Received: 6 May 2022 | Revised: 25 July 2022 | Accepted: 15 August 2022

DOI: 10.1111/ejn.15801

RESEARCH REPORT

EJN European Journal of Neuroscience FENS WILEY

Long-term modulation of the axonal refractory period

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Funding information
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Edited by: Francisco Alvarez

Abstract

The main question addressed in this study was whether the refractoriness of nerve fibres can be modulated by their depolarisation and, if so, whether depolarisation of nerve fibres evokes a long-term decrease in the duration of the refractory period as well as the previously demonstrated increase in their excitability. This was investigated on nerve fibres within the dorsal columns, dorsal roots and peripheral nerves in deeply anaesthetised rats in vivo. The results revealed major differences depending on the sites of fibre stimulation and polarisation. Firstly, the relative refractory period was found to be shorter in epidurally stimulated dorsal column fibres than in fibres stimulated at other sites. Secondly, the minimal effective interstimulus intervals reflecting the absolute refractory period were likewise shorter for nerve fibres within the dorsal columns even though action potentials evoked by the second of a pair of stimuli were similarly delayed with respect to the preceding action potentials at all the stimulation sites. Thirdly, the minimal interstimulus intervals were reduced by epidurally applied cathodal direct current polarisation but not at other stimulation sites. Consequently, higher proportions of dorsal column fibres could be excited at higher frequencies, especially following their depolarisation, at interstimulus intervals as short as 0.5–0.7 ms. The results demonstrate that epidural depolarisation results in long-lasting effects not only on the excitability but also on the refractoriness of dorsal column fibres. They also provide further evidence for specific features of afferent fibres traversing the dorsal columns previously linked to properties of their branching regions.

KEYWORDS
afferent fibres, epidural stimulation, polarisation, spinal cord

1 | INTRODUCTION

Previous studies have revealed a considerable degree of both short- and long-term plasticity in nerve fibre properties (for recent references, see Bucher & Goaillard, 2011; Debanne, 2004; Debanne et al., 2011; Jankowska &

Abbreviations: DC, direct current; GABA, gamma-aminobutyric acid; L, lumbar; Na, sodium; $NaHCO_3$, sodium hydrogen carbonate; PAD, primary afferent depolarisation; Per, peroneal nerve; Th, thoracic; Tib, tibial nerve.

Hammar, 2021; Suminaite et al., 2019). Our own interest has been in the long-lasting increase in the excitability of afferent nerve fibres providing input to the spinal cord following their short-lasting epidural direct current (DC) polarisation (Bączyk & Jankowska, 2018; Jankowska et al., 2017; Li et al., 2020) which might enhance effects of clinical rehabilitation procedures combined with spinal polarisation. However, whether any sustained effects of DC on the refractoriness of nerve fibres are or are not related to the DC-evoked changes in the excitability but might be of similar clinical interest remained unexplored.

In order to examine the possibility of such effects, we compared the refractory period of dorsal column fibres before and after epidural DC polarisation. The duration of the refractory period was assessed from effects of paired stimuli applied at decreasing intervals. The minimal interval at which the second stimulus was able to excite the fibres was used as a measure of the absolute refractory period, while the range of intervals over which the effectiveness of the second stimulus increased was used to evaluate the relative refractory period. In the case of single cells or fibres, the absolute and the relative refractory periods are assessed from the threshold intensity of the second of a pair of stimuli applied at varying interstimulus intervals. However, when nerve fibres in a tissue volume at different distances from the electrode tip are excited, the same stimulus intensity may be subthreshold for some of these fibres but at threshold or suprathreshold for others. We, therefore, applied two suprathreshold stimuli of the same intensity to the surface of the dorsal columns and evaluated the effectiveness of these stimuli by comparing the compound action potentials they evoked. To this end, we measured the areas of nerve volleys propagated from the dorsal columns to hindlimb nerves. Interstimulus intervals at which minimal nerve volleys were evoked by the second of a pair of stimuli at twice threshold intensity were considered as reflecting the absolute refractory period while the intervals at which both stimuli started to evoke nerve volleys of the same size as the end of the relative refractory period (Bucher & Goaillard, 2011).

It will be shown that following epidural polarisation, larger nerve volleys are evoked during the relative refractory period and that the minimal intervals between the two stimuli are reduced, indicating that the epidural depolarisation shortens both the relative and the absolute refractory periods. This allows dorsal column fibres to be excited at higher frequencies by repetitive stimuli, for example, be activated at 800 Hz as faithfully as originally at 400–600 Hz. It will also be shown that the refractory period of afferent fibres traversing the dorsal columns is nearly half of that of fibres in either peripheral nerves or in the dorsal roots and is modulated to a much greater extent. These specific properties of the dorsal column fibres relate the long-lasting effects of DC on the refractoriness to those on the excitability of afferent fibres traversing the dorsal columns and thereby to their branching regions (for references, see Jankowska & Hammar, 2021; Li et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments were approved by the Regional Ethics Committee for Animal Research (Göteborgs Djurförsöksetiska Nämnd) and followed guidelines for animal care set by the EU and the NIH. The animals were housed under veterinary supervision at the Laboratory of Experimental Biomedicine at Sahlgrenska Academy, Göteborg University. Measures were taken to minimise both animal discomfort and the number of animals used (37 adult Sprague–Dawley rats, 200–360 g).

2.2 | Preparation

Anaesthesia was induced with isoflurane (Baxter Medical AB, Kista, Sweden) (4.5% in air) followed by intraperitoneal administration of pentobarbital sodium (Apoteksbolaget, Göteborg, Sweden; 30 mg/kg) together with α-chloralose (Acros Organics, Geel, Belgium, 30 mg/kg). During the experiment, the anaesthesia was supplemented with additional doses of α-chloralose (3 mg/kg, up to 40 mg/kg) at 3–4 h intervals. Neuromuscular transmission was blocked by Gallamine triethiodide (Sigma Aldrich, G8134) injected intravenously (via the tail vein) at an initial dose of 10 mg/kg supplemented with 5 mg/kg. Gallamine was not administered until approximately 3 h after anaesthesia induction, by which time deep and stable anaesthesia was established. Artificial ventilation was applied by a respiratory pump (CWE; model SAR-830/P) to maintain the end-tidal CO_2 level at 3%–4%. To compensate for fluid loss and changes in blood pH, 10–20 ml of a buffer solution (100 mM $NaHCO_3$ with 5% glucose, (Edgley & Jankowska, 1987; Haglund & Lundgren, 1972) was injected subcutaneously or intraperitoneally at the beginning of the experiments. The experiments were terminated by a lethal injection of pentobarbital causing cardiac arrest.

Following tracheal intubation and tail vein cannulation, the peroneal (Per) nerve including nerve branches to pretibial flexors and the tibial (Tib) nerve including nerve branches to the medial and lateral gastrocnemius

on the left side were dissected free and mounted on pairs of electrodes in a paraffin oil pool. The Th13-L4 spinal segments were exposed by laminectomy after the vertebral column had been stabilised by a clamp and covered by a paraffin oil pool. Core body temperature was monitored continuously and kept at ~38°C by servo-controlled heating lamps. The temperature in the hind limb and vertebral oil pools was monitored either continuously or intermittently and allowed to be lower than the body temperature by a few degrees (usually 32–36° in the leg pool and 35–37° in the vertebral pool).

2.3 | Epidural stimulation and polarisation

The setup used in the reported experiments is illustrated in Figure 1. The dorsal column was stimulated monopolarly via tungsten needle electrodes insulated except for 20–30 μm at the tip (Microneurography active needle, UNA35FNM, FHC, Bowdoin, ME, USA; impedance 30–400 kΩ) against a 2 cm long wire reference electrode inserted in midline back muscles just rostral to the laminectomy or a subcutaneous abdominal electrode. Single 0.2 ms rectangular stimuli were delivered at intensities up to 60 μA. Peripheral nerves were stimulated bipolarly at 2–5 times threshold intensity using constant voltage stimuli.

