SAFETY MARGIN AT MAMMALIAN NEUROMUSCULAR JUNCTION - AN EXAMPLE OF THE SIGNIFICANCE OF FINE TIME MEASUREMENTS IN NEUROBIOLOGY

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Key words: neuromuscular transmission, neuromuscular junctions, single fibre electromyography, jitter, safety factor.

Abstract: Single fibre electromyography with axonal microstimulation was used to study the margin of safety of neuromuscular transmission in human and in rat muscle. The latter has been claimed to have a significantly wider safety margin compared to man. There is an inverse relationship between the jitter of the neuromuscular junction (NMJ) and its safety factor. Jitter was measured at 498 NMJs of the extensor communis muscle of 16 healthy human volunteers and at 177 NMJs in the tibialis anterior of 6 Wistar rats. The mean jitter expressed as MCD was 17.1 μs (SD 8.2) and 17. 7 μs (SD 6.1) for the human and the rat muscle, respectively. Moreover, the scatter of the individual jitter values was remarkably similar. These closely similar findings ($P < 0.5$) demonstrate that no significant difference exists in the safety margin of neuromuscular transmission between the two muscles in man and in the rat. An essential condition for studies of this kind has been adequate resolution of time measurement, < 1 μs. This could have been achieved by using a home-designed system for finely adjustable microstimulation amplitude at pulse width ≤ 0.01 ms and for jitter measurement at a resolution of 0.0001 ms, both of which is still unsurpassed by the commercially available equipment for single fibre electromyography.

Varnostni rob na živčno-mišičnem stiku sesalcev, primer pomembnosti natančnega merjenja časa v nevrobiologiji

Kjučne besede: živčno-mišični prenos, živčno-mišični stik, elektromiografija posamičnih vlaken, živčno-mišični drget, varnostni koeficient

Izvleček: V raziskavi varnostnega roba živčno–mišičnega prenosa v človeški in podganji mišici je bila uporabljena elektromiografija posamičnih vlaken z mikrostimulacijo motoričnih aksonov. Ta je po splošnem prepričanju pri podgani mnogo širši kot pa pri človeku. Obstaja obratno sorazmerje med razponom drgeta živčno–mišičnega stika (NMJ) in varnostnim koeficientom. Razpon drgeta je bil merjen v mišici extensor digitorum communis na 498 motoričnih ploščicah 16 zdravih prostovoljcev in na 177 motoričnih ploščicah v mišici tibialis anterior 6 podgan rase Wistar. Povprečni drget, izražen kot MCD, je bil 17.7 μs (SD 8.2) pri človeški mišici in 17.8 μs (SD 6.1) pri podganji mišici. Tudi raztros posameznih vrednosti drgeta je bil zelo podoben. Ti zelo podobni rezultati ($P<0.5$) kažejo, da ni pomembne razlike med varnostnim koeficientom za pregledani mišici pri človeku in podgani. Ključni pogoj za take raziskave pa je ustrezna ločljivost in natančnost merjenja časa, ki mora biti boljša od ene mikrosekunde. To je bilo možno doseči z lastno zasnovo fino nastavljive amplitude mikrostimulacije pri širinah impulza ≤ 0.01ms in merjenje latenc z ločljivostjo 0.0001 ms, kar je veliko boljše, kot je dosegljivo s komercialno dostopnimi napravami za elektromiografijo posamičnih mišičnih vlaken.

1. Introduction

Single fibre EMG is a method which allows recording of action potentials of single muscle fibres in humans or animals, *in vivo* and *in situ*. The method, originally developed by Ekstedt and Stålberg (1964 and 1966) and later supplemented by axonal microstimulation (Trontelj and Stålberg 1992), has been introduced in clinical neurology as a highly sensitive and specific diagnostic technique, mainly for the diagnosis of disorders of neuromuscular transmission. In research, it has contributed many new observations regarding the microphysiology and structure of the motor unit (Stålberg, Trontelj 1994).

