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RESEARCH ARTICLE | *Control of Movement*

Long-lasting increase in axonal excitability after epidurally applied DC

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Jankowska E, Kaczmarek D, Bolzoni F, Hammar I. Long-lasting increase in axonal excitability after epidurally applied DC. *J Neurophysiol* 118: 1210–1220, 2017. First published May 17, 2017; doi:10.1152/jn.00148.2017.—Effects of direct current (DC) on nerve fibers have primarily been investigated during or just after DC application. However, locally applied cathodal DC was recently demonstrated to increase the excitability of intraspinal preterminal axonal branches for >1 h. The aim of this study was therefore to investigate whether DC evokes a similarly long-lasting increase in the excitability of myelinated axons within the dorsal columns. The excitability of dorsal column fibers stimulated epidurally was monitored by recording compound action potentials in peripheral nerves in acute experiments in deeply anesthetized rats. The results show that *1*) cathodal polarization (0.8–1.0 μA) results in a severalfold increase in the number of epidurally activated fibers and *2*) the increase in the excitability appears within seconds, *3*) lasts for >1 h, and *4*) is activity independent, as it does not require fiber stimulation during the polarization. These features demonstrate an unexplored form of plasticity of myelinated fibers and indicate the conditions under which it develops. They also suggest that therapeutic effects of epidural stimulation may be significantly enhanced if it is combined with DC polarization. In particular, by using DC to increase the number of fibers activated by low-intensity epidural stimuli, the low clinical tolerance to higher stimulus intensities might be overcome. The activity independence of long-lasting DC effects would also allow the use of only brief periods of DC polarization preceding epidural stimulation to increase the effect.

NEW & NOTEWORTHY The study indicates a new form of plasticity of myelinated fibers. The differences in time course of DC-evoked increases in the excitability of myelinated nerve fibers in the dorsal columns and in preterminal axonal branches suggest that distinct mechanisms are involved in them. The results show that combining epidural stimulation and transspinal DC polarization may dramatically improve their outcome and result in more effective pain control and the return of impaired motor functions.

nerve fibers; epidural stimulation; direct current polarization; spinal cord excitability

EPIDURAL STIMULATION and transspinal direct current stimulation (tsDCS) are both used in clinical practice for pain relief as well as for restoring motor functions after spinal cord injuries (for recent reviews see Priori et al. 2014; Ramasubbu et al. 2013). However, the use of epidural stimulation is restricted by the low stimulus intensity tolerated by the patients (Ramasubbu et al. 2013), and, consequently, a smaller number of nerve fibers than would be optimal are activated by epidural stimuli. According to Holsheimer (Holsheimer 2002; Holsheimer and Buitenweg 2015), epidural stimuli below the discomfort threshold would only activate fibers within a thin outer layer of the dorsal columns, as the current density is much higher within the layer of subdural cerebrospinal fluid than within the spinal cord (see Fig. 1*D*). The first aim of the present study was therefore to examine whether the number of skin and muscle afferent fibers stimulated within the dorsal columns might be increased by increasing their excitability by epidural polarization to the same extent as the excitability of preterminal branches of sensory fibers is increased by intraspinal polarization (Bolzoni and Jankowska 2015; Jankowska et al. 2016). The second aim was to establish whether DC-induced facilitation outlasts the period of DC application and to determine the circumstances under which the facilitation may or may not be evoked. In light of the relatively short-lasting effects of epidural stimulation (Ramasubbu et al. 2013), a long-lasting facilitation induced by DC polarization would be clinically relevant, as it might prolong the pain-relieving effects as well as significantly increasing the probability of restoring deficient functions after spinal injuries.

The reported results show that the increases in excitability of myelinated fibers in the dorsal columns evoked by epidural DC are even more potent than the increases in excitability of preterminal axonal branches of these fibers and that their time course is different. Thus these results suggest that the mechanisms underlying DC effects on myelinated nerve fibers in the dorsal columns and on intraspinal preterminal nerve branches are not necessarily the same and that the reported effects indicate a hitherto unexplored form of axonal plasticity.

METHODS

All the main experimental procedures were performed as described in detail in previous publications of our group (Bolzoni and Jankowska 2015; Jankowska et al. 2016; Kaczmarek et al. 2017).