The dura mater remained intact to maintain the protection of the spinal cord by the cerebrospinal fluid. The Per and Tib group I muscle afferent fibres were stimulated and polarised epidurally at the level of Per and Tib motor nuclei where these afferents branch most extensively and give off the largest number of axon collaterals targeting motoneurons. The location of the Per and\or Tib motor nuclei was defined at the beginning of each experiment as corresponding to the 2–3 mm length of the spinal cord over which the largest afferent volleys were evoked when Per and Tib nerves were stimulated at intensities supramaximal for group I afferents, as illustrated in Figure 1b in Li et al., 2020. A tungsten needle electrode used for the subsequent epidural stimulation and polarisation was positioned at the centre of this region, about halfway between the posterior spinal vein and the dorsal root entry zone (Figure 1a). In order to prevent changes in contact between the electrode and the

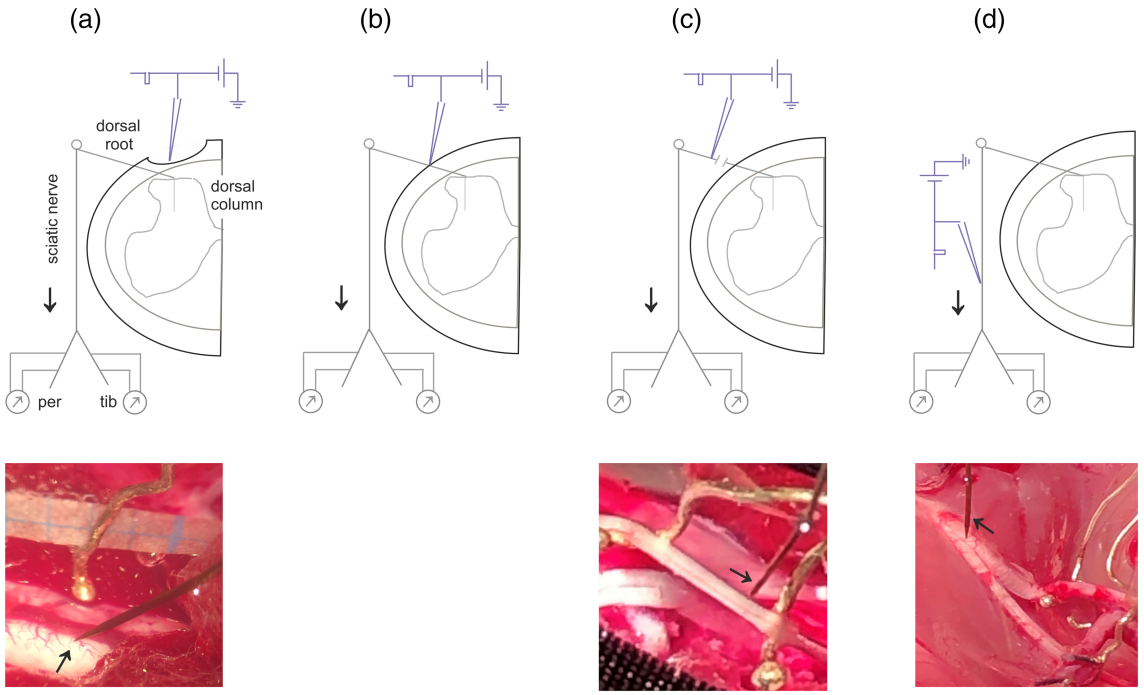

FIGURE 1 Diagrams of the experimental setup. Schematic drawings indicating the experimental setup with the location of the stimulating (purple) and recording (grey) electrodes illustrated with images as seen through the dissection microscope. The direction of the propagation of action potentials induced by stimulation at the indicated sites is shown by vertical arrows. (a) Epidural stimulation with indentation of the dura mater by the stimulating tungsten electrode and reduction of the local subdural space. (b) As in (a) but for stimulation of intact dorsal roots. (c) Stimulation of transected dorsal roots lifted and mounted on silver electrodes in the paraffin oil pool. (d) Stimulation of the sciatic nerve at a site proximal to the site of recording from the peroneal and tibial nerves, transected distally and mounted on recording electrodes. Black arrows in photographs indicate the tungsten electrode used for stimulation and polarisation.

dura matter, any fluid collecting around the electrode tip was continuously removed via a thin plastic catheter connected to a pump.

The effects of epidural polarisation (1 μA for 1 min) were tested both on the excitability and the refractory period of dorsal column fibres (Figure 1a). Changes in the excitability were defined as described previously, using nerve volleys evoked by near-threshold stimuli (20–30 μA) (for references, see Jankowska & Hammar, 2021). The degree of excitability was established during a 5 min control period and subsequently during 1 min of cathodal DC application followed by a 15 min post-polarisation period. We used the same parameters of DC polarisation as in previous studies on the effects of DC on the excitability of nerve fibres to allow a comparison of the effects of polarisation on the excitability and on the refractory period. The intensity of epidurally applied DC of 1 μA was chosen as it results in a near-maximal increase of the excitability of dorsal column fibres, the radius of the resulting effects not exceeding a fraction of a millimetre and the duration of DC application of 1 min as sufficient for a maximal increase in the excitability of dorsal column fibres during their cathodal polarisation with or without a decline during the postpolarisation period under optimal conditions (Jankowska et al., 2017).

The refractory period was defined by using paired stimuli at twice threshold intensity (40–80 μA) at a series of standard interstimulus intervals (0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 1.0, 1.1 and 1.2 ms). When the refractory period of fibres in the sciatic nerve was investigated (see below), the range of the interstimulus intervals was increased by adding intervals of 1.4, 1.6, 1.8, 2.0, 2.2, 2,4, 2.6, 3.0, 3.4 and 3.8 ms. The stimulus intensity of twice threshold was selected to ensure a sharp onset and synchronisation of the nerve volleys. Stronger stimuli were avoided considering that they might affect nerve fibres over larger distances from the surface of the spinal cord than that of the effective epidural DC. The effects of the resulting changes in the refractory period were estimated from the ability of the stimulated fibres to be excited by 10 ms long trains of stimuli delivered at 200, 250, 333, 400, 500, 600 and 800 Hz.

2.4 | Stimulation of the sciatic nerve and of the dorsal roots

In order to ensure that experimental conditions of stimulation and polarisation of fibres within the sciatic nerve would be comparable with those in the dorsal columns, the sciatic nerve was likewise stimulated and polarised, monopolarly (Figure 1d), via a tungsten needle electrode against the same large reference electrode and using the same parameters of DC (1 μA for 1 min). In control experiments, the sciatic nerve was also stimulated bipolarly, after it had been transected within the proximal part of the thigh and mounted on a pair of silver–silver chloride electrodes, using twice threshold stimuli. The polarising current was then passed via the same tungsten needle electrode positioned between the cathode and anode. However, as no differences were found in the effects of DC on monopolarly and bipolarly stimulated fibres, the results from these experiments were pooled together. The peroneal and tibial nerves were stimulated bipolarly at 2–5 times the threshold using constant voltage stimuli.

Intact dorsal roots were stimulated epidurally, using the same tungsten needle electrode as for the dorsal column but positioned over a dorsal root (Figure 1b), 3–7 mm caudal to their entry region at a site where afferent volleys from either the tibial or peroneal nerves were maximal. Transected dorsal roots (Figure 1c) were stimulated bipolarly via a pair of silver–silver chloride electrodes in the paraffin oil pool over which the most proximal segment was placed. The roots were transected within 1–2 mm from their entry zone.

2.5 | Recording

The number of fibres excited by epidural, sciatic and dorsal root stimulation was estimated from changes in the size of nerve volleys in Per and Tib nerves (see Figure 1 and below in Section 2.8). The nerve volleys were recorded bipolarly, via a pair of silver–silver chloride electrodes about 3–4 mm apart. Afferent volleys evoked by peripheral nerve stimulation were recorded using a silver–silver chloride ball electrode in contact with the cord dorsum close to the dorsal root entry zone against a reference electrode inserted into one of the back muscles or a subcutaneous abdominal electrode. Both single records and averages of 10 consecutive nerve volleys were stored online using a sampling frequency of 33 kHz (resolution of 0.03 ms), with a low-pass filter set to 15 or 1 Hz and a high-pass filter set to 5 or 3 kHz.

2.6 | DC polarisation

DC (1 μA of cathodal current) was applied using a custom-designed, battery-driven, constant current stimulator (D. Magnusson, University of Gothenburg) or a DS3 Isolated Current Stimulator (Digitimer, UK). The current was passed via the same tungsten electrode that was used for the stimulation of the fibres, as we have previously demonstrated that DC does not interfere with the

stimulating current pulses under our experimental conditions (see Bączyk & Jankowska, 2014; Li et al., 2020). Even though a few seconds of epidural polarisation suffices for inducing post-polarisation increases in fibre excitability (Bączyk & Jankowska, 2018), the reported results were routinely evoked by 1 min polarisation.

2.7 | Drug solutions

The following solutions were administered either intraperitoneally or intravenously: chloralose 3 mg/ml (in distilled H_2O), gallamine 20 mg/ml and pentobarbital 10 mg/ml in 0.9% NaCl. Buffer solution (100 mM $NaHCO_3$ with 5% glucose) was injected subcutaneously or intraperitoneally.

2.8 | Analysis

The size of nerve volleys was estimated by measuring their areas, using an analysis programme designed by E. Eide (University of Goteborg; see Jankowska et al., 1997). The areas of nerve volleys in the fastest conducting fibres (expressed in arbitrary units) were measured within a time window of 0.4–0.7 ms from the onset of their earliest components to exclude a non-linear summation with later components in slower conducting fibres. The early components were routinely verified to be evoked at latencies corresponding to the latencies of afferent volleys in group I afferents in muscle nerves from the respective stimulus artefacts. As group II and cutaneous afferents in mixed muscle nerves display 0.7–0.8 ms longer conduction time (compare, for example, latencies of afferent volleys in group I and II afferents recorded under similar experimental conditions in Figure 1b of Li et al., 2020), the early components of nerve volleys recorded from the Per and Tib nerves could be attributed to group Ia or Ib muscle afferents in contrast to later components most likely evoked in group II and skin rather than group I afferents. For technical reasons, the later components were not analysed but were similarly affected by DC.

The results are illustrated with data from single experiments as well as with averaged data from several experiments. As no differences were found between the effects on Per and Tib afferents, data for these afferents were pooled together for statistical analysis.

The statistical significance was analysed in Statistica 13 (TIBCO Software Inc.). The differences between the normally distributed experimental data for each interstimulus interval were assessed using Student's *t* test. For the data where normality was not met, Student's *t* test was replaced by the Wilcoxon test (for comparisons before and after DC) or by the Mann–Whitney *U* test (for comparisons under different experimental conditions, following epidural stimulation and stimulation of the sciatic nerve or stimulation of the intact and transected dorsal roots). The normality of the data sets was verified by the Shapiro–Wilk normality test ($p > 0.05$). The significance level indicated by * was set at <0.05 level.

3 | RESULTS

The results of this study demonstrate that epidural polarisation affects not only the excitability but also the refractoriness of nerve fibres stimulated within the dorsal columns and indicate a long-lasting shortening of both the absolute and the relative refractory period in these fibres. The results also provide evidence for major differences between the refractoriness of fibres within and outside the dorsal columns.

3.1 | The refractory period of the dorsal column fibres is shortened by epidural polarisation

The refractory period of nerve fibres stimulated within the dorsal columns was assessed from changes in the resulting nerve volleys recorded from peripheral nerves when intervals between epidurally applied paired stimuli were gradually decreased. The stimuli were at 2–2.5 times the threshold and thus supramaximal for the fastest conducting fibres. Stronger stimuli were avoided in view of the risk of a surround block (for review, see, e.g. Bhadra & Kilgore, 2004; Tai et al., 2009) or spread of current outside the region of the dorsal column that was affected by their DC polarisation.

When evoked separately, the nerve volleys following the two stimuli were of similar size, with examples in Figure 2a,b. As the intervals between the stimuli were reduced, the second volley gradually decreased and ultimately failed to be induced. As indicated in Section 1, the size of the second volley was considered to reflect the excitability of the stimulated fibres at these intervals, the intervals at which the second volley started to decrease and disappeared defining the relative and the absolute refractory period, respectively.

When the two volleys overlapped at short interstimulus intervals, the procedure illustrated in Figure 2a–d was applied to allow measurements of the area of the unveiled second volley. The first volley (a) was subtracted from the compound potentials following the paired stimuli (c), and the difference (d) was used for the

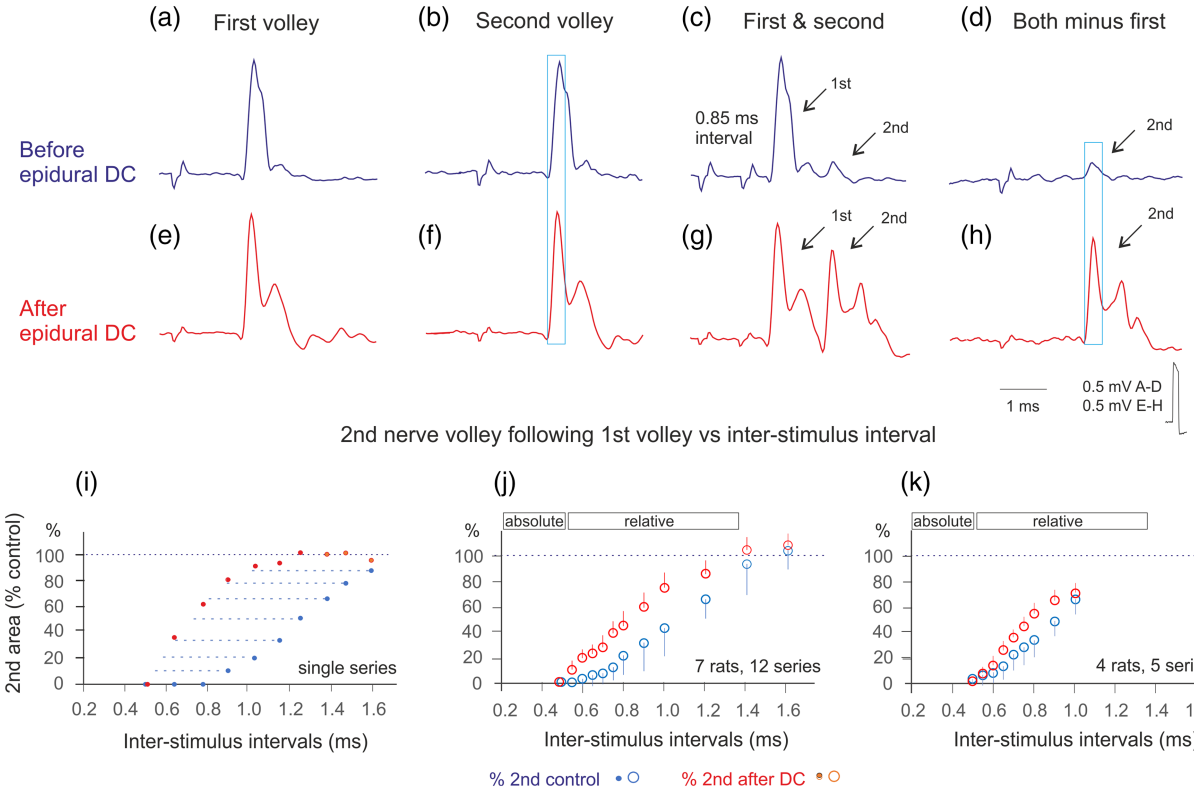

FIGURE 2 Decreases in responses of nerve fibres stimulated within the dorsal column to the second of paired stimuli at decreasing interstimulus intervals before and after epidural polarisation. (a–d) and (e–h) Examples of nerve volleys in the peroneal nerve evoked by epidural stimulation using the set op of Figure 1a. They were recorded before and 20 min after cathodal polarisation, respectively. The nerve volleys were evoked by the same stimulation intensity (60 μA) stimuli. (a, b) Nerve volleys evoked by the first and the second stimuli applied separately. (c) Nerve volleys following both stimuli at a 0.85 ms interstimulus interval. (d) The responses to the second stimulus (boxed) visualised by subtracting (a) from (c). (e)–(h) As in (a)–(d) but 20 min after epidural polarisation, taken at a lower amplification as the volleys increased following epidural polarisation. (i) Comparison of mean areas of the second nerve volley evoked at 0.5–2 ms interstimulus intervals before and following epidural polarisation (1 μA cathodal DC for 1 min) in a single experiment. The areas are expressed in percentages of the areas of volleys not preceded by the first stimulus at the same interstimulus interval, measured within the boxed time windows. Dotted lines indicate the estimated differences in the latency of nerve volleys of sizes represented by the blue circles. (j, k) As in (i) but means and *SD* for 12 and 5 series of records into which these data were subdivided depending on whether the minimal effective interstimulus intervals were shortened by ≥0.1 ms during the post-polarisation period (j) or remained unchanged (k). Statistically significant differences between areas of the second volleys before and after DC were found for intervals 0.55–1.2 ms in J ($p = 0.043$, 0.012, 0.028, 0.018, 0.005, 0.005, 0.008, 0.002 and 0.002) and for intervals 0.65–0.8 ms in K ($p = 0.043$, 0.043, 0.044 and 0.020).

measurements. The records in Figure 2d also illustrate common, although not quantified observations, that the later components of the epidurally evoked nerve volleys, in group II muscle afferents or skin afferents in mixed muscle nerves, were affected similarly if not more potently than the first components in group I muscle afferents.

The relationship between the decrease in the area of the second volley and the interstimulus intervals in the illustrated records is plotted in Figure 2i, and the average data from two series of experiments are in Figure 2j,k (blue symbols). In order to restrict the analysis to the excitation of the fastest conducting fibres, the plots are for the earliest components of the volleys, within 0.4–0.7 ms from their onset (boxed in Figure 2b,f,d,h).

The deviation between the second volleys evoked before and after polarisation (Figure 2i–k; blue and red data points, respectively) occurred at interstimulus intervals ranging from 0.5 to 1.4 ms. At these intervals, the volleys evoked during the post-polarisation period were much larger and volleys of equal size were evoked at considerably shorter interstimulus intervals (as indicated by the horizontal dotted lines in Figure 2i). This demonstrates that DC significantly shortened the relative refractory period of the dorsal column fibres. In addition, following DC, the second stimulus often evoked nerve

volleys at intervals of 0.5–0.65 ms at which it previously failed to do so. As judged by the size of these volleys, 10%–30% of nerve fibres could be excited post-DC, indicating that epidural polarisation might shorten not only the relative but also the absolute refractory period of a considerable proportion of fibres.

Epidural polarisation increased the nerve volleys to a varying extent, but in four of the 11 experiments, the effects were as marked as those shown in Figure 2i, and in seven experiments, the minimal effective interstimulus intervals were shortened by at least 0.1 ms (plots in Figure 2j). In the remaining experiments, the post-polarisation increase in the nerve volleys was weaker and restricted to the longer intervals (Figure 2k) but likewise statistically significant.

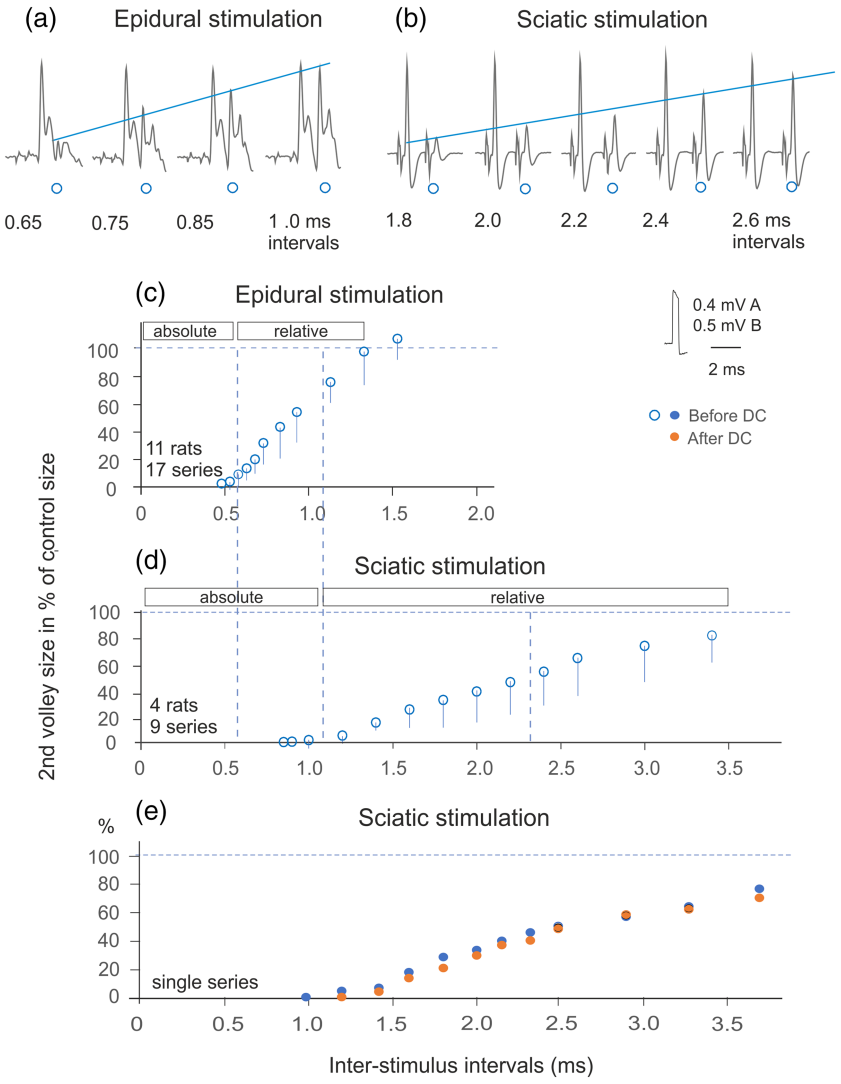

FIGURE 3 Comparison of changes in responses to paired stimuli evoked by epidural and peripheral nerve stimulation as a function of interstimulus intervals. (a) Examples of nerve volleys evoked by epidural stimulation at four intervals between the paired stimuli. Those following the second stimulus are indicated by blue circles. The nerve volleys were recorded from the peroneal nerve. (b) As in (a) but for nerve volleys evoked by stimulation of the sciatic nerve. Note that the decrease of the second volleys began at much longer interstimulus intervals. (c) Means and *SD* of the areas of nerve volleys evoked by the second epidural stimuli at varying interstimulus intervals, estimated as in Figure 2c,d. They are expressed in percentages of the areas of the volleys that were not preceded by the first stimuli. At interstimulus intervals exceeding 1.5 ms, the areas of the second volleys were sometimes less than 100% but in about half of the sample exceeded 120% or even 140%. (d) As in (c) but for nerve volleys evoked by stimulation of the proximal part of the sciatic nerve using set up of Figure 1d. Note that they became minimal and at about half size between the second and third rather than between the first and second vertical dotted lines, reflecting much longer refractory periods than the epidurally evoked nerve volleys. (e) Comparison of the sizes of nerve volleys evoked by the second of paired stimuli applied to the sciatic nerve before and after its polarisation (1 μA, 1 min) in the same experiment. Note that the polarisation failed to alter them. Statistically significant differences between areas of the second volleys, evoked epidurally (c) or by stimulation of the sciatic nerve (d), were found at all intervals ≤1.6 ms (at $p < 0.001$)

Changes in the refractory period were routinely tested 15–30 min after DC application and provided evidence for relatively long post-polarisation effects. In addition, in five experiments, the period of testing was extended revealing that epidural polarisation may shorten the refractory period for at least 80–90 min.

3.2 | Effects of polarisation on the refractory period of nerve fibres stimulated within and outside the dorsal columns

Comparison of the refractory period of nerve fibres stimulated within the dorsal columns and at sites distal to their entry into the spinal cord, that is, within peripheral nerves or dorsal roots, revealed several major differences. These differences concerned (i) the minimal interstimulus intervals at which the fibres responded to the second stimulus reflecting the duration of the absolute refractory period, (ii) the duration of the relative refractory period and (iii) the latency of responses evoked during the relative refractory period.

Figure 3 shows examples of nerve volleys evoked by paired stimuli at different interstimulus intervals when the stimuli were applied either epidurally (a) or to the sciatic nerve (b). Both series of records were from fibres in the peroneal nerve but stimulated either within or outside the dorsal columns, as indicated in the diagrams in Figure 1b,c. Mean areas of the second nerve volleys evoked under these two conditions are plotted in Figure 3c,d, respectively. The plots show that the nerve volleys evoked by stimulation of the sciatic nerve began to decline at intervals at least twice longer (3–4 ms) than those following epidural stimuli (1.2–1.4 ms). The minimal intervals at which the second volleys were only marginal were likewise more than twice longer following sciatic (≥1.2 ms) than epidural stimuli (≤0.6 ms), indicating longer absolute as well as relative refractory periods.

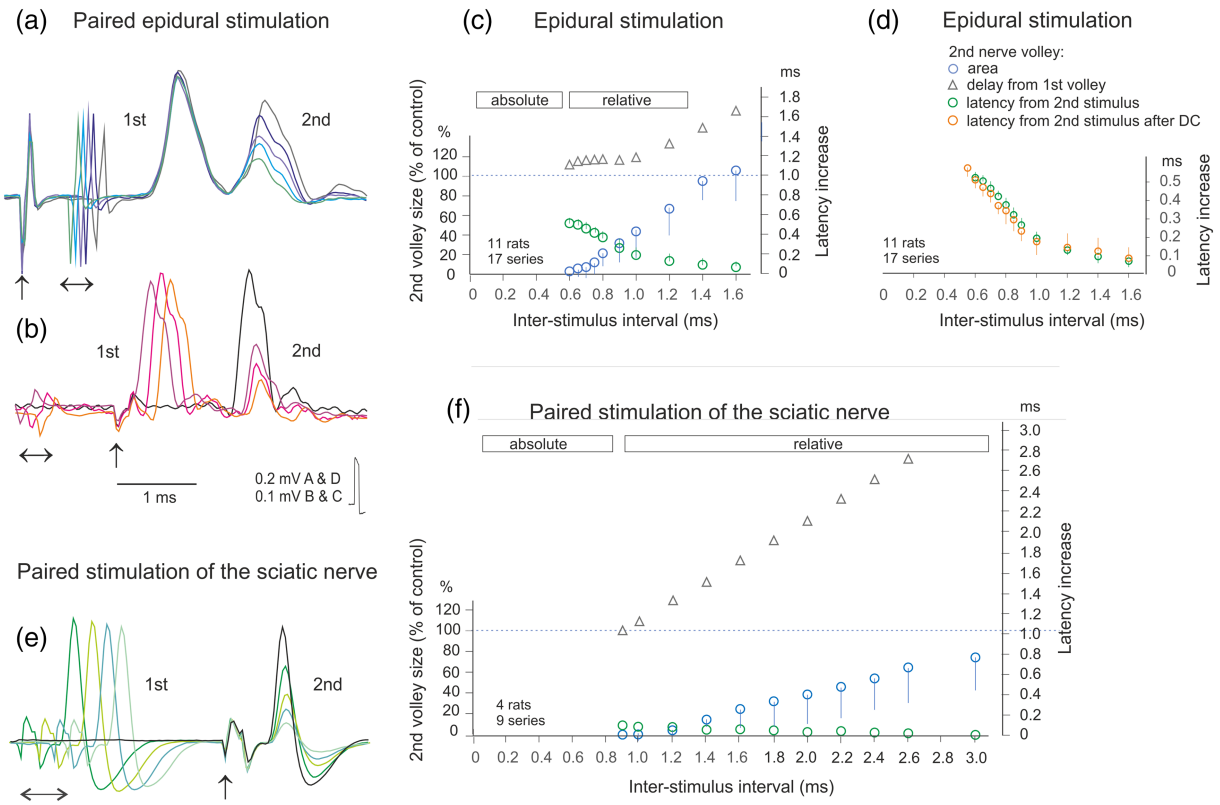

FIGURE 4 Latencies of responses evoked by epidural and peripheral nerve stimulation. (a, b) Examples of nerve volleys evoked by paired epidural stimuli. Both were evoked at interstimulus intervals <1 ms but aligned by either the first (a) or the second (b) stimulus. Note that all second volleys in (a) were similarly delayed with respect to the first volley despite the decreasing interstimulus intervals. Note also that the four second volleys in (b) were evoked at increasing latencies from the second stimulus when the interstimulus intervals were decreased; the control second volley that was not preceded by the first volley is in black. (c) Means and *SD* of the areas of nerve volleys evoked by the second epidural stimuli at varying interstimulus intervals and the corresponding changes in the latencies (blue and green circles, respectively) with respect to the second stimuli as in (b). Triangles indicate delays of the same nerve volleys with respect to the first volley, as in (a). (d) Comparison of latencies of nerve volleys evoked by epidural stimuli with respect to the second stimuli, before and after epidural polarisation (1 μA, 1 min). Note that they were practically unchanged by DC. (e) As in (b) but for stimulation of the sciatic nerve. (f) As in (c) but for stimulation of the sciatic nerve.

Consequently, at interstimulus intervals when the twice threshold stimulation of the sciatic nerve was ineffective (circles between the first two vertical dotted lines in Figure 3d), epidural stimuli at a similar intensity excited more than half of the fibres (Figure 3c).

Figure 4 illustrates a major difference in the timing of nerve volleys evoked by epidural and peripheral stimuli. As shown in Figure 4a, nerve volleys evoked by the second epidural stimuli were similarly delayed *with respect to the first volleys* at several interstimulus intervals, irrespective of the duration of these intervals (see the range of the second stimulus shock intervals indicated by the double-headed arrow). Both the smallest and the larger of these volleys were initiated 1.12–1.17 ms from the onset of the first volley (first seven triangles in the plot of the latencies in Figure 4c coinciding with the end of the first volley and thus the end of the absolute refractory period. These intervals were in keeping with the original evidence that the action potentials are generated only once the membrane potential has repolarised at the end of the declining phase of a preceding action potential. Shorter minimal intervals between the effective epidural stimuli thus indicate that these stimuli can initiate the second action potential before the repolarisation of the fibres following the first action potential has been completed even though the second action potentials are evoked after a much longer initiation period. As the stimuli evoking these volleys were applied increasingly closer to the first stimuli (within the range of 0.5–1.2 ms intervals), the latencies of the second volleys *measured from the second stimuli* considerably increased. This is illustrated in Figure 4b, where the records are aligned with the second stimulus, and by green circles in Figure 4c. The timing of the second volleys with respect to either the first volley or the second stimulus was not changed by the epidural DC polarisation (Figure 4d).

As nerve fibres in the sciatic nerve were characterised by longer absolute as well relative refractory periods than the epidurally stimulated afferent fibres (Figure 3c,d), the latencies of peripherally evoked nerve volleys could only be analysed for intervals exceeding 1 ms. As shown in Figure 4e, the latencies of these volleys with respect to the second stimulus did not change within the range of intervals at which the successively weaker responses were evoked. Any changes in the delays from the first volley reflected thus only changes in interstimulus intervals similarly as for >1 ms interstimulus intervals for epidural stimulation (Figure 4f, triangles).

These results thus indicate that following polarisation stimuli applied during the absolute refractory period are capable of initiating the delayed action potentials in fibres stimulated within but not outside the dorsal columns. In contrast to epidurally polarised nerve fibres, nerve volleys evoked in peripheral nerves remained greatly unchanged by DC. In the majority of the experimental series (in three of five experiments; 5/7 series), data points sampled before and following polarisation of the sciatic nerve practically overlapped (Figure 3e). A small and inconsistently occurring increase in responses evoked during the relative refractory period was seen in only two of five experiments (2/9 series) and at only some (1.6–2.2 ms) interstimulus intervals.

3.3 | The refractory period of nerve fibres stimulated within the dorsal roots

Effects of stimulation of dorsal roots by paired stimuli depended on whether the dorsal roots were intact or transected (close to their entry to the spinal cord; see Figure 1b,c). When interstimulus intervals were decreased, stimulation of nerve fibres in an intact dorsal root during the relative refractory period evoked responses at latencies increasing with respect to the second stimulus (Figure 5a,c, green symbols) but similarly timed with respect to the nerve volleys evoked by the first stimulus, as in the dorsal columns. In contrast, latencies of responses of fibres stimulated within the transected dorsal roots increased only marginally when the interstimulus intervals changed (Figure 5b,d), as in the case of responses evoked from the sciatic nerve (Figure 4e,f). A comparison of nerve volleys evoked at these intervals (Figure 5b,d) reveals similar differences in the timing of the relative refractory period, 0.6–1.2 ms for the intact dorsal roots and 1.0–4.0 ms for the transected roots, replicating timing of the relative refractory period following epidural stimulation and stimulation of the sciatic nerve. Effects of DC polarisation on these volleys were likewise similar, in that only those evoked at interstimulus intervals < 1.0 ms from intact dorsal roots were increased.

These comparisons thus lead to the conclusion that afferent fibres stimulated within intact dorsal roots are affected by electronic spread from the sites at which they branch within the dorsal columns, even though axon collaterals entering the spinal grey matter may be given off a few mm rostral to the site of stimulation of the dorsal roots.

3.4 | Comparison of the ability to follow high-frequency stimulation of fibres in the dorsal columns and in peripheral nerves

Considering that a shorter refractory period would allow nerve fibres to be excited at higher frequencies, we compared nerve volleys evoked by repetitive stimulation of

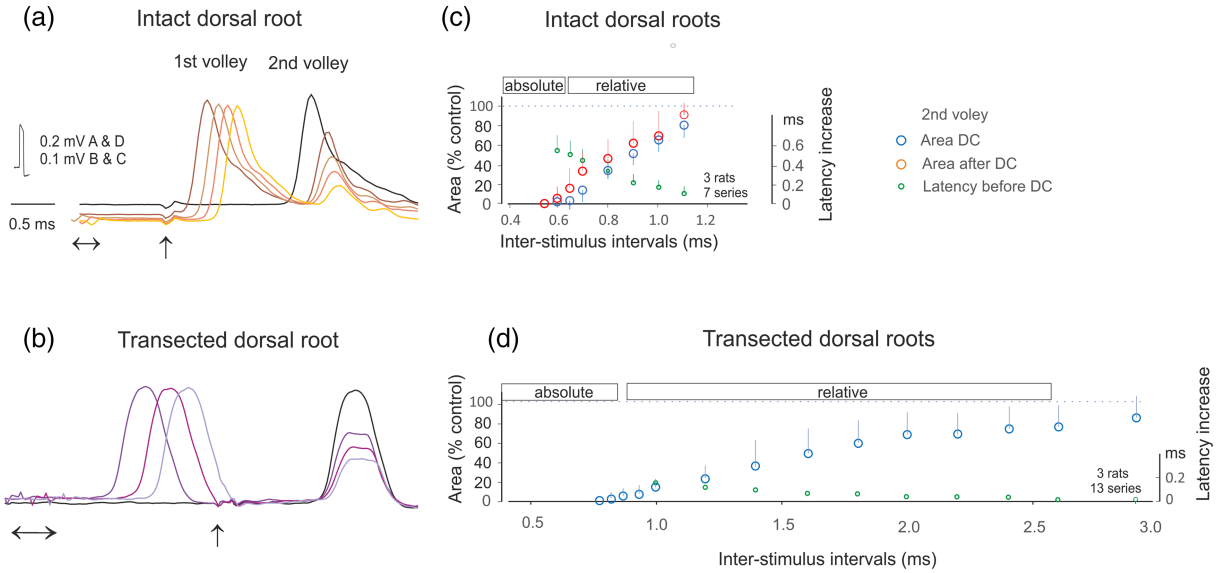

FIGURE 5 Comparison of responses evoked during relative refractory periods in intact and transected dorsal root fibres. (a) Changes in the size and the latency of nerve volleys evoked in the peroneal nerve by stimulation of an intact dorsal root alone (black) and when preceded by another stimulus (at 0.8–1.0 ms interstimulus intervals (shades of orange). The records were overlayed by aligning them with the second stimulus (indicated by the vertical arrow). (b) As in (a) for nerve volleys evoked by stimulation of a transected dorsal root (at 1.6–2.2 ms interstimulus intervals). (c, d) Mean changes and *SD* in the size and the latency of nerve volleys evoked by stimulation of the intact and transected dorsal roots, respectively, as a function of interstimulus intervals. Differences between the pre-DC and post-DC data for the area size in (c) were statistically significant for intervals 0.65 and 0.7 ms ($p = 0.021, 0.040$). Statistically significant differences between the volleys evoked by stimulation of intact and transected dorsal roots were statistically significant at all intervals <1.4 ms in (c) and (d) at $p = 0.004–0.0004$.

epidural or peripheral nerves. To this end, we used 10 ms long trains of stimuli at twice threshold intensity at 5.0, 3.33, 2.5, 1.66 and 1.25 ms intervals. Under control conditions, epidurally stimulated nerve fibres followed repetitive stimuli not only at lower frequencies, as illustrated in Figure 6 for 400 Hz (2.5 ms intervals) but also at 800 Hz (1.25 ms intervals) even though later stimuli in a train at 800 Hz only activated a proportion of these fibres. Following epidural polarisation, the same fibres followed 800 Hz stimuli more faithfully. This is indicated by a similar amplitude of nerve volleys evoked by successive stimuli 1.25 ms apart (compare green shaded areas in Figure 6b,d), including the last stimuli in the train.

In contrast, nerve fibres stimulated within the transected dorsal roots and in peripheral nerves were only able to faithfully follow frequencies below 400 Hz. As illustrated in Figures 6e,g and in 7, amplitudes of nerve volleys evoked at 400 Hz tended to decline, especially after every second stimulus and their decline resembled that of dorsal column fibres at 800 Hz (compare Figure 6e,g with Figure 6b). Alternatively, only every second stimuli were effective (Figures 6f and 7f); that is, the nerve volleys evoked at 2.5 ms interstimulus intervals were effective but those at 1.25 ms interstimulus intervals falling within the refractory periods of these fibres were not. Similar patterns of responses were found when terminals of single afferent fibres were stimulated by natural stimuli (at an increasing rate of vibration although at somewhat higher frequencies (Johnson & Murray, 1991). The failures to follow stimuli at 800 Hz occurred both when these stimuli were applied bipolarly (to the transected dorsal roots (Figure 6) and Tib and Per nerves (Figure 7b,d) and when they were applied monopolarly (to the sciatic nerve in situ (Figure 7f), indicating that the difference between effects of the stimulation within and outside the dorsal columns is unlikely to depend on how the fibres were stimulated.

In an attempt to quantify the differences in the ability to follow high-frequency stimulation of afferent fibres within and outside the dorsal columns, we compared nerve volleys evoked by the first and the last stimulus in the train without taking into account whether all or only every second of these stimuli were effective. Depending on the stimulus frequency, 3–10 nerve volleys were evoked by these trains but only the first and the last volleys could be reliably measured because of the overlap between them at higher frequencies. In Figure 8, the mean areas of the earliest components of these volleys ranged from those at 800 Hz (1.25 ms interstimulus intervals) to 200 Hz (5 ms interstimulus intervals) demonstrating the relationship between the last and the first volleys. Figure 8a shows that the last nerve volleys evoked by

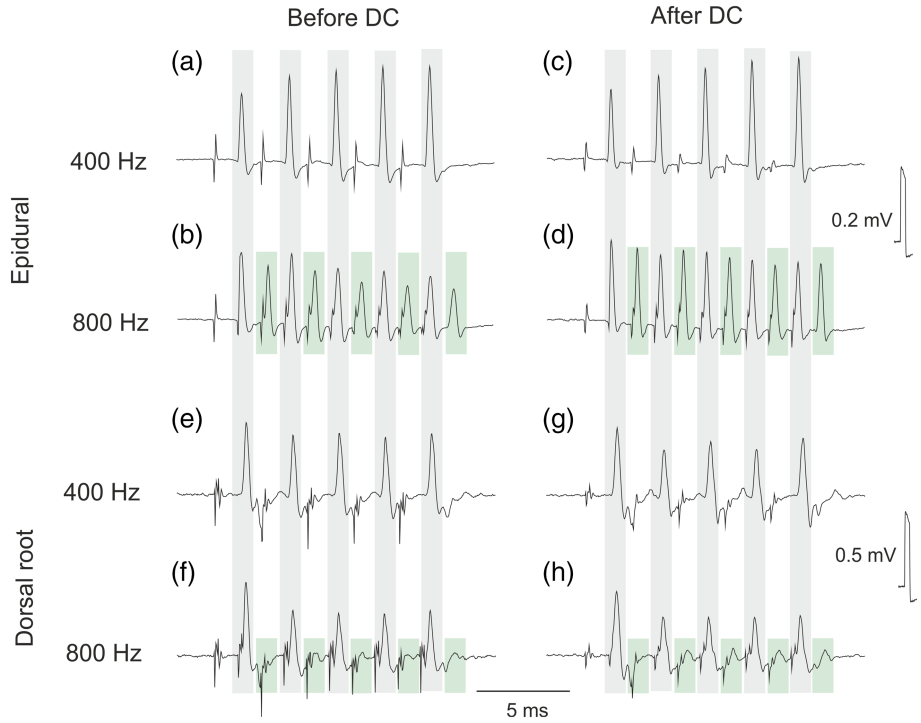

FIGURE 6 Repetitive activation of nerve fibres in the dorsal columns and in the transected dorsal roots. (a–d) Examples of records of nerve volleys evoked by epidural stimulation at two frequencies, before as well as following polarisation of the stimulated fibres. (a, c) Effects of a train of stimuli 2.5 ms apart. (b, d) Effects of stimuli at twice shorter intervals, the interstimulus intervals of 1.25 ms exceeding the refractory period of the majority of dorsal column fibres. Note that after DC (right column), the response at the grey background similarly increased at 400 Hz and that their decrease at 800 Hz was less pronounced than before DC. Note also that responses evoked by the intervening stimuli (at the green background) decreased much less at 800 Hz after DC than before. (e–h) As in (a)–(d) but for nerve volleys evoked by stimulation of a dorsal root transected close to its entry to the spinal cord and isolated from it using set up of Figure 1c. Note that the dorsal root fibres followed the stimuli in the train less faithfully than the epidurally stimulated dorsal column fibres, as every second volley at 400 Hz was slightly smaller, and that the volleys failed to a great extent to be evoked at 800 Hz. Also, note the lack of effect of DC on the ability of dorsal root fibres to be excited by high-frequency stimulation.

epidural stimulation were generally larger than the first volleys. In contrast, amplitudes of last nerve volleys evoked by stimulation outside the dorsal columns (Figure 8b–d) decreased as the frequency of the stimulation increased. The range of frequencies at which the nerve fibres failed to be faithfully activated was in keeping with the range of the relative refractory periods of these fibres (see above). Polarisation of these fibres at the site of their stimulation did not alter their ability to follow repetitive stimulation within these frequencies as is shown by the overlap of the blue and red symbols in Figure 8a–c.

3.5 | Are DC-evoked changes in the excitability and in the refractory period of the dorsal column fibres related?

Effects of epidurally applied DC were routinely tested at the segmental levels at which the most intense branching of muscle afferents occurs and at which the most marked sustained increase in the excitability is evoked (Li et al., 2020). A persistent shortening of the refractory period of the polarised fibres was found at all these sites but without any obvious relationship with the degree of DC-evoked increase of the excitability. Two arbitrarily selected measures were chosen for this comparison: the degree of the excitability 10 min after the termination of DC and the size of the nerve volleys evoked by the second of paired stimuli 0.75 ms apart. Both varied after as well as before epidural polarisation, but the shortening of the refractory period described in the first section of the results was found both when the post-polarisation increase in the excitability of the fibres remained high (exceeding 300%) and when it was relatively low (150–300%).

4 | DISCUSSION

The results of this study revealed that the refractory period of nerve fibres may be modulated by their

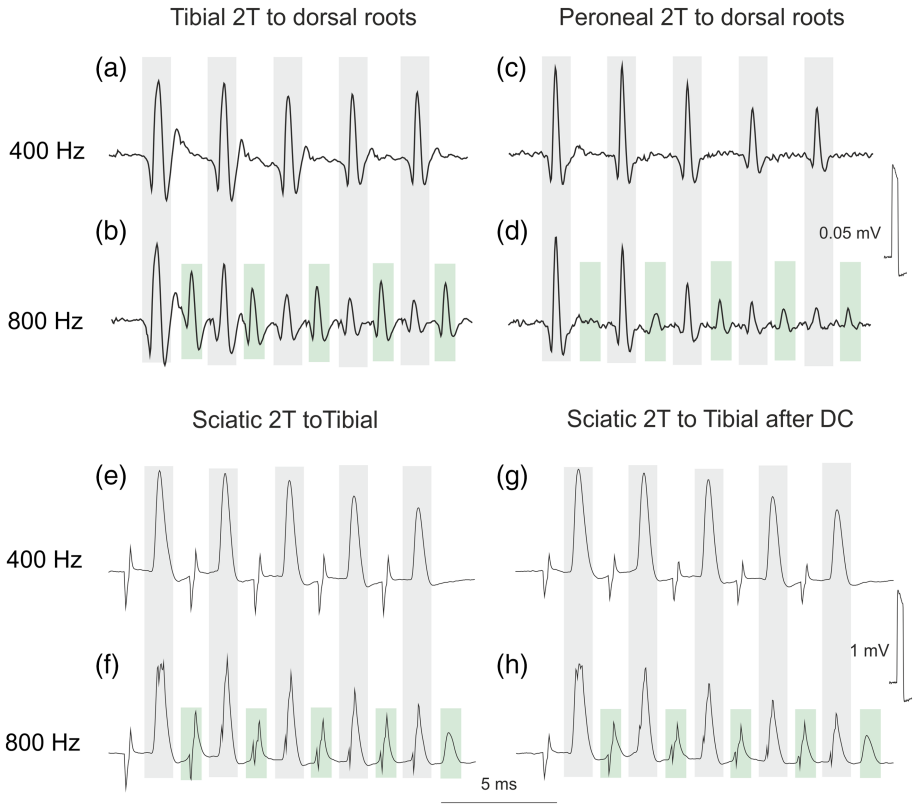

FIGURE 7 Repetitive activation of nerve fibres in peripheral nerves. (a–d) Centripetal afferent nerve volleys evoked at two frequencies of tibial and peroneal stimulation. Note the relatively weakly declining amplitudes of successive volleys at 400 Hz (2.5 ms apart; grey background) and much smaller nerve volleys following stimuli at 800 Hz, especially those delivered at twice shorter interstimulus intervals (green background). (e–h) Nerve volleys evoked by stimulation of the sciatic nerve, using set up of Figure 1d, recorded from the tibial nerve before and after its polarisation (1 μA, 1 min)

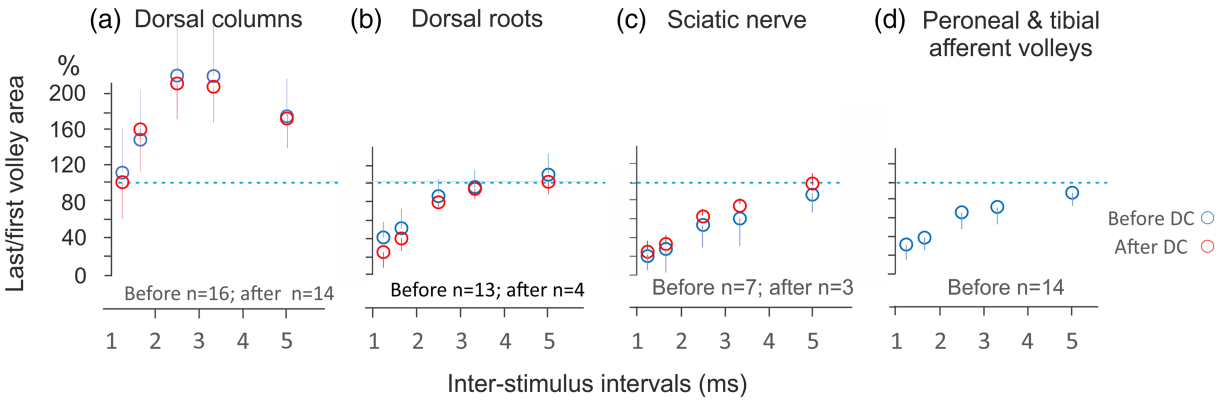

FIGURE 8 Comparison of nerve volleys evoked by a train of stimuli. (a–c) Relationship between nerve volleys evoked by the first and last stimuli in a 10 ms long train of stimuli delivered 1.25, 1.67, 3.3 and 5 ms apart, applied epidurally and to a transected dorsal root and the sciatic nerve respectively, as illustrated in Figures 6 and 7; all were recorded in the peroneal and tibial nerves. (d) As in (a)–(c) but for nerve volleys evoked by peroneal and tibial stimulation and recorded from the cord dorsum. Blue symbols, mean areas and *SD* of early components of nerve volleys measured as indicated in Figure 2. Red symbols, data for the same nerve volleys following polarisation of the fibres at the site of their stimulation. No statistically significant differences were found between before and after DC data in (a)–(c).

depolarisation and that the resulting changes are long-lasting. However, the refractory period was affected in different ways depending on where along their trajectory the fibres were stimulated and polarised. The refractory period of nerve fibres stimulated within the dorsal columns was significantly shortened, while effects on nerve fibres in the dorsal roots and peripheral nerves were practically negligible.

This difference appeared to be related to the much shorter duration of the absolute and the relative refractory period of afferent fibres within than outside the dorsal columns. The difference was in particular reflected in

the up to 0.5 ms shorter minimal interstimulus intervals at which the dorsal column fibres were excited by paired stimuli. While such a difference may appear not to be very marked, it amounts to about half of the absolute refractory period of nerve fibres and would allow them to be activated at a much smaller risk for propagation failures (see Debanne et al., 2011; Hari et al., 2021). The advantages of the lower risk for failures might manifest themselves not only when these fibres are excited by electrical stimuli but also under natural conditions when nerve impulses are evoked at short intervals, for example, in muscle spindle afferents, or by short trains of stimuli, and not only by prolonged stimulation at a high frequency.

When the refractory period was shortened by epidural depolarisation, the second of the paired stimuli applied during the relative refractory period evoked significantly larger nerve volleys and thus activated larger numbers of dorsal column nerve fibres, indicating that the shortening of the refractory period was combined with an increase in the excitability of the dorsal column fibres. The DC-evoked shortening of the refractory period might accordingly, as the DC-evoked increase of the excitability of dorsal column fibres (see Jankowska & Hammar, 2021; Li et al., 2020), be linked to their branching regions.

4.1 | Differences in the duration of the refractory period in different compartments of the same nerve fibres

At the level of single fibres or cells, the absolute refractory period coincides with the duration of the action potentials and inactivation of transient voltage-gated Na^+ channels until these channels are de-inactivated by repolarisation of the membrane. Similar minimal intervals between nerve volleys found to be evoked by paired epidural and more distal stimuli would thus be in keeping with a similar period of inactivation of transient voltage-gated Na^+ channels along nerve fibres within the dorsal columns, dorsal roots and peripheral nerves. However, even though the shortest intervals between action potentials evoked by the paired stimuli were similar within these three fibre compartments, the second stimuli could initiate the propagated action potentials before their repolarisation was completed within but not outside the dorsal columns. This is indicated by shorter minimal intervals between paired epidural stimuli (0.52 ± 0.01 ms) than between the nerve volleys that they evoked (1.1 ± 0.01 ms).

The relative refractory period of nerve fibres stimulated within the dorsal columns was likewise much shorter as indicated by the faster increase in the proportions of the de-inactivated dorsal column nerve fibres, possibly due to differences in the time required for repolarisation of afferent fibres within their different compartments. However, despite the knowledge of several factors affecting the duration of action potentials (see recent reviews by Bucher & Goaillard, 2011; Debanne et al., 2011), little is known to what degree they are involved in different fibre categories and even less in different compartments of the same fibres. For instance, the decisive role of the potassium channels was questioned in some mammalian myelinated axons (Brismar & Schwarz, 1985; Corfas et al., 2004), the repolarisation in these fibres being also linked to changes in sodium channels and leakage current of the persistent channels (see Nodera & Kaji, 2006). Furthermore, the great variability of membrane properties of nerve cells and axons (see e. g. Hu & Bean, 2018) precludes generalisations from one of these to the other.

The comparison of the duration of action potentials evoked in intraspinal and extraspinal compartments of afferent fibres has been hampered by technical difficulties of intra-axonal recording from the critical regions of these fibres. Nevertheless, records of action potentials from fast conducting afferent fibres at different sites along their trajectory support the possibility that the differences in the duration of the refractory period within and outside the dorsal columns depend on the speed of the repolarisation at these sites. Thus, intra-axonal recordings from the largest muscle and skin afferents penetrated at short distances from the surface of the dorsal columns (in the anaesthetised cat in vivo) revealed the duration of action potentials of these fibres of 0.5–0.7 ms (Eccles & Krnjevic, 1959; Figures 1 and 3; Hongo et al., 1972; Figure 7a,b), while action potentials with a somewhat longer duration (about 1–1.3 ms) were recorded in the dorsal horn in an In vitro ('ex vivo') rat preparation (Hari et al., 2021; Figures 2 and 3 and supporting information Figures 2 and 3). In addition, when these fibres were stimulated within the dorsal roots, the second of the paired stimuli failed to excite them at intervals of 3–5 ms (Hari et al., 2021; Figure 2), in keeping with the longer refractory period of fibres in transected dorsal roots in rats investigated in this study.

The longer refractory period of electrically stimulated axons within peripheral nerves would likewise be matched by a longer duration of action potentials. Intra-axonally recorded action potentials in the rat sciatic nerve in vitro (Figure 4 in Waxman & Ritchie, 1993) showed a duration about twice longer than that in axons penetrated in the spinal cord. However, these were recorded in unspecified motor axons, and it has not been

established whether the duration of action potentials in motor nerve fibres in this nerve is the same as in the fastest sensory fibres. Estimates of the refractory period of motor axons in the rabbit sciatic nerve (Ritchie, 1982; Figure 6) were close to those found in the present study as the compound action potentials evoked by the second of two stimuli started to decline at >4 ms interstimulus intervals and failed to be evoked at approximately 0.8 ms intervals. In sensory fibres in the sciatic nerve, the range of intervals at which the responses to the second stimulus were smaller than to the first stimulus was also of several seconds (Brunton et al., 2018).

The most marked differences between fibres stimulated within and outside the dorsal columns will probably lie in the morphology of afferent fibres at their branching sites, as the denuded areas of the axons at the sites where axon collaterals are issued are much larger than the nodes of Ranvier in peripheral nerves (see Jankowska & Hammar, 2021; Lucas-Osma et al., 2018; Nicol & Walmsley, 1991). These differences in morphology may be combined with differences in membrane properties and the voltage-gated and other ion channels, but the features of the branching regions of dorsal column fibres have only recently become the focus of investigation (Hari et al., 2021; Lucas-Osma et al., 2018).

Provided that the repolarisation of afferent fibres at the sites of origin of axon collaterals within the dorsal columns is faster than within the peripheral nodes of Ranvier, one might expect different proportions of rapidly ($Na_v1.6$) and more slowly (Na_v 1.7) de-inactivated Na_v channels (see, e.g., Alsaloum et al., 2020; Bucher & Goaillard, 2011) as well as different proportions of fast and slow potassium channels (see, e.g., Waxman & Ritchie, 1993). The various Na_v channels might also be differently modulated, for example, by GABA released from axons of GABAergic interneurons projecting to dorsal columns (Hari et al., 2021; Lucas-Osma et al., 2018) and increasing the excitability of dorsal column fibres. Tonic central actions of GABA could thus contribute to the shortening of the relative refractory period of fibres in the dorsal column by depolarising these fibres, thereby reducing the threshold for excitation at different interstimulus intervals as well as shortening the de-inactivation period. Long latencies of the second volleys measured with respect to the second of the paired epidural stimuli at the shortest interstimulus intervals would be consistent with slower development of voltage-gated sodium channel spikes when the membrane potential is further from the threshold within the refractory period (Hari et al., 2021; extended Figure 3). As indicated by one of the reviewers of this paper, the effects of stimuli applied during the refractory period may be related to a subthreshold slow regenerative current that is resistant to inactivation and produces a persistent sodium current leading to a subthreshold plateau potential that contributes to spike initiation. Our preliminary observations suggest that any subthreshold plateau potentials evoked in dorsal column fibres may involve partly the same but also partly different membrane mechanisms than those in motoneurons or other neurons. We are awaiting further data from current experiments for the discussion of this issue. It may nevertheless be considered that a subthreshold slow regenerative current that is resistant to inactivation, likely a persistent sodium current, helps trigger the spikes centrally but not peripherally. If it accelerates the depolarisation near threshold, it may enable there the bulk of the sodium channels to escape inactivation and cause a full spike. Initiation of action potentials at the extreme short interstimulus intervals within the branching regions of the dorsal column fibres might also be due to the GABA-related depolarisation reducing the threshold of these fibres when they are partially inactivated, replicating effects of PAD at longer intervals (Hari et al., 2021; extended Figure 8).

4.2 | Differences in effects of DC on the refractory period within and outside the dorsal columns

As DC shortened the relative refractory period of nerve fibres stimulated within the dorsal column but not of fibres stimulated within peripheral nerves, differences in central and peripheral actions of DC were first considered as an explanation. However, the timing of the effects of DC suggests a more plausible explanation. As indicated above, the shortening of the refractory period following epidural DC depolarisation occurred during a critical range of interstimulus intervals <1 ms, during which the initiation of action potentials evoked by the second epidural stimulus was delayed with respect to this stimulus in fibres within but not outside the dorsal column. DC did not shorten the latency of nerve volleys evoked during this period with respect to the preceding volleys as the second volleys were delayed until the propagated action potentials could be initiated when the de-inactivation of Na_v channels became completed. During this period, the effects of DC might have supplemented effects of the pre-existing tonic depolarisation by GABAergic or other modulatory actions referred to in the previous section, further decreasing the threshold of dorsal column fibres and thereby allowing a larger proportion of fibres to be excited. The absence of background modulation of the refractory period of fibres outside the dorsal columns would not allow a similar summation

with DC effects to occur during the critical period of the refractory period in peripheral nerves and would explain the differences in effects of DC on fibres stimulated within the dorsal columns and peripheral nerves.

Bennett and co-workers revealed modulation of Na^+ channels within branching regions of afferent fibres by extra-synaptic membrane receptors for $alpha_5GABA_A$ that flank Na_v channels (Hari et al., 2021; Lucas-Osma et al., 2018). GABA released from terminals of GABAergic interneurons close to dorsal column fibres was shown to decrease the threshold of activation of these fibres. Continuing this line of reasoning, one might further postulate that the tonically active modulatory interneurons are excited by epidurally applied DC (Hari et al., 2021; Lucas-Osma et al., 2018) as well as by other stimuli used to excite the dorsal column fibres, thereby enhancing their actions on the dorsal column fibres.

In view of these possibilities, it would be plausible that the DC-evoked shortening of the refractory period of dorsal column fibres is closely related to the DC-evoked increase in their excitability (see Jankowska & Hammar, 2021; Li et al., 2020). However, it might be an oversimplification to postulate that the same factors are critical for both these effects of DC as no direct relationship between them has yet been demonstrated.

It would be likewise a simplification to expect that DC affects the refractory period and/or the excitability of nerve fibres via only one mechanism. Even relatively simple effects of DC on membrane channels may involve a number of effects. For instance, they may include changes in the opening of both fast and slow membrane channels and induce both short- and long-lasting conformational changes in their components. Long-lasting changes in slowly inactivating sodium channels might be particularly important for DC effects lasting not only milliseconds but minutes or even hours (for the review, see, e.g., Debanne et al., 2011; Kiernan et al., 2004; Kiernan & Bostock, 2000; Krishnan et al., 2009; Nodera & Kaji, 2006). In future analysis, it would thus be important to take into account that a long duration of changes in any membrane properties would be a necessary, although not a sufficient condition for the involvement in the long-lasting DC-evoked changes in the excitability and the refractoriness of dorsal column fibres.

Some of the differences between the effects of DC found in the present and previous studies might be related to the conditions under which the analysed nerve fibres were polarised. For instance, transcutaneously applied depolarising current was reported to increase the axonal excitability, as in the present study, but to prolong rather than shorten the relative refractory period in the peripheral nerves (Boerio et al., 2011). It may also be noted that the term 'refractory period' has often been used in a more descriptive way than at the level of single fibres or cells to denote any period during which an investigated reaction failed to be evoked, or required stronger stimuli, depending not only on the refractory period sensu stricto but also on the subsequent subexcitability periods. This might, in particular, be the case when the refractory period following stimulation of a peripheral nerve in humans was estimated from the resulting muscle activity, based on the joint responses of the stimulated nerve fibres and their target muscles (see Boerio et al., 2011).

Despite the many unsolved issues still remaining, the results of this study fully support the conclusions by Debanne that ... "the axon displays a high level of functional flexibility that was not expected initially. Thus, it may allow a fine tuning of synaptic strength and timing in neuronal microcircuits" (Debanne, 2004). The presented results provide also further evidence for long-term changes reflecting the flexibility of nerve fibres induced by their polarisation.

ACKNOWLEDGEMENTS

We wish to thank Prof. B. Gustafsson and Prof D. Bennett for their helpful comments on an earlier version of this article. The study was supported by the University of Gothenburg.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

All of the authors contributed to the study design, collection of the data and data analysis as well as the drafting of the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.15801.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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How to cite this article: Jankowska, E., Kaczmarek, D., & Hammar, I. (2022). Long-term modulation of the axonal refractory period. *European Journal of Neuroscience*, *56*(7), 4983–4999. https://doi.org/10.1111/ejn.15801