Sensitivity to ischaemia of single sympathetic nerve fibres innervating the dorsum of the human foot.

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Key points summary

- Changes in nerve conduction velocity following an impulse (i.e. velocity recovery cycles) reflect afterpotentials, and can provide an indication of altered nerve membrane properties
- This study used microneurography to assess the effects of ischaemia on single human sympathetic fibres innervating the dorsum of the foot
- It was found that velocity recovery cycles can distinguish whether a sympathetic nerve fibre is depolarized or not
- The method may be used to detect membrane depolarization of sympathetic nerve fibres in human patients when autonomic neuropathy is suspected

Abstract

The aim of this study was to determine whether velocity recovery cycles (VRCs) could detect the effects of ischaemia on sympathetic nerve fibres. VRCs of human sympathetic nerve fibres of the superficial peroneal nerve innervating the dorsum of the foot were recorded by microneurography in 7 healthy volunteers. Sympathetic nerve fibres were identified by studying their response to manoeuvres increasing sympathetic outflow and by measuring activity-dependent slowing at 2 Hz stimulation. VRCs were assessed at rest, during 30 minutes of induced limb ischaemia and during 20 minutes of recovery after ischaemia. From each VRC was measured the relative refractory period (RRP), the supernormality and the time to peak supernormality (SN@). During ischaemia, RRP increased from the baseline value of 37.4±8.7 ms (mean ± SE) to 67.1±12.1 ms ($P < 0.01$) and SN@ increased from 68.6±9.8 ms to 133.8±11.0 ms ($P < 0.005$). The difference between SN@ and RRP separated ischaemic from non-ischaemic sympathetic nerve fibres. It is concluded that these sympathetic nerve fibres are sensitive to ischaemia, and that VRCs provide a method to study changes of axonal membrane potential of human sympathetic nerve fibres *in vivo*.

Abbreviations. CV, conduction velocity; ISI, interstimulus interval; RRP, relative refractory period; SN, supernormality; SN@, time to peak supernormality; VRC, velocity recovery cycle.

Introduction

Changes of membrane potential and alterations of ion channel function of unmyelinated axons and of muscle fibres can be assessed by measuring velocity recovery cycles (VRCs) (Bostock et al., 2003; Z'Graggen and Bostock, 2009). The technique of recording VRCs is based on the principle that an evoked action potential is followed by a characteristic sequence of changes in the membrane potential, which decay over a variable period. These afterpotentials affect the conduction velocity of a second action potential. For human sympathetic nerve fibres innervating the hairy skin it has been shown, that RRP is followed by a depolarizing afterpotential leading to an acceleration of the conduction velocity of a second action potential and causing a phase of supernormality (SN) with a duration of about 1000 ms (Bostock et al., 2003). In addition to this short-lasting phase of SN, these fibres also exhibit a long-lasting phase of altered conduction velocity (Campero et al., 2004). The latter leads to a distinct 'Type 4' pattern of activity-dependent slowing at 2 Hz-stimulation, which can be used to distinguish this fibre type from other C-fibres (Campero et al., 2004).

To our knowledge, the technique of recording VRC has not yet been used for studying alterations of sympathetic nerve membrane potential. The aim of this study was to assess nerve membrane potential changes of sympathetic nerve fibres during ischaemia by recording repeated VRCs. Describing a set of parameters that could distinguish normal versus "depolarised" sympathetic nerve fibres could be a useful diagnostic method with clinical application where specific sympathetic peripheral nerve fibre pathology is suspected.

Methods

Ethical approval

Twelve healthy subjects (7 female, 5 male; mean age 29.4, range 22-45) volunteered for this study. Approval was obtained from the local ethics committee (Kantonale Ethikkommission, Bern, Switzerland) and conformed to the Declaration of Helsinki. All participants provided written informed consent.

Microneurography

Microneurography was performed as described earlier (Serra et al., 1999; Campero et al., 2004). In brief, volunteers rested comfortably on an armchair in a warm room. An isolated constant-current stimulator (DS7; Digitimer, Ltd., Welwyn Garden City, Hertfordshire, UK) was used for stimulation of the cutaneous receptive fields with a pair of non-isolated needle-electrodes placed on the skin. Stimulus duration was set at 0.05 ms.