Ethical Approval

All experiments were approved by the Regional Ethics Committee for Animal Research (Göteborgs Djurförsöksetiska Nämnd) and fol-

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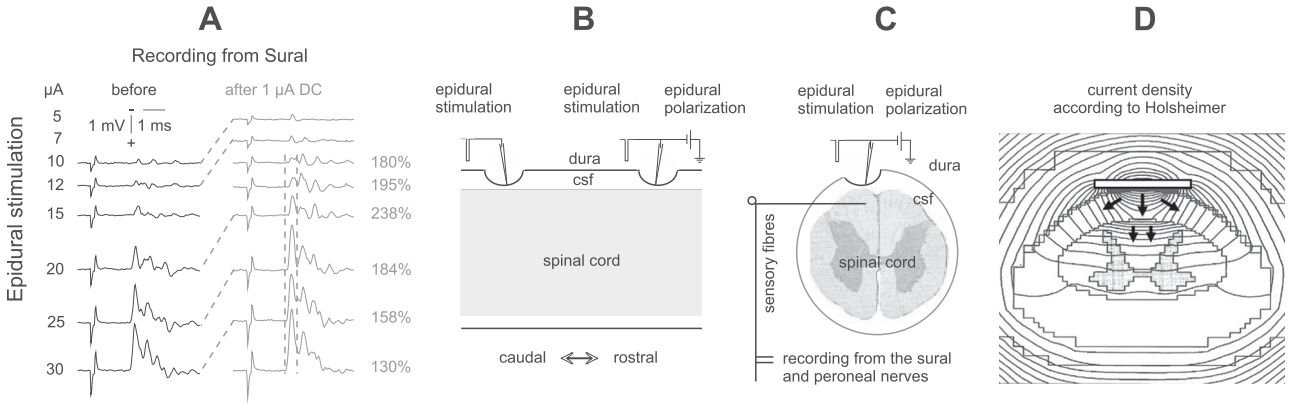

Fig. 1. Diagram of the basic experimental setup used in the study and examples of nerve volleys evoked by epidural stimuli before and after DC application. *A*: examples of series of nerve volleys evoked by epidural stimuli at increasing intensities before (*left*) and 1 h after 2 min (*right*) of DC application (averages of 10 consecutive single records). Note that after the DC application lower stimulus intensities sufficed to evoke similar volleys (as indicated by dashed lines). Vertical lines indicate the time windows within which the areas were measured. *B*: setup of epidural electrodes in the rostrocaudal plane, when the caudal electrode was used for stimulation and the rostral electrode for both stimulation and DC application, the distances between their tips being adjustable. *C*: arrangements of the stimulating and recording electrodes, under conditions when DC was applied via the rostral stimulating epidural electrode. *D*: reconstruction of current densities in a model of current flow around an epidural electrode in human subjects according to Holsheimer (Holsheimer and Buitenweg 2015, © 2015 International Neuromodulation Society; with permission). In this and subsequent figures the negativity in the records from the peripheral nerves is upward and the positivity is downward.

lowed European Union and National Institutes of Health guidelines for animal care. The animals were bred and housed under veterinary supervision at the Laboratory of Experimental Biomedicine at Sahlgrenska Academy, University of Gothenburg, where the experiments were carried out. Particular measures were taken to minimize animal discomfort and the number of animals used.

Preparation

The experiments were performed on 17 adult rats of both sexes (Wistar, 2–6 mo old, 200–450 g). Anesthesia was induced with isoflurane (4% in air) (Baxter Medical, Kista, Sweden) followed by intraperitoneal administration of α-chloralose (Acros organics, Geel, Belgium,) at a dose of 30–40 mg/kg together with pentobarbital sodium (Apoteksbolaget, Gothenburg, Sweden) at a dose of 20–25 mg/kg. During the course of the experiment, the anesthesia was supplemented at regular intervals with additional doses of α-chloralose (10 mg/kg; up to 60 mg/kg). The preliminary dissection included tracheal intubation, cannulation of one or two tail veins, dissection of the sural and peroneal nerves, as well as exposure of the second to fifth lumbar (L_2–L_5) spinal segments by laminectomy. Mineral oil pools were constructed by skin flaps above the dissected tissues. When the neuromuscular transmission was blocked by gallamine triethiodide (Sigma-Aldrich, G8134-5G), artificial ventilation was applied with a respiratory pump (CWE; 65–80/min and 0.2–0.4 ml/min volume depending on animal weight), maintaining the expired CO_2 level at 3–4%. Gallamine was administered intravenously (via the tail vein) at an initial dose of about 10 mg/kg and supplemented, when needed, with about 5 mg/kg. CO_2 level and heart rate were continuously monitored. The experiments were continued only for as long as these remained within physiological ranges.

The core body temperature was maintained at ~38°C by servo-controlled heating lamps. To compensate for fluid loss and to prevent the deterioration of the state of the animals, 10–20 ml of acetate buffer was injected subcutaneously during the initial surgical procedures. The experiments were terminated by a lethal dose of pentobarbital followed by excision of the heart.

Recording

The effects of DC on the excitability of sensory fibers were estimated from changes in antidromic compound action potentials reflecting responses evoked in these fibers by epidural stimuli. Responses recorded from the sural nerve would primarily be evoked in skin afferents, while those recorded from the peroneal nerve would include responses of both skin and muscle afferents. They were recorded via pairs of silver-silver chloride electrodes in a mineral oil pool. Both original records and averages of records evoked by 10 or 20 stimuli were stored online with a time resolution of 30 μs per address and were analyzed off-line with software for sampling and analysis developed by E. Eide, T. Holmström, and N. Pihlgren (Univ. of Gothenburg).