The essence of the technique is in its high selectivity of recording, higher than with the conventional needle electrodes used in standard clinical electromyography. The physical dimensions and the input impedance of the active electrode, 25 mm platinum surface exposed in a side port of a cannula, provide a happy compromise resulting in relatively high amplitudes of the recorded single muscle fibre action potentials (most often 1-7 mV, and up to 15 mV). On the other hand, the size of the recording area is comparatively small (a hemisphere of about 300 μm) and is well suited to record from only a limited number of closely adjacent muscle fibres (1-3, rarely up to 10). Later it became apparent that this number does not change much in muscle atrophy in spite of the smaller fibre size, since atrophic fibres are weaker electric generators, and the recording area also becomes smaller. Thus a restricted, more or less standard fraction of the motor unit territory can be investigated. With some experience, the intramuscular position of the electrode can be manually adjusted and maintained for periods of up to more than an hour, thus allowing prolonged recordings from individual muscle fibres. With constant position, the amplitude, shape, and duration of single fibre action potentials clear-cut biphasic spikes remain remarkably constant on consecutive discharges. This makes it possible to accurately identify the moment of depolarization of muscle fibre membrane at the

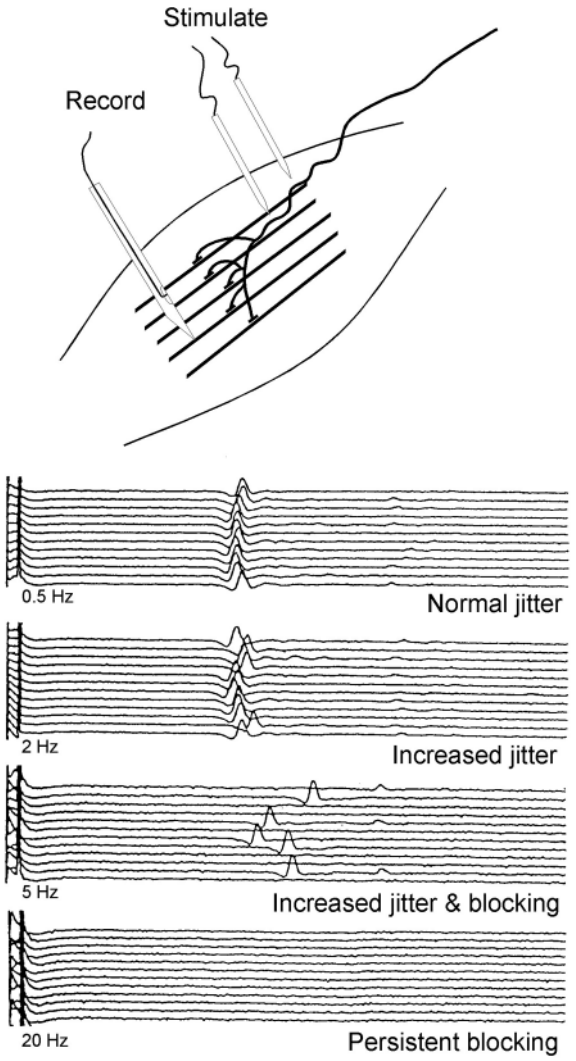

Fig. 1. Jitter study with axonal stimulation technique. The jitter is measured between the stimulus and the action potentials from one muscle fibre. Different degrees of jitter at the same myasthenic NMJ are produced in this case by changing stimulation rate. With 50 % block in the 3rd pane the safety margin of neuromuscular transmission is zero. (From Trontelj et al. 2001, with permission).

electrode surface. Time measurements, e.g. between stimulus and response or between two action potentials from neighbouring fibres with a resolution as high as 100 nanoseconds are not only possible but may, in certain cases, have a physiological significance (Trontelj et al, 1990).

When a motor axon is stimulated repetitively above its threshold and responses are recorded from a single muscle fibre there is latency *variability* of the order of tens of microseconds. This phenomenon is called *the jitter*. The term jitter was introduced to denote variation of neuromuscular transmission time at a single or a pair of neuromuscular junctions (NMJs). It is usually expressed as *mean of consecutive differences* of delay, the *MCD* (Stålberg, Trontelj 1994).

The main source of the jitter in normal muscle is at the NMJ. The jitter is discussed in detail elsewhere (e. g., Stålberg and Trontelj 1994). At the normal NMJ, it is mainly due to small fluctuations in the firing threshold of the subsynaptic sarcolemma, which result in variable neuromuscular transmission time. Moreover, minor variations in amplitude and therefore slope of the end-plate potential, which are due to the variable number of released ACh quanta, contribute to the variability of this synaptic delay in normal muscle (Fig. 2a).

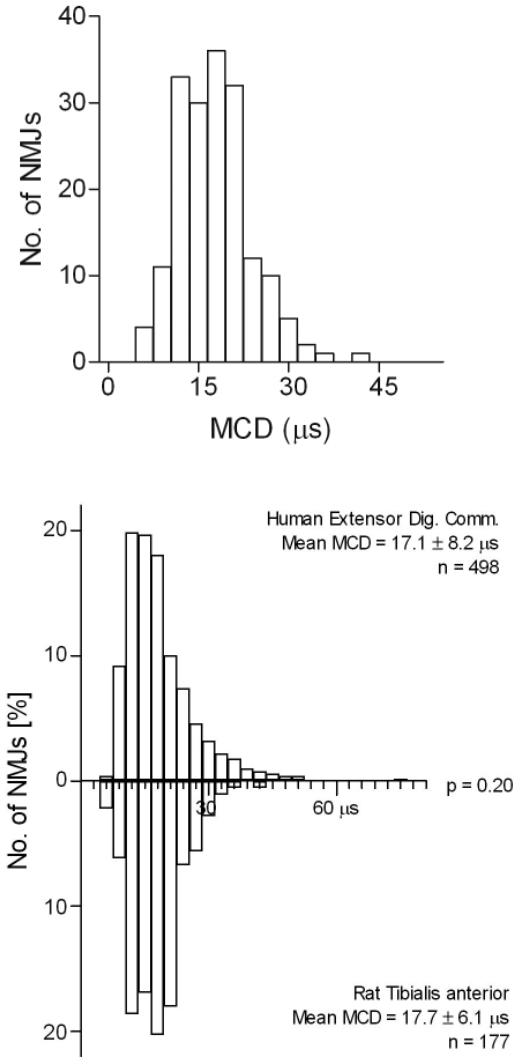

Fig. 2. A. Jitter values of the 177 rat tibialis anterior NMJs. B. Jitter of 498 NMJs in the human extensor digitorum muscle of 16 subjects plotted against jitter values at 177 tibialis anterior NMJs of 6 rats. The means and distributions are closely similar.

Normal jitter varies among different NMJs in the same muscle, and the normal range of mean jitter values varies among muscles. In a study of the effects of regional curarization (Schiller, Stålberg and Schwartz 1975) it was shown that the magnitude of the jitter is related to *the safety margin* of neuromuscular transmission. In other words, the jitter depends on the height of the tip of the endplate potential above

Muscle	Number of NMJs	Minimum [µs]	95% centile [µs]	Mean [µs]	SD [µs]
Extensor dig. comm. (man)	498	5	35.5	17.1	8.2
Tibialis ant. (rat)	177	6	30.5	17.7	6.1

the muscle fibre firing threshold, i.e., on the excess of the acetylcholine receptors activated per nerve impulse. This means that in a normal muscle, the NMJs have a rather different safety margin. When a disease process, such as myasthenia gravis, affects the structure and function of the NMJs, the jitter becomes increased and at a certain point, most often with jitter values between 60 and 120 µs, intermittent blocking of transmission sets in. At this point, the safety margin is close to zero. With increasing jitter values, for example during continued activity, the blocking becomes more frequent and may finally persist.

This safety margin can be semi-quantitatively estimated in vivo in experiments with axonal stimulation during ischaemia or treatment with neuromuscular blocking agents (Dahlbäck et al. 1970; Ekstedt, Stålberg 1969; Schiller et al. 1975; Trinkaus et al. 2007).

Jitter measurement was originally described during voluntary muscle contraction, where the phenomenon is displayed as changing intervals between action potentials of two muscle fibres of the same motor unit during repetitive discharges. The technique was later made simpler and independent of patient's cooperation by replacing voluntary contraction with axonal microstimulation. In this way it became possible to use it also in animals (Trontelj et al. 1986). Moreover, the perfect control of firing patterns and rates over a wide range exceeding that of physiological discharge rates combined with high resolution time measurement made possible new types of in vivo research into physiology and pathology of neuromuscular transmission in intact man or animal.

The purpose of this study was to compare the jitter, and thus the safety margin of neuromuscular transmission, in a human and the rat limb muscle.

2. Materials and Methods

2.1. Human subjects

Sixteen normal subjects participated in the study, their ages ranging between 25 and 45 years. All were in good health and without evidence or past history of neurological problems. The jitter measurement was performed at 10 Hz stimulation at 498 NMJs in the extensor communis muscle. The details of the technique are described elsewhere (Trontelj, Stålberg 1992, Stålberg, Trontelj 1994). Part of the data has been published (Trontelj et al. 2002).

2.2. Animals

Adult male rats (Wistar strain, 200-300 g) were used. The animals were maintained on a standard diet with food and water ad libitum and all efforts were made to assure their comfort. Uretan (Fluka, EU) in 25 % normal saline solution was applied i.p. in a dose of 1.75 g per kg of animal weight for anaesthesia. Tibialis anterior muscle was used; the jitter was measured at 177 NMJs of 6 animals.

The same standard stimulation SFEMG technique was used on the human subjects as well as in the animals (Trontelj, Stålberg 1992). A pair of Teflon coated monofilar needle electrodes were inserted into the belly of the muscle just proximally to its middle, so that the uninsulated tips were 3-6 mm apart. Stimuli were 0.04 µs rectangular pulses of 0.1 - 5.0 mA, adjusted to be suprathreshold for the studied motor axon. They were presented at 10 Hz, although rates of 0.5, 1, 15 and 20 Hz were tried on some NMJs to examine the presence of intratetanic potentiation or exhaustion. Recording was made with a standard SFEMG electrode inserted proximally or distally into the twitching portion of the muscle, between 5 and 12 mm away from the tips of the stimulating needle electrodes (Fig. 1). The stimulation, recording and jitter measurement were performed on a Keypoint EMG system by Medtronic. The jitter programme in this equipment uses a peak-detection algorithm, so the jitter is measured between the stimulus and the negative peak of the single muscle fibre action potential. The jitter is expressed in the standard way as the mean of absolute latency differences between consecutive responses (MCD) to a series of 100 stimuli. The accuracy of measurement was tested by measuring the jitter on simulated single fibre action potentials delivered by a pulse generator with zero jitter, and the measurement error for the conditions used was < 3 µs (MCD). This degree of time resolution was considered less than an optimum, but still adequate for recognising so called low jitter, which indicates direct stimulation of muscle fibres (i.e., not via the nerve and the NMJ). Responses with jitter < 5 µs (MCD) were excluded (Trontelj et al. 1990), but they were infrequent with the position of the stimulating and recording electrodes used.

In accordance with the national guidelines, the study was approved by the National Medical Ethics Committee of Slovenia and the Veterinary Administration of the Slovene Ministry for Agriculture, Forestry and Food, for the human and animal part, respectively.

3. Results

The results obtained are shown in Fig. 2 and in Table. The mean of all MCD values for the 498 NMJs of the human EDC muscle was 17.1 µs (SD 8.2), and for the 177 NMJs of the rat tibialis anterior muscle it was 17.7 µs (SD 6.1). The difference between the two sets of values is nonsignificant (P = 0.20). Even the distribution of the individual values within the two muscles closely resembled each other (Fig. 2). It is obvious that the jitter in the rat tibialis anterior muscle is nearly identical with that in the human extensor digitorum communis muscle. The scatter of the results among the individual animals was small (Fig. 3), again similar to the findings in the human subjects.

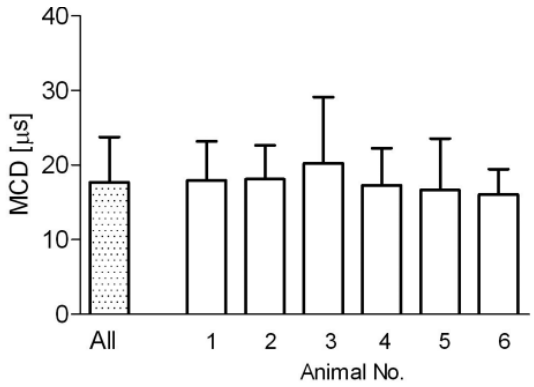

Fig. 3. The variation of the mean jitter values and the SD between the individual animals is relatively small.

With the position of the stimulating and recording electrodes used, there were few responses that could be suspected to be due to direct stimulation of the muscle fibres. As indicated above, the equipment used was barely satisfactory to measure the jitter with the precision required, so some doubt remained with the responses with the jitter in the neighbourhood of 5 µs. Experience gained with the original equipment that served during the development of the technique and the criterion to exclude any slightly 'noisy' action potentials with jitter just above 5 µs were helpful in eliminating direct responses, and were not considered to have introduced a bias towards higher jitter values.

4. Discussion

The jitter of normal neuromuscular transmission at NMJs of human muscles is between 5 and 55 ms. It has been shown that the size of the jitter at a NMJ reflects the amplitude of the end-plate potentials and is inversely related to the safety margin of impulse transmission at that NMJ.

A MCD value of less than 5 ms measured between two different single fibre action potentials or between a stimulus and the response is termed *low jitter*; the impulse in this case has not crossed a NMJ, as neuromuscular jitter always exceeds this value (Trontelj et al. 1986). An example of this is the jitter between action potentials of branches of a split muscle fibre. The jitter of a directly stimulated muscle fibre is low, provided that the stimulus is above the threshold. This could be reliably confirmed by using specially designed equipment and measuring technique (Mihelin et al. 1975) allowing an accuracy of 100 ns (Trontelj et al. 1990). With stimulation pulse width of 10 ms (Trontelj et al. 1967) and good recording conditions this system makes it possible to accurately identify cases of direct muscle fibre stimulation. Commercially available EMG equipment does not provide this degree of temporal resolution. Yet, the test of the equipment indicated an acceptable error of measurement, provided that the noise was kept at a low level.

On the other hand, the jitter of a directly stimulated muscle fibre activated at threshold may be large, between a few tens and a few hundreds of ms, when the discharge rhythm is disturbed by intermittent drop out of the responses and the discharge rate is higher than 1 Hz. This variation in latency is due to changes in muscle fibre conduction velocity (Stålberg 1966, Trontelj et al. 1990).

A similar situation arises when stimulation is via the motor axon and threshold stimulus intensity is used, so that some of the responses fail (false blocking due to insufficient stimulus). Such situations were carefully avoided by adjusting the stimulus strength well above the threshold.

The jitter of neuromuscular transmission results from variability of the time needed for the end-plate potential (EPP) to reach the depolarization threshold of the juxta-junctional sarcolemma (Fig. 4). A part of this variability is due to oscillation of the depolarization threshold of the muscle fibre (the *JUXTA-junctional* component of the jitter). This is not known to be associated with any pathology and is assumed to represent a significant share of the normal jitter.

Moreover, the EPP slope varies from discharge to discharge in a random fashion. The EPP slope in the region of threshold depends on the (extrapolated) EPP amplitude. The mean EPP amplitude and the variation of the EPP slope determine the *junctional* component of the jitter. This component depends on the amount of ACh released per nerve impulse and on the sensitivity of the postsynaptic membrane. The junctional part of the jitter thus reflects the safety margin of neuromuscular transmission, and is proportional to the (excess) amplitude of the extrapolated EPP potential above the level of muscle fibre discharge threshold. The *effect* of fluctuation of the discharge threshold (the juxta-junctional component of jitter) is inversely proportional to the EPP slope. The combination of these factors actually determines the safety margin of neuromuscular transmission (Stålberg, Trontelj 1994).

The *postjunctional* part of the jitter, mainly due to changes in muscle fibre conduction velocity resulting from different degrees of "supernormality" following previous activity, is largely avoided by the uniform discharge rates during electrical stimulation. (The contribution of this factor may be large when intermittent blocking occurs, resulting in disrupted rhythm.)

There is a possibility for a pre-junctional contribution to the jitter, seen as latency variation of the end-plate potential. The *end-plate potential jitter* is usually negligible (<1-2 µs); it may however become large after intoxication with some organophosphates (De Blaquiere et al. 1998). A large EPP jitter may also be seen in botulism or experimental intoxication with botulinum toxin B (Maselli et al. 1992; Gansel et al. 1987).

Neither pre- nor postjunctional factors could have influenced the measured jitter in this study. The measured values can therefore be safely considered to reflect the true variation of time taken for transmission at the NMJ itself, and thus its safety margin.

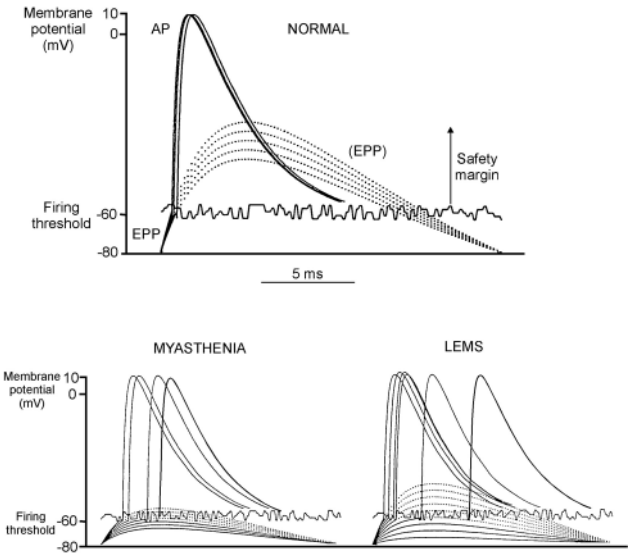

Fig. 4. Schematic explanation of the relationship between the jitter and the safety margin. At the normal NMJ, the variation in neuromuscular transmission time (the jitter) is generated mainly by oscillations of the firing threshold at which muscle fibre action potential (AP) is triggered. High amplitude EPPs have a steeper slope and a shorter course through the firing threshold range, and the jitter generated is smaller. Another source of the jitter is the variation in endplate potential (EPP) amplitude and therefore slope. The amplitude of the extrapolated EPPs exceeding the firing threshold represents the safety margin. In cases of postsynaptic dysfunction (such as myasthenia gravis), the variation of neuromuscular transmission time is increased due to lower EPPs, which take a longer path through the firing threshold range; some EPPs do not reach the threshold and the transmission is blocked. In cases of presynaptic dysfunction (such as the Lambert Eaton syndrome, LEMS), the EPP amplitude is not only low but is also excessively variable.

The safety margin at the individual NMJs has been semi-quantitatively estimated *in vivo* in experiments with axonal stimulation during ischaemia or treatment with neuromuscular blocking agents (Schiller et al. 1975; Stålberg et al. 1975). However, the exact relationship between the magnitude of the jitter and the safety factor is difficult to establish. A computer simulation study suggested an exponential-like relationship, which was supported by the actual data from myasthenic NMJs (Lin, Cheng 1998b, Trontelj et al. 2002). It suggested a safety factor of 8-10 for human NMJs. In contrast, it was estimated at up to 20 or more in some animals (Waud, Waud 1975; Lin, Cheng 1998b).

In this study, the rat tibialis anterior muscle has been found to have practically identical jitter as the human extensor digitorum muscle and therefore equally wide safety margin. On the other hand, the NMJs in the rat tibialis anterior muscle have a narrower safety margin than those of the human orbicularis oculi or mentalis muscle.

One might argue that comparison should be made between identical muscles of man and the rat. However, the human tibialis anterior muscle has rather large jitter values. This has been suggested to be due to subclinical microtraumatisation of the peroneal nerve at the head of the fibula, for example while sitting with crossed legs. As a result, some denervation and reinnervation is going on in the peroneal supplied muscle. New NMJs are known to have larger jitter (Stålberg, Trontelj 1994). This mechanism is unlikely to operate in the rat.

Our results are actually similar to those of Lin and Cheng (1998a), who studied the rat gastrocnemius muscle. Yet they found some NMJs with small jitter (about 5 µs) which they took as evidence of extremely high safety factor of neuromuscular transmission in the rat. However, such values can be recorded in human muscles and are seen in a similar (small) proportion of the NMJs in a muscle. Indeed, the jitter and thus the safety margin of neuromuscular transmission may vary in one and the same muscle of a single individual quite considerably. In the human extensor digitorum communis muscle, about 20 % of the NMJs are found to have relatively larger jitter (Trontelj et al 2002).

Slightly smaller jitter was reported for 81 gluteus medius NMJs of 8 Lewis rats (Verschuuren et al. 1990). The mean MCD at 10 Hz stimulation in this study was 11.5 µs (SD 4.0). Considerably smaller jitter was found in the mouse gastrocnemius muscle: MCD 5-15 µs; mean 7.9, 11.3 and 6.1; SD 3, 4 and 2, respectively, for mice of three different strains (Gooch, Mosier 2001). Such low values would be compatible with a significantly wider safety margin.

However, one has to exclude technical reasons. The gastrocnemius muscle in the mouse is small and the position of both stimulating and recording electrodes is more critical. One possibility for obtaining small jitter values is unrecognized direct stimulation of muscle fibres (rather than through their axons), which would result in 'low' jitter. With less than optimal recording conditions, and in particular with unsatisfactory resolution of time measurement, the values may exceed the 5 µs criterion and be erroneously considered as normal NMJ jitter. As a result, false-low readings will shift the calculated mean towards lower figures.

Another possible explanation for relatively small mean jitter values found in some studies could be superimposition of action potentials from several fibres. The resulting spike, when well synchronised, may in fact resemble a single fibre action potential. However, the jitter of the composite potential may be smaller than that of the individual muscle fibre components, as has been demonstrated by a computer simulation (Stålberg et al. 1992). In our experience, such recordings are common in the rat tibialis anterior muscle, and their inadvertent inclusion could result in a significantly underestimated mean jitter. Good recording

conditions, including absence of noise are an essential prerequisite for a reliable distinction between an axonal and a direct muscle fibre stimulation. Single fibre action potentials used for measurement of jitter should have a peak-to-peak amplitude of at least 1.0 mV. Criteria for single fibre action potentials must be strictly observed, in order to avoid measurements on composite spikes. The effective resolution of latency measurement should be at least 1 µs. Jitter measurement based on peak detection algorithm used in some equipment for diagnostic SFEMG, is reliable and convenient for clinical use, where the aim is to detect and measure large jitter, but may, near the low jitter values, become unreliable.

In conclusion, this study failed to confirm the assumption of a wider safety margin of neuromuscular transmission in the limb muscle of the rat compared to a similar muscle in man. On the contrary, there does not seem to be any difference between the mean safety factors of human and rat NMJs.

Acknowledgements

This study is part of a research project on the protection and treatment of intoxication with neurochemical warfare agents (M3-0142). The contributions of Dr. Janez Sketelj, Dr. Miha Trinkaus, Dr. Špela Glišovič, Dr. Marjan Mihelin, Mrs. Tatjana Trontelj, Mr. Ignac Zidar and Tone Žakelj to the experimental work, data collection and analysis are gratefully acknowledged.

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Prispelo (Arrived): 01.09.2008 Sprejeto (Accepted): 15.09.2008