Action potentials from sympathetic nerve fibres were recorded using tungsten microelectrodes (200 μm diameter, impedance 1 MΩ) placed intraneurally in the superficial peroneal nerve at ankle level. Signals were amplified (gain 1000, bandwidth 1.6 Hz to 2 kHz) with an isolated high-input impedance amplifier (NeuroAmpEx, ADInstruments, Bella Vista, Australia) and digitized (NI DAQCARD-6062E; National Instruments Europe Corp., Debrecen, Hungary) at a sampling rate of 20 kHz. Stimulation and recording were controlled by Qtrac software (written by H. Bostock, copyright Institute of Neurology, London, UK), using the menu-driven recording protocol 750RC5K.QRP. Responses were digitally filtered, and the largest peaks displayed as a latency profile or raster plot (Serra et al., 2014). Sympathetic fibres were identified in the raster plots by their characteristic profile of activity-dependent slowing of conduction velocity, which reached a plateau within one minute when stimulation rate was increased from 0.25 Hz to 2Hz, and by their responses to manoeuvres increasing sympathetic outflow (Campero et al., 2004).

Recording of velocity recovery cycles

After identification of a sympathetic nerve fibre action potential with adequate signal-to-noise ratio, the stimulation rate was increased to 1 Hz, and VRCs with single conditioning stimuli were recorded repeatedly. An unconditioned stimulus was followed after an interval of 1s by a conditioned stimulus, and an interval of 2s elapsed before the next unconditioned stimulus, to give an average stimulation rate of 1 Hz. There were 40 interstimulus intervals (ISI), which decreased from 375 to 5 ms, in an approximately logarithmic series, so that one VRC was completed every two minutes. Two VRCs were recorded at rest, and then ischaemia was induced by inflating a sphygmomanometer cuff on the lower leg above systolic blood pressure for 30 minutes. Fifteen VRCs were recorded during ischaemia, and another 10 during 20 minutes of the recovery phase after ischaemia.

Temperature

Skin temperature close to the superficial peroneal nerve was recorded continuously with an infrared thermometer (PCE-IR10, PCE Ibérica, Spain) and an electric radiator adjusted to maintain temperature in the range 30-32˚C. Although it was possible to minimize changes in skin temperature during the ischaemia, it was usually not possible to avoid a small rise in skin temperature during the reactive hyperemia that followed cuff release (Figure 1).

Data analysis and statistics

VRCs were analysed using the Qtrac software. In the first stage, the 'latency profile' of an identified sympathetic unit was generated by 'latency tracking' in which a latency 'window' was re-centred on the peak of the action potential during successive sweeps, as described by Serra et al. (1999). The latency profiles of the unconditioned and conditioned spikes were tracked separately, but shown superimposed in Figure 1. In the second stage of the analysis, VRCs were selected to represent each of the three phases of the recording: pre-ischaemia (the last two VRCs before cuff inflation), end-ischaemia (the last 3 VRCs before

cuff release), and recovery (the 3 VRCs closest to 15 m after cuff release)(see Figure 2). Each VRC was measured separately, and the measurements averaged for each phase. In addition to conduction velocity (CV), the following VRC measurements were analysed: (i) relative refractory period (RRP) = the interpolated ISI at which velocity first reached its unconditioned value; (ii) supernormality (SN) = peak percent increase in velocity at ISIs longer than RRP; and (iii) the ISI at which supernormality reached its peak (SN@). One unit with very little resting supernormality, lost this supernormality entirely during ischaemia. In this case the supernormality was registered as 0% and the RRP as 100 ms rather than ∞. The changes in these measurements during and after ischaemia were compared by paired *t*-tests. $P < 0.05$ was considered significant.

Results

Successful complete recordings as in Fig. 1B were made from 7 sympathetic units in 7 different healthy control subjects (5 female, 2 male, mean age 27.4 years, range 22-40), and the average recordings are illustrated in Figure 3. The width of the standard errors shows there was considerable variability in RRP and SN of the units, as well as in their latency, but the changes with ischaemia were consistent, especially during the recovery from ischaemia, as shown in Figure 4. Although RRP became shorter on average post-ischaemia than it was pre-ischaemia (Fig. 4B), this difference was not significant, and was probably accounted for by the slight post-ischaemic rise in temperature (from 31.16 ± 0.66 to 32.20 ± 0.56˚C, mean ± SE).

None of the variables illustrated in Fig. 4 was able, on its own, to accurately determine whether a given recording was from an ischaemic or a non-ischaemic sympathetic nerve fibre. However, the plot of RRP vs. SN@ in Fig. 5A, shows that this combination of variables achieved a good separation between both conditions. Figure 5B shows that the difference

SN@ - RRP fully discriminated pre-ischaemia and post-ischaemia recordings from the end-ischaemia recordings.