Stimulation

Epidural stimuli were delivered via needle tungsten electrodes (200–500 kΩ) (microneurography active needle, UNA35FNM, FHC, Bowdoin, ME), insulated except for a tip of 20–30 μm. The electrodes were mounted in a double-headed manipulator, the caudal electrode being used for stimulation and the rostral electrode for both stimulation and polarization, as indicated in Fig. 1*B*. The electrodes were positioned in contact with the dura mater within the L_1–L_3 segments, at approximately equal distances between the central vein and the dorsal root entry zone. The reference electrode consisted of a 2-cm-long tungsten electrode inserted into back muscles along the vertebral column close to the rostral edge of the laminectomy or a crocodile clip attached to the first spinous process rostral to the laminectomy, in both cases 10–20 mm rostral to the stimulation site. In experiments in which stimuli were applied after the cerebrospinal fluid was drained via a small opening in the dura mater, the epidural stimuli were applied 2–3 mm rostral or caudal to this opening. When the dura remained intact, epidural stimuli were applied either just at the surface or at a slightly deeper position, with the dura indented to a point where the distance between the dura and the surface of the dorsal columns was reduced to the minimum or contact between them occurred, as indicated in Fig. 1, *B* and *C*. The latter procedure was adopted to reduce the current flow through the cerebrospinal fluid in the subdural space rather than via the dorsal columns, as in the situation illustrated in Fig. 1*D* with a reconstruction of current densities during epidural stimulation in humans by Holsheimer (Hernández-Labrado et al. 2011; Holsheimer 2002; Holsheimer and Buitenweg 2015) The conditions under which the long-lasting changes in excitability of epidurally activated nerve fibers appear and some of the implications of these changes are discussed in the first and second parts of DISCUSSION, respectively. Single 0.2-ms constant-current stimuli were applied at 0.5–1 Hz, at intensities between 10 and

50 μA. The final rostrocaudal position of the stimulating electrodes was guided by a preliminary localization of the area where the largest afferent volleys were evoked by stimulation of afferent fibers in the peroneal and sural nerves, thereby increasing the probability of antidromic activation of these fibers by epidural stimuli. When several series of records were possible during an experiment, the explored areas of the spinal cord were separated by at least 2 mm.

Epidural Polarization

The polarization was applied with a custom-designed, battery-driven, constant current stimulator (D. Magnusson, Univ. of Gothenburg). The stimulator supplied a continuously monitored current within a range of intensities of 0–1.1 μA. It allowed the selection of either cathodal or anodal DC, but only cathodal DC was used in the present series of experiments. The polarizing current was applied for 1, 2, 5, or 25 min but in a few experiments only for a period of seconds. The current was manually turned on and off (not ramped). It was applied via one of the two tungsten electrodes used for epidural stimulation. Unless specified, the current was passed with the dura mater almost in contact with the surface of the spinal cord.

Analysis

Compound action potentials recorded in peripheral nerves after epidural stimulation were compared when they were evoked before, during, and after joint DC and epidural stimulation or before and after DC application alone. Both single records and averages of 10 successive records obtained online were stored for further off-line analysis. The comparison concerned changes in the area and/or the latencies of the earliest and most distinct components of averages created from 10 successive records with software developed by E. Eide, T. Holmström, and N. Pihlgren (Univ. of Gothenburg). The areas were measured within time windows of 0.3–1.4 ms from their onset (as illustrated in Fig. 1*A*), excluding any later components and taking into account only the first phase of any potentials resembling biphasically recorded potentials. All analyzed nerve volleys were evoked at the same latencies as the afferent volleys evoked by stimulation of the sural and peroneal nerves at the beginning of each experiment (or at ≤0.3 ms longer latencies), consistent with the direct activation of the nerve fibers and precluding records from axons of transsynaptically activated motoneurons. In each set of measurements, the areas were normalized with respect to the mean values of the areas sampled under control conditions. Thereafter, the mean values of normalized areas from all experiments were calculated for each test period and comparisons between these periods were performed with repeated-measures ANOVA. When a significant effect was found ($P < 0.05$), Tukey honestly significant difference (HSD) post hoc comparison was performed. Student′s paired *t*-test for two samples assuming equal variance was also used to estimate statistically significant differences within selected time periods and control values. The samples were obtained from at least three or four experiments, with at least four experimental series in each animal, and were considered adequate when paired *t*-test and repeated-measures ANOVA allowed their comparison.

Experimental Design

Nerve volleys were evoked by epidural stimuli during three periods, before, during, and after DC application, but the number and duration of periods of DC application were variable. To maximize the outcome from each experiment, nerve volleys evoked by epidural stimuli were recorded simultaneously from the sural and peroneal nerves. During each of the stimulation periods, we also used stimuli at three different intensities to allow a comparison of effects evoked at threshold and at two suprathreshold intensities. One of these stimuli was delivered by the same electrode that was used for DC application, while the remaining two stimuli were delivered by the epidural electrode used exclusively for stimulation. Therefore, each sequence of records provided data for afferents in two separate nerves and for responses evoked at different current intensities via two epidural electrodes. Sequences of 10 stimuli at each of these intensities were applied at 1–2 Hz and repeated every 2 or 3 min. The main differences in the stimulus parameters in experimental paradigms included the intensity, duration, and number of periods of DC application, the timing between DC and epidural stimuli, the intensity of epidural stimuli, the distances between the two epidural stimuli, or the distances between the surface of the dura mater and the surface of the dorsal columns. In control experiments, effects of epidural stimuli applied via a silver ball electrode or a tungsten wire with a larger (0.5–1 mm^2) contact area were compared with effects evoked via needle tungsten electrodes. If several sequences of observations were made in an animal, the subsequently explored spinal cord areas were located at least 2 mm apart. We refer to the data obtained in a particular experimental variant as a "series," all conclusions being based on at least six to eight series in at least two or three animals.

RESULTS

The study revealed that DC applied via epidural electrodes potently increases the number of sensory fibers activated within the dorsal columns. Even more importantly, the number of activated fibers remains elevated not only during DC application but also during postpolarization periods lasting >1 h. DC-evoked changes in the number of sensory fibers excited by epidurally applied stimuli were estimated using records from these fibers at the level of a skin nerve (sural) and a muscle nerve (peroneal), as indicated schematically in Fig. 1*C*. The area of nerve volleys evoked by the epidural stimulation was used as the measure of the number of excited fibers, as illustrated in Fig. 1*A*, even though no strict linear relationship could be expected between the two.

Higher Efficacy of Epidural Stimulation After DC Polarization

DC-evoked changes in the efficacy of epidural stimulation were first analyzed when the parameters of DC were kept constant while intensities of epidural stimulation were altered. The epidural stimuli were delivered by two electrodes, the caudal electrode being used only for stimulation and the rostral electrode for both stimulation and polarization, as indicated in Fig. 1*B*. Figure 1A illustrates effects of a series of epidural stimuli of increasing intensities before (Fig. 1*A*, *left*) and 1 h after (Fig. 1*A*, *right*) the termination of DC polarization (1 μA for 2 min). The records show that DC reduced the threshold for activation of the fibers from ~10 μA to ~5 μA and that considerably larger nerve volleys were evoked at all stimulus intensities during the postpolarization period. However, the degree of facilitation varied, as nerve volleys evoked by weaker stimuli, e.g., 10–15 μA, were increased by DC to a greater extent (2- to 3-fold) than nerve volleys evoked by stronger stimuli (< twice). As similar differences were consistently found whenever effects of DC were tested on nerve volleys of different sizes, submaximal nerves corresponding to those evoked by 15–20 μA in the series illustrated in Fig. 1*A* were routinely selected for the analysis. Figure 1*A* also illustrates a common finding that the increases in the earliest components of the analyzed nerve volleys were not associated with a shortening of their latencies. This is taken to indicate the recruitment of fibers located deeper within the dorsal column

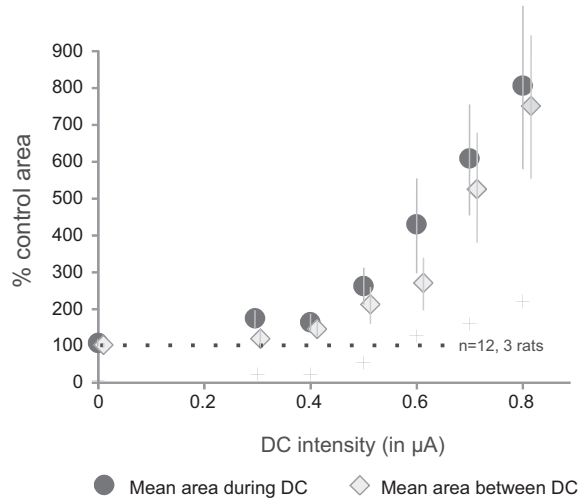

Fig. 2. Relationship between the intensity of DC used for the polarization and the increases in nerve volleys evoked by epidural stimulation. Mean increases in nerve volleys (*y*-axis) evoked in 12 experimental series by DC ranging between 0.3 and 0.8 μA (*x*-axis). Dotted horizontal line indicates the control level.

rather than fibers with a higher conduction velocity or over some distance in the rostrocaudal direction. No differences were seen in effects of DC related to the age, weight, or sex of the animals. The analysis was therefore not restricted to a particular sample of the rats.

DC Parameters for Enhancing Effects of Epidural Stimulation

Threshold DC. To define the lowest intensities of DC needed to increase nerve volleys evoked by epidural stimuli, the DC intensity was increased stepwise between 0.3 and 1.0 μA during successive 1-min periods while the intensity of the epidural stimulation remained constant. Such cumulative effects of DC were analyzed in 12 series of records obtained in three rats. The first signs of a facilitation during DC application were found when the DC was delivered at intensities of 0.3–0.5 μA, while more potent facilitation was evoked when DC increased to 0.8 μA (filled circles in Fig. 2). A somewhat weaker facilitation was observed during the between-polarization periods of 1 min immediately following periods of DC stimulation (diamonds in Fig. 2). After a polarization of 0.8–1.0 μA a similar degree of facilitation was found during a postpolarization period lasting 10 min, and 1.0-μA DC was therefore routinely applied. Similar ranges of DC intensities were needed for facilitation of responses evoked in the sural and peroneal nerves and for facilitation of responses evoked by both the rostral and the caudal epidural electrodes.

Minimal duration of DC application. When DC was applied at sufficiently strong intensities (0.8–1.0 μA), the duration of the polarization period did not appear to be critical for eliciting facilitation of effects of epidural stimulation, whether during or after DC application. During DC polarization, the facilitation appeared within the first 2–3 s. This is illustrated in Fig. 3*B* with a series of records of nerve volleys evoked every second, where already the second volley was larger than those evoked during the control period (Fig. 3*A*). The peak amplitudes of these volleys continued to increase, reaching a maximum ~7–9 s after the onset of DC and thereafter remaining unaltered until the DC application was terminated (Fig. 3*C*). The same temporal development of DC-evoked facilitation was found in all 12 series of records, irrespective of whether the nerve volleys were evoked in the peroneal or sural nerves (Fig. 3, *top* and *bottom*, respectively) and whether they were evoked via the rostral or caudal epidural electrode. The increase in the areas of the averaged records of control volleys following the polarization (D/A) is indicated as a percentage in Fig. 3*C*.

The postpolarization facilitation was found after a single period of epidural DC application and when the duration of this period was reduced to 1 min (5 rats, 23 series, as in Fig. 3) or even to 15–30 s (3 rats, 12 series). The effects of the shortest tested period (15 s) of polarization are shown in Fig. 4. Records in Fig. 4, *B* and *C*, show that after such a brief polarization the same stimuli evoked much larger responses and that the latter resembled the responses in Fig. 4*A* that were originally evoked by much stronger stimuli. The records also illustrate the similar degree of facilitation of responses evoked via the caudal and rostral electrodes, the DC having been applied via the rostral electrode. The plots in Fig. 4*A* show in addition that the decline of the postpolarization facilitation during the 35-min-long postpolarization period was only moderate and illustrate the

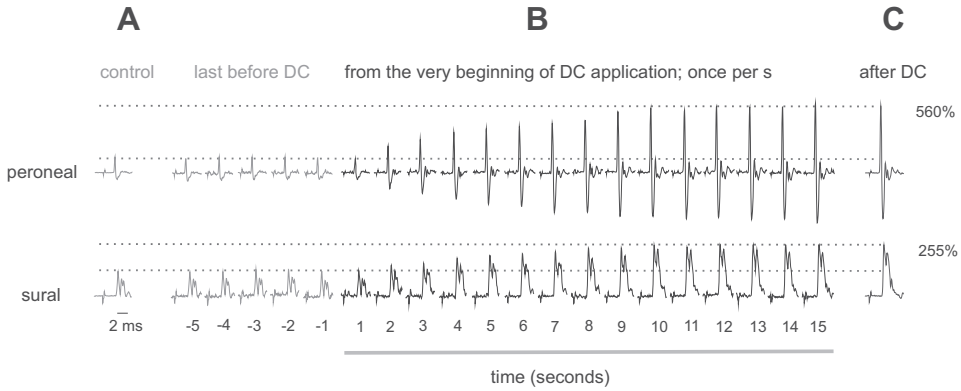

Fig. 3. Timing of facilitation of nerve volleys evoked by epidural stimulation during polarization. *A*: examples of averaged records of nerve volleys (n = 10) evoked by epidural stimuli during the prepolarization period in the peroneal and sural nerves. *B*: a continuous series of records of nerve volleys following those illustrated in *A*, evoked by the same intensity stimuli once per second just before and during DC application. The period of the polarization is indicated by the horizontal line below the *x*-axis. Dotted horizontal lines indicate peak amplitudes of the control volleys and maximal amplitude of the volleys after the first 10 s of polarization. *C*: averaged records of nerve volleys (n = 10) evoked by the same stimuli 1 min after the termination of DC application (for 1 min). Nerve volleys in the peroneal and sural nerves were recorded simultaneously in the same series of records.

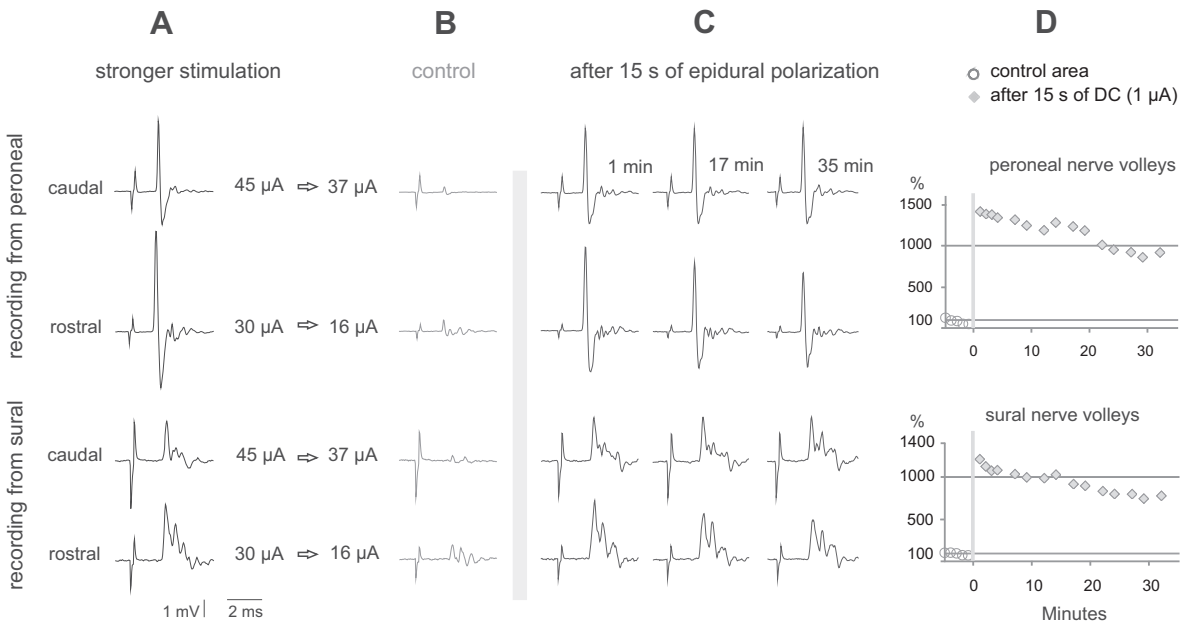

Fig. 4. Facilitatory effects evoked after 15 s of DC application. *A*: examples of records of nerve volleys in peroneal and sural nerves evoked by suprathreshold epidural stimuli applied via either the caudal or rostral epidural electrodes. *B*: as in *A* but with the intensity of the stimuli considerably reduced. *C*: nerve volleys evoked by the same stimulus intensities, as in *B* but after 15 s of 1 μA DC application through the rostral epidural electrode. The volleys were recorded during the postpolarization period, 1, 17, and 35 min after the polarization. *D*: full-time course of changes in the areas of the first components of nerve volleys evoked by the rostral epidural electrode (within time windows of 0.35 and 0.45 ms from the onset for the volleys recorded in the peroneal and sural nerves respectively). Gray bar between *B* and *C* indicates DC application for 15 s. No epidural stimulation was applied during the polarization period, so that increases in *C* and *D* illustrate activity-independent DC facilitatory effects.

similar time course of facilitation of responses of peroneal and sural afferents.

Epidurally evoked postpolarization facilitation thus did not require the repeated periods of DC application needed for slowly developing facilitation via tDCS (Bączyk et al. 2014; Bolzoni et al. 2013) or via locally applied DC (Bączyk and Jankowska 2014; Bolzoni and Jankowska 2015; Jankowska et al. 2016; Kaczmarek et al. 2017), illustrated in Fig. 5*D* for comparison. In addition, the degree of facilitation of nerve volleys following the various periods of polarization was comparable. After 10 min, the mean increase in the volley area exceeded 400% irrespective of whether these nerve volleys were evoked after application of DC for 15–30 s (411 ± 97%; $n = 10$; illustrated in Fig. 5*A*), 1 min (733 ± 251%; $n = 23$), 2 or 5 min (502 ± 94%; $n = 13$; illustrated in Fig. 5*B*), or 5 × 5 min (419 ± 125%; $n = 9$; illustrated in Fig. 5*C*). No statistically significant differences between effects evoked by different durations of DC application were indicated by *t*-test (unpaired, assuming equal variance). This comparison was made by pooling together the data from the experiments in which DC application was either combined with epidural stimulation or applied alone. In all experimental series in which the postpolarization periods lasted 30–60 min ($n = 30$), the facilitation of the nerve volleys evoked by the epidural stimulation exceeded 200% throughout these periods. This is illustrated in Fig. 4*D* for single series of records and in Fig. 5*B* for averages of 13 series.

DC Effects Depend on Distance Between Epidurally Stimulated Fibers and Site of DC Application

Effects of indentation of dura mater. The effects of DC described in the preceding sections were evoked under conditions expected to be optimal for activation and polarization of fibers in the dorsal columns They were thus evoked either with the epidural electrodes indenting the dura mater (positioned under microscope control), to reduce the layer of the cerebrospinal fluid between the surface of the spinal cord and the dura, or when the cerebrospinal fluid was drained by an intentional or unintentional small opening in the dura. In this way, the proportion of current passing the cerebrospinal fluid with a much lower resistivity than that of the white matter (Holsheimer and Buitenweg 2015) (see Fig. 1*D*) was reduced, the density of DC within the dorsal columns increased, and the effect of epidural stimulation potentiated.

The thresholds for activating afferent fibers in either the peroneal or sural nerves were more than two times higher when the epidural electrode just touched the dura surface (62.5 ± 4.8 μA; $n = 8$) than when it nearly touched the surface of the dorsal columns (28.7 ± 2.5 μA; $n = 8$). The postpolarization effects of 1-μA DC applied with the electrode just touching the dura were also weaker (190 ± 51%; $n = 8$; 20–25 min after DC application). The degree to which the nerve volleys were enhanced when the dura was indented is shown in Fig. 6*A*.

Effect of changes in distance between the two epidural electrodes. The effectiveness of DC would be expected to be highest within a small radius from the site of its application, not only in the vertical but also in the rostrocaudal plane. In the original study of Bindman (Bindman et al. 1979), DC applied locally similar to the present study was found to affect cortical neurons within the radius of ~100 μm from the electrode tip. Under the conditions of the present study, the highest degree of facilitation of responses evoked by epidural stimuli was accordingly expected to be evoked by DC applied via the same electrode (distance = 0) and to decrease with increasing dis-

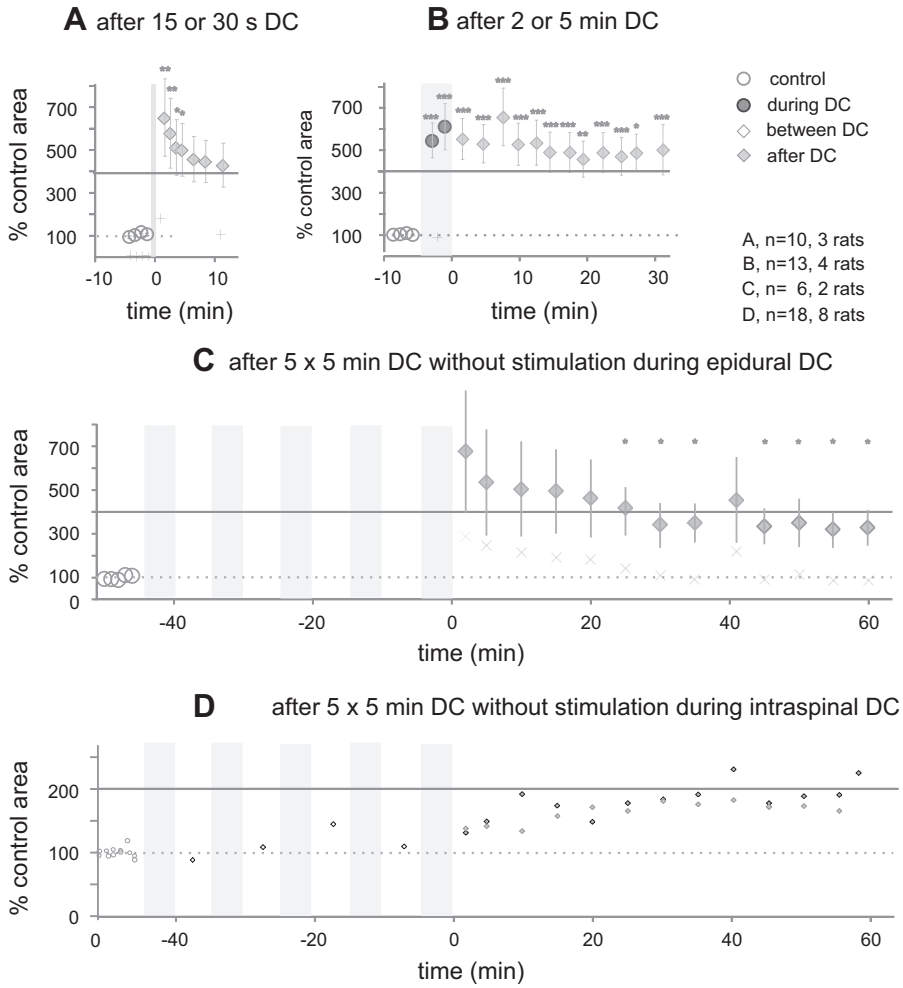

Fig. 5. Comparison of postpolarization facilitation of nerve volleys evoked by epidural stimulation after DC application of different durations. *A*: time course of postpolarization facilitation following 15 or 30 s of 1 μA DC polarization not associated with the epidural stimulation. Data for changes in nerve volley areas (mean and SE) from 10 series of records in 3 rats, pooling together changes in nerve volleys recorded in the peroneal and sural nerves as well as those induced by the 2 epidural electrodes. *B*: as in *A* but after 2 or 5 min of DC application concomitant with epidural stimulation in 13 series of records in 4 rats. *C*: as in *A* but after 5 periods of 5 min of DC application without concomitant epidural stimulation in 5 series of records in 2 rats. *D*: as in *A* and *C* but after 5 periods of 5 min of DC application without concomitant intraspinal stimulation (replotted data from Fig. 5A in Jankowska et al. 2016). Gray bars indicate the timing of the polarization. Horizontal dotted lines indicate the control level. Solid lines indicate a steady-state level following the early decrease in the DC effect in *A*–*C* or an increase in *D*. One-way repeated-measures ANOVA indicated a significant effect of epidural stimulation on nerve volleys in both *A* [$F(10, 80) = 6.44$, $P < 0.001$] and *B* [$F(17, 102) = 11.10$, $P < 0.001$]. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; differences revealed by Tukey's HSD post hoc comparisons in relation to the last control. In *C*, *t*-test (for paired 2-tailed samples) indicated differences at $P = 2.4–4.9$ at time intervals 22–60 ms.

tances. To define the distances within which DC of 1 μA would be effective, the caudal epidural electrode remained stationary while DC was applied via the rostral electrode over a range of decreasing distances beginning at 1 mm. The facilitatory effects of 1 μA of cathodal DC were only evoked at distances of 400 μm or less. As illustrated in Fig. 6*B*, cumulative effects of DC found under these conditions fell within quite a large range but consistently exceeded the size of the control volleys by at least 200%. The effects of DC were comparable with effects on responses induced by the same electrode only at distances of ~100–200 μm.

Sustained Increases in Excitability of Epidurally Stimulated Fibers Are Activity Independent

Previous studies revealed that intraspinal polarization of preterminal branches of afferent fibers evokes sustained increases in excitability that do not require concomitant fiber activation during DC application (Jankowska et al. 2016). These effects may thus be classified as activity independent, in contrast to long-lasting effects of tDCS with features of activity-dependent long-term potentiation (LTP)-like plasticity in both mouse and human motor cortex (Fritsch et al. 2010; Kim et al. 2017; Monte-Silva et al. 2010, 2013). The question therefore arose of whether fibers running in the dorsal columns, i.e., at a distance from their terminals, are affected by DC in the same manner as their preterminal branches.

As shown in Fig. 4, *B*–*D*, and Fig. 5, *A* and *C*, DC applied without concomitant stimulation of dorsal column fibers considerably increased the number of fibers activated by epidural stimuli during the postpolarization period. Postpolarization facilitation evoked under such conditions was found in a total of 19 series of records after 15-s to 2-min DC application in four rats. No statistically significant differences were found (unpaired *t*-test, assuming equal variance, $P = 0.2448$) between the degree of postpolarization facilitation after 10 min in these 19 series and in 29 series in which epidural stimuli were applied during DC application. In addition, irrespective of whether the facilitation following polarization was or was not associated with epidural stimulation, the facilitation remained within the postpolarization periods of 35–60 min and exceeded 200% of control at the end of these postpolarization periods.

Control Data

As the most potent effects of epidural polarization were found when DC was applied through the electrode also used for epidural stimulation, precautions were taken to avoid unspecific effects of DC caused by this arrangement. Previously, it was verified that stimulus pulses applied during DC application did not differ from those evoked either before or after DC (Bączyk and Jankowska 2014). In the present study, effects of epidurally applied DC were examined in parallel on nerve volleys evoked by the two epidural electrodes, one of which

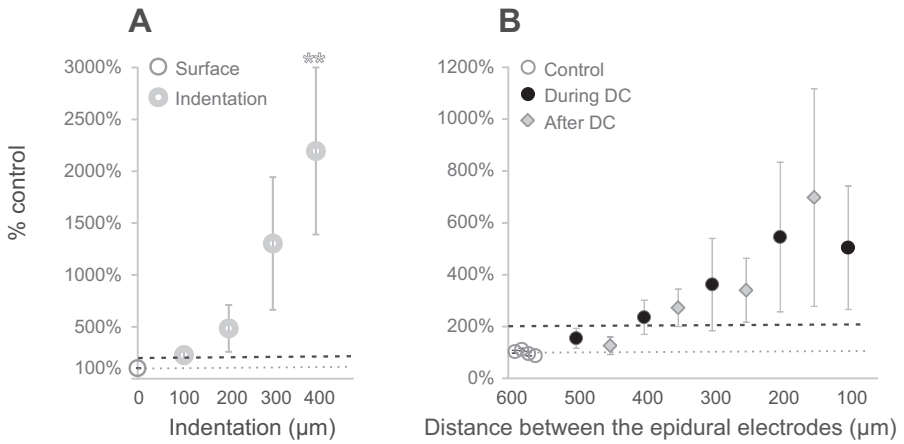

Fig. 6. