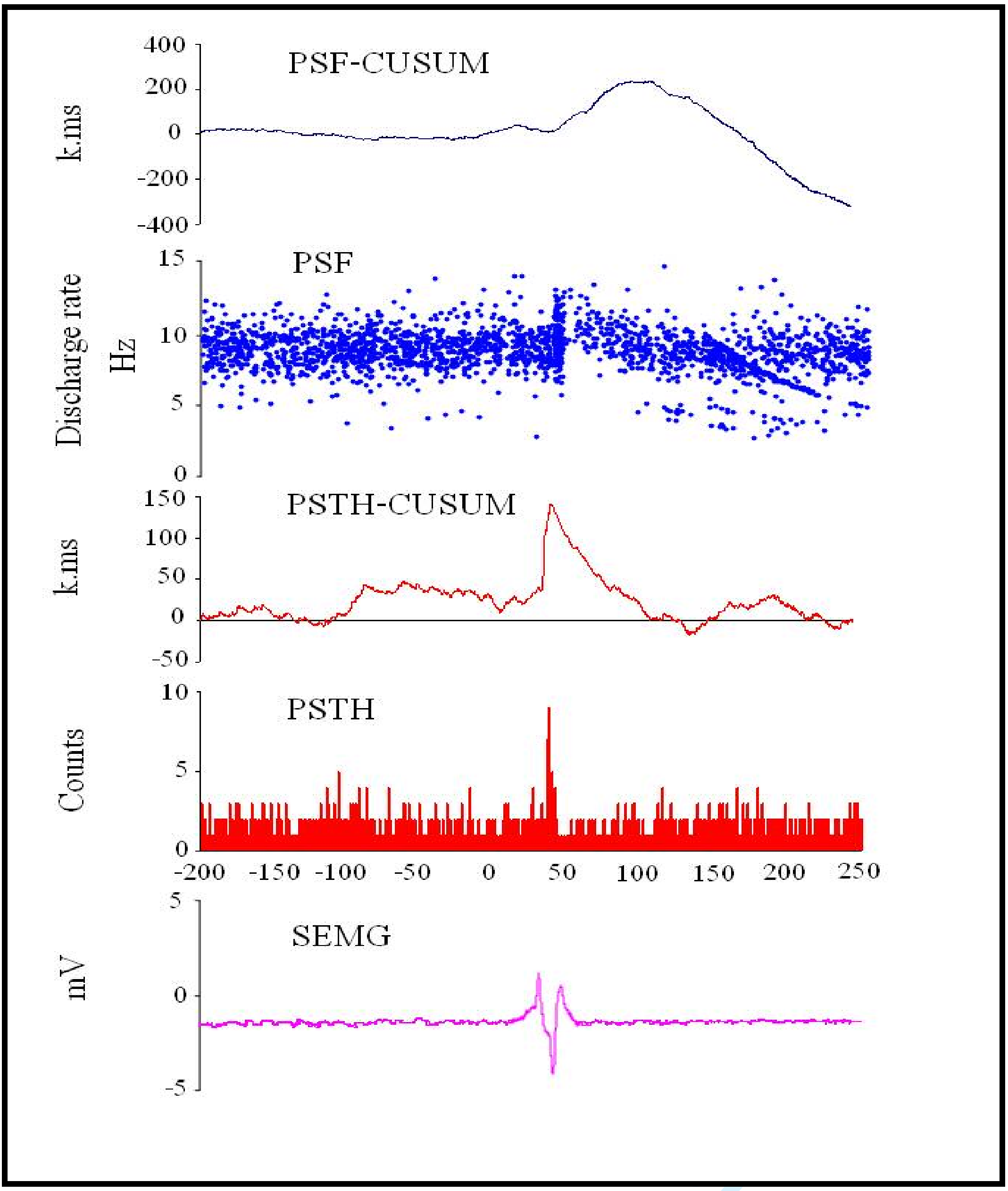

Figure 2 illustrates an example of the H-reflex only response results as can be seen in the last panel for surface electromyogram (SEMG). Top panel is the cumulative sum of peristimulus frequencygram (PSF-CUSUM) which indicates a long-lasting excitatory postsynaptic potential (EPSP) effect. The second panel shows the peristimulus frequencygram (PSF) which clearly shows the existence of increased discharge rates after H reflex response. In contrast, the third panel (CUSUM of peristimulus time histogram, PSTH-CUSUM) and the fourth panel (PSTH) show a reduction in number of spikes immediately after the H-reflex response and a large increase later which is termed the late excitation.

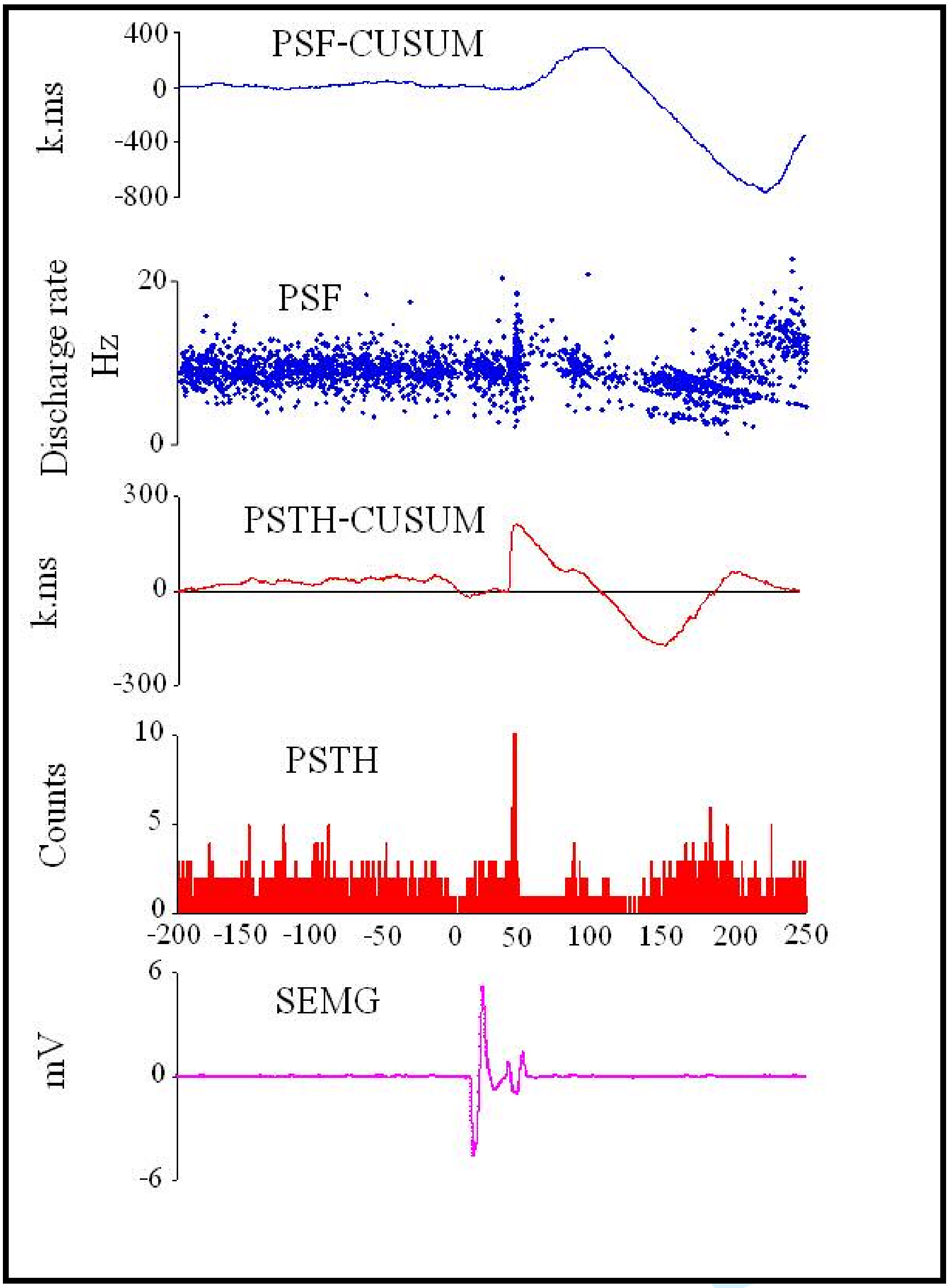

Figure 3 illustrates an example of the M + H response results as can be seen in the last panel for surface electromyogram (sEMG). Top panel is the PSF-CUSUM which indicates a long-lasting excitatory effect. The second panel shows the PSF which clearly shows the existence of discharge rates after the H-reflex response. In contrast, the third panel (PSTH-CUSUM) and the fourth panel (PSTH) indicate a clear reduction in spike counts immediately following the H-reflex (during the silent period) and a significant increase in spike counts later which induces a peak in the PSTH and an upswing in the PSTH-CUSUM (Fig 3).

DISCUSSION

Current results can be interpreted in two different ways depending on the analyses used. With classical PSTH analysis, an increase followed by a decrease could imply that following the stimulus there was an excitatory postsynaptic potential followed by inhibitory postsynaptic potential (EPSP+IPSP) (Ashby and Zilm 1978; Miles et al. 1989). On the other hand, with the PSF analysis, during the same period, there is only an increase in the discharge rate, which should be interpreted as a single long EPSP.

However, we prefer that the PSF interpreted as illustrating the net effect of the stimulation on the motoneurone pool rather than a single distinct pathway. This means that the pool had received several post synaptic potentials but the end product (the net effect) was a single long lasting excitation. If the EPSP and IPSP occur around the same time, depending on the dominant PSP, one could have seen a net excitation, a net inhibition, or no change at all as the end product. We therefore cannot rule out the occurrence of IPSP as a result of the stimulation on the common peroneal nerve in this study especially when the stimulus intensity induces both the M and H responses.

When the stimulus induced both the M and H responses, the PSF analyses were not as clear cut as the ones obtained when the stimulus induced only the H-reflex response (Tables 1 and 2). In deed, when a stimulus induces an M response, one would expect it to generate recurrent inhibition on the motoneurone pool (Bussel and Pierrot-Deseilligny 1977) and hence the net effect may reflect the sum of the Ia EPSP and the Renshaw cell IPSP. Hence, in 6 out of 13 units the discharge rate was not significantly different than the prestimulus. Therefore, involvement of the IPSPs cannot be ruled out in the PSF work as it represents the net effect.

Our conclusion as it stands today is that the activity in the low threshold fibres in the common peroneal nerve generates a single long lasting excitation in the motor pool of the tibialis anterior muscle if the stimulus intensity only generates the H-reflex response on the sEMG. However, even this single long excitation may be due to simultaneous stimulation of Ia and Ib fibres in the nerve. However, until we can distinctly suppress one of these pathways and determine the synaptic potential, we cannot conclude that a single long lasting excitation that we have observed means a single distinct excitatory pathway. Therefore, we cannot confirm or reject our first hypothesis as we are observing the net effect of the stimulus but not a pure PSP from one source.

We can however confirm our second hypothesis, since, during silent period, discharge rate of most motor units were significantly higher than the prestimulus average indicating that during this ‘silent’ period, the net excitation on the motoneurone pool continued. However, this conclusion may only apply to the units obtained when the stimulus only induced the H-reflex response. With the other 13 units that are studied with the stimulus that induced both M and H responses, we may have a combination of excitation and inhibition occurring around the same time. We can also confirm our third hypothesis since we found that the discharge rate of motor units actually significantly declined during the “long-latency excitatory reflex response” illustrating that this period is an artefact of the synchronous discharge of the spikes that generated the H-reflex.

Why do the classical methods contain errors: The classical techniques are based on the principle that a significant increase in the occurrence of discharge (spike) probability at a time after the stimulus represents an excitatory postsynaptic potential (**EPSP**) and a significant decrease indicates an inhibitory postsynaptic potential (**IPSP**). This assumption has led to serious errors in descriptions of connections between various elements of the central nervous system. These arise from the fact that the peaks and troughs in averaged surface EMG or PSTH can reflect not only direct synaptic effects, but also secondary effects arising from the discharge statistics of the pre- and postsynaptic cells (Moore et al. 1970). Despite this early warning about the problems in the probabilistic analysis method, the peaks and troughs in an averaged response to a stimulus are still defined as synaptic excitation and inhibition, respectively (e.g. Brooke et al. 1999; Okdeh et al. 1999; Sonnenborg et al. 2000).

To show directly that the probability based analyses contain inherent errors and to put forward an error free method for the estimation of synaptic potential, and hence the wiring diagram, Türker and Powers have conducted a series of experiments on regularly discharging motoneurons in rat brain slices. The brain slice experiments clearly illustrated that the frequency-based analysis gave a better indication of the underlying synaptic potentials than the probability-based analyses (Türker and Powers 1999; 2001; 2002; 2003;2005).

Significance: This study will correct apparent errors in the most commonly studied and firmly 'established' pathway in the central nervous system. This correction needs to be done urgently since the existence, stability, strength and sign of pathways in the brain and spinal cord are used for diagnosis, treatment and assessment of the success of treatment of many neurological disorders (Cruccu et al. 1989; Chen et al. 1998; Nadler et al. 2004).

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