Discussion

Recovery cycles, which indirectly reflect post-spike afterpotentials, have been used as indicators of membrane potential in both axonal and muscle preparations. The basic rationale is that afterpotentials reflect the imbalance between inward sodium charge movement and outward potassium charge movement during the action potential, the resulting charge difference (ΔQ) causing a change in membrane potential determined by the membrane capacitance (C) as ($\Delta Q/C$). The depolarizing afterpotential is associated with a post-spike increase in excitability (superexcitability) and an increase in conduction velocity (supernormality). With depolarization of the resting membrane potential, sodium channel inactivation increases, so that sodium currents are reduced, whereas potassium currents may be increased, depending on whether or not the depolarization is caused by an increase in extracellular potassium concentration. In any case, the net inward charge movement is reduced by membrane depolarization, so that the depolarizing afterpotential is reduced (or becomes hyperpolarizing). Consequently, excitability recovery cycles show less superexcitability (or become subexcitable), while velocity recovery cycles show less supernormality (or become subnormal). Thus in human motor axons, depolarization by ischaemia or applied current produces a dramatic increase in relative refractory period and reduction in superexcitability, so that already within 5 minutes of applying a pressure cuff there is no overlap with the pre-ischaemia values (Kiernan & Bostock, 2000). In C fibres and muscle fibres, recovery cycles are more conveniently recorded in terms of velocity changes (Weidner et al., 2000; Bostock et al., 2003; Z'Graggen & Bostock, 2009). Muscle velocity recovery cycles resemble motor axon excitability recovery cycles in showing an increase in RRP and reduction in supernormality within 5 minutes of applying a pressure cuff (Z'Graggen

& Bostock, 2009), and in both motor axons and muscle fibres, these potential-dependent changes in recovery cycles have been used to reveal and assess membrane depolarization in disease states. For example, reduction in superexcitability has provided evidence for membrane depolarization of motor axons in renal failure (Kiernan et al., 2002; Krishnan et al., 2005), Fabry disease (Tan et al., 2005), focal compression (Ikemoto et al., 2009) and critical illness polyneuropathy (Z'Graggen et al., 2006), and of sensory axons in Bortezomib-induced neuropathy (Nasu et al., 2014), while reduction in supernormality has similarly provided evidence that muscle fibres are depolarized in renal failure (Z'Graggen et al., 2010) and Andersen-Tawil syndrome (Tan et al., 2012) .

In C fibres, the simple argument above for the basis of supernormality is complicated by the enhanced effects of intracellular sodium accumulation and, in C-nociceptors, by the importance of changes in inactivation of the TTX-resistant $Na_v1.8$ sodium channels, so that a detailed model of post-spike excitability changes found that a depolarizing afterpotential is not necessary for supernormality (Tigerholm et al., 2015). Nevertheless, the modelling suggested that depolarization should still reduce supernormality, and sympathetic fibres lack the complication of $Na_v1.8$. Therefore it was hoped that this study would provide evidence that velocity recovery cycles could be used, as in muscle fibres, to detect membrane depolarization in neuropathies affecting sympathetic nerve fibres. We were at first disappointed that although each sympathetic fibre recorded showed the expected change in RRP with ischaemia (Fig. 4B), because of the variability in resting values, there was considerable overlap between the normal and ischaemic fibres. None of the recovery cycle measurements on its own was sensitive enough to identify an individual measurement as coming from an ischaemic or non-ischaemic fibre. We found, however, that the combination SN@ - RRP does enable a recording to be identified unambiguously as ischaemic or not (Fig. 5B).

The sample in this study was limited, because of the difficulty of following single sympathetic fibres for up to an hour during the period of ischaemia and recovery (Fig. 1B). However, a check on the normal sympathetic fibres in an earlier study (Bostock et al., 2003) showed that in those 9 fibres also the value of SN@ - RRP never exceeded 50 ms. We therefore propose that this measure may provide a useful clinical biomarker for assessing neuropathic membrane depolarization in human patients where an autonomic neuropathy is suspected.

Nature of the sympathetic fibres recorded

There are three subtypes of sympathetic fibre that innervate the skin: vasomotor, sudomotor and pilomotor, although the latter are rarely encountered in microneurography (Macefield & Wallin, 1999; Macefield, 2013). Unfortunately, the sympathetic manoeuvres used in this study do not permit separation of vasomotor and sudomotor fibres, and they cannot be distinguished by their firing patterns (Macefield & Wallin, 1999). The only established method for achieving this is by warming or cooling the whole body (Bini et al., 1980; Macefield & Wallin, 1999). A possible clue to the nature of the 7 sympathetic fibres is their conduction velocity, which averaged 0.66 ms-1 (range 0.56 to 0.82 ms^{-1}). These values are closer to Fagius and Wallin's (1980) estimate of 0.77 ms-1 for the peroneal fibres mediating reflex vasoconstriction, than their estimate of 1.27 ms^{-1} for fibres mediating the sudomotor reflex. However, it is uncertain how well velocities estimated from reflex latency correspond to those measured over the distal few centimetres, so this comparison must be treated with caution. Also, in a microneurographic study in which whole-body warming and cooling was used to help identify sympathetic fibre type, Schmelz et al. (1998) tentatively classified one unit conducting at 0.78 ms^{-1} as vasomotor, and one conducting at 0.68 ms^{-1} as sudomotor. Disappointingly, two recent reviews of single sympathetic fibre recordings make no mention of conduction velocity (Lambert et al., 2012; Macefield, 2013). Other possible

characteristics that might provide a clue to sympathetic subtype are the pattern of activity-dependent slowing (Campero et al., 2004) and action potential shape, which has been reported as 'usually biphasic' for sudomotor, as against 'often triphasic' for vasomotor fibres (Macefield & Wallin, 1996). However, neither feature has been shown to provide a reliable means of classification. Since our 7 fibres all had triphasic action potentials and were slowly conducting, it seems likely that they were all vasomotors, but this is uncertain, and there is a clear need for a more convenient method of identifying sympathetic functionality than whole-body heating and cooling.

In conclusion, we have used ischaemia to demonstrate changes in the recovery cycle of single human sympathetic fibres in the superficial peroneal nerve, that may help to determine whether such fibres are depolarized in disease states. However, because of uncertainty in the subtype(s) of sympathetic fibre studied, we caution against expecting this relationship to hold for sympathetic fibres recorded elsewhere, or which have biphasic rather than triphasic action potentials, or conduction velocities outside the range studied (0.56 to 0.82 ms^{-1}).

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Author contributions

All authors contributed to the conception and design of the experiments, collection and interpretation of data, and critical revision of the manuscript. RS and HB analysed the data, and WZ and HB drafted the article. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Competing interests

HB receives royalties from UCL for sales of the Qtrac software used in this study.

Figures

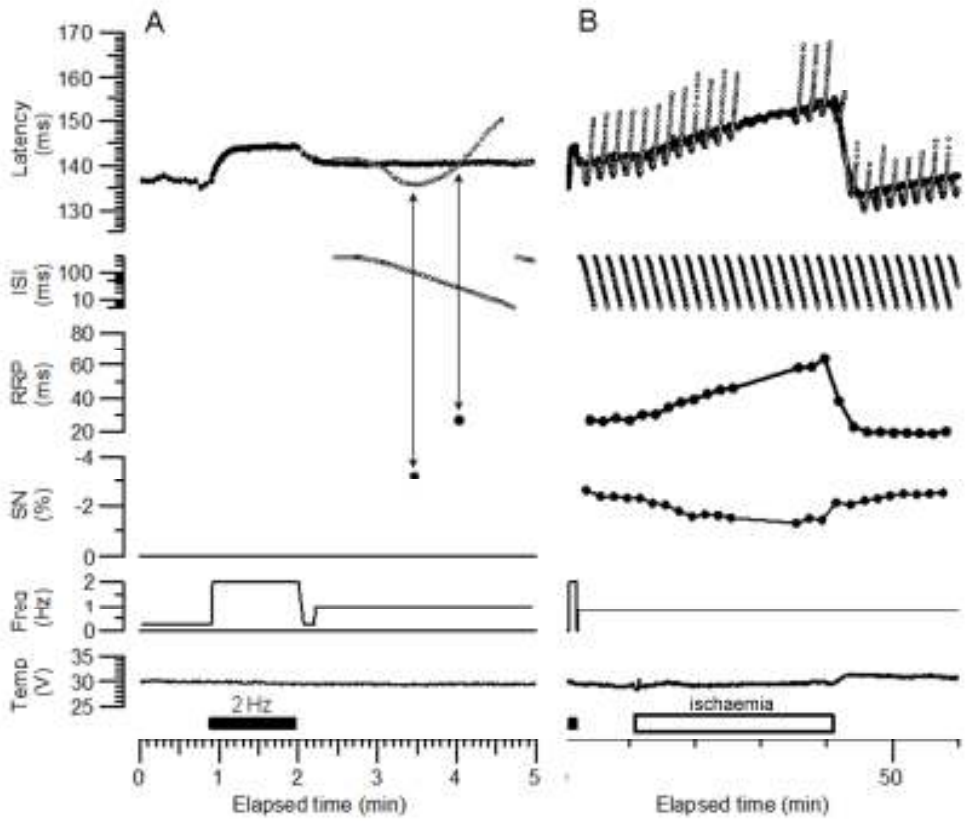

Figure 1. Recording of velocity recovery cycles of single sympathetic fibres during ischaemia. A: Detail of response to 2 Hz stimulation and first baseline recovery cycle. B: Full recording of ischaemia and recovery over 60 minutes. *Top row*: latency of unconditioned (filled circles) and conditioned (open circles) responses to electrical test stimuli. *2nd row:* Interstimulus interval between conditioning and test stimuli (log scale). *3rd row:* Relative refractory period, or shortest ISI at which latencies of unconditioned and conditioned responses were equal. *4th row*: Supernormality, calculated as greatest % difference between conditioned and unconditioned responses. *5th row*: Mean stimulation frequency. *Bottom row*: Skin temperature (˚C). Filled bar below plots indicates period of 2 Hz repetitive stimulation. Open bar indicates period during which cuff was inflated. The first vertical arrow links (*top row*) the point of maximum latency difference, to (*4th row*) the calculated supernormality for this recovery cyle. The second vertical arrow links (*top row*) the point at which conditioned and unconditioned latencies cross, via (*2nd row*) the ISI at which this occurs, to (*3rd row*) this ISI plotted as relative refractory period. In B, not all conditioned response latencies could be measured easily because of overlap between different units.

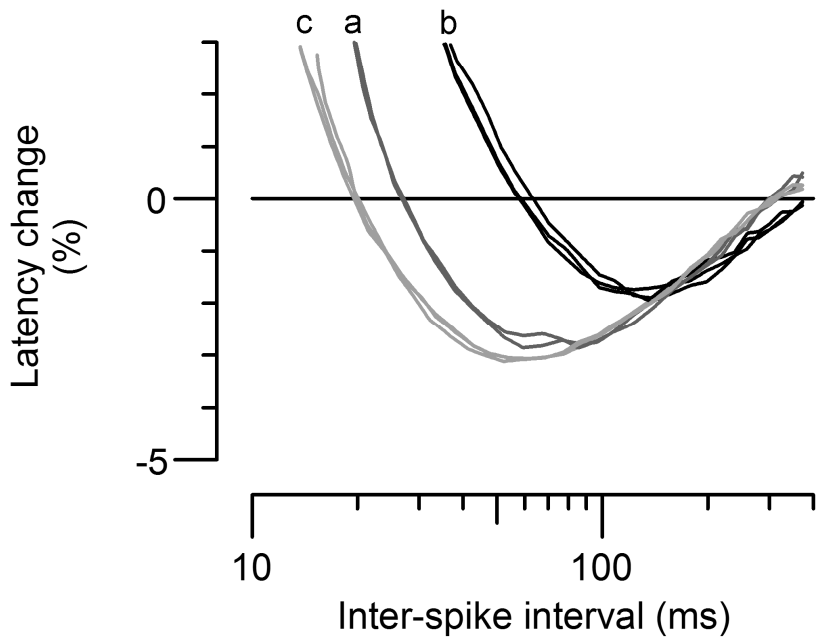

Figure 2. Velocity recovery cycles extracted from data in Fig. 1. a: 3 pre-ischaemia, b: 3 end- ischaemia, c: 2 ca. 10m post-ischaemia.

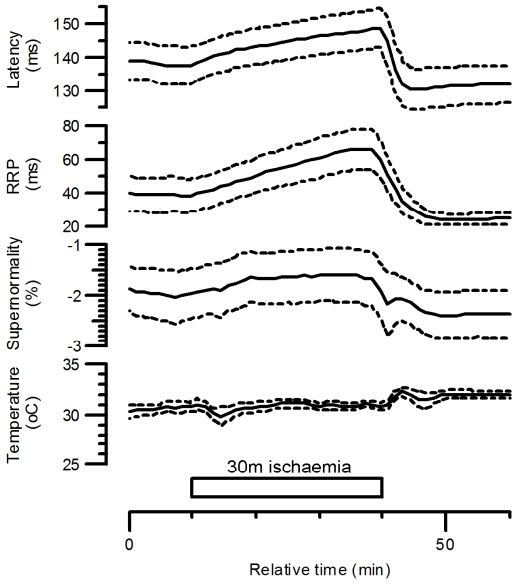

Figure 3. Average responses to ischaemia. Traces show means ± SE of recordings as in Fig. 1B from single sympathetic C fibres in 7 different subjects.

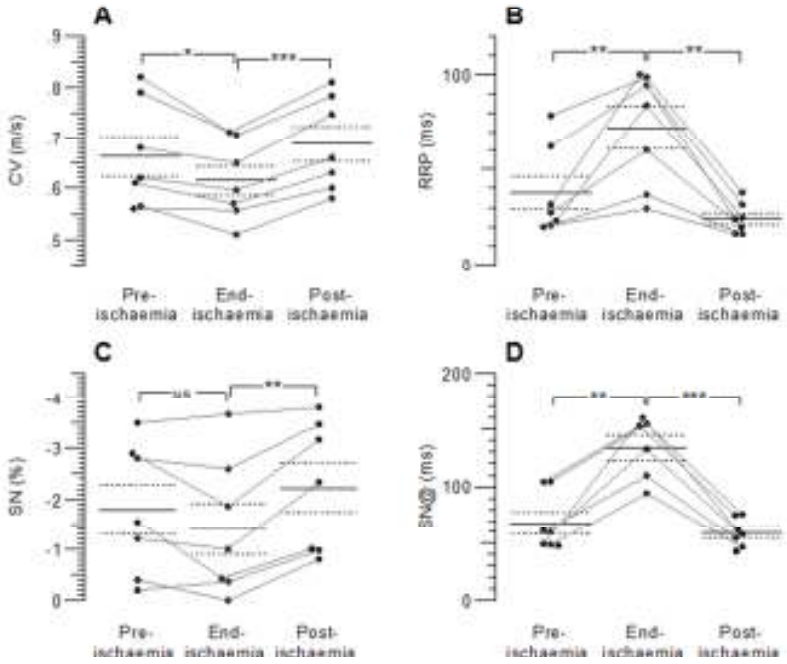

Figure 4. Changes in conduction velocity and velocity recovery cycles for 7 sympathetic units. **A:** Conduction velocity. Lines join measurements for the same single sympathetic fibre. **B**: Relative refractory period. **C**: Supernormality. **D**: Time to peak supernormality. P values indicated are for paired t-tests: NS = P>0.05, * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001. In each case, the difference between pre-ischaemia and post-ischaemia values was not significant.

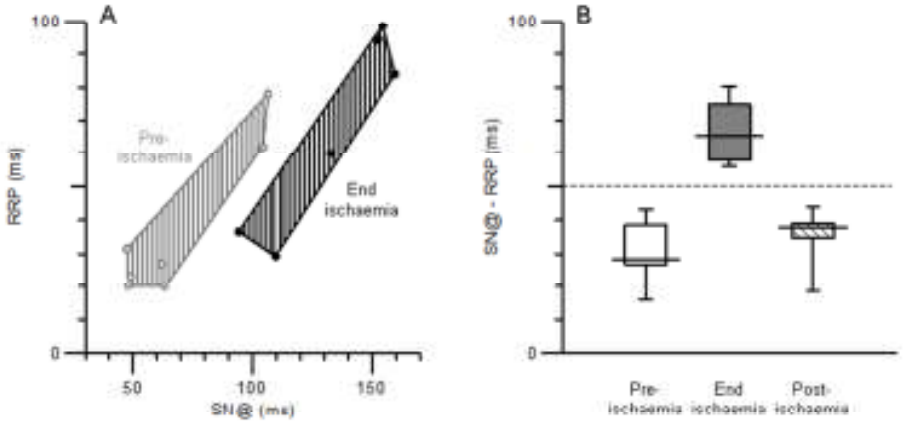

Figure 5. Discrimination of ischaemia recordings by combining two measurements. A: Plot of relative refractory period against time to peak supernormality for pre-ischaemia (open grey circles) and end ischaemia (filled black circles) recordings. B: Box and whisker plots for SN@ - RRP values for 3 groups, showing complete separation by dashed line at 50 ms. (Boxes show interquartile ranges, whiskers show ranges, and horizontal lines show medians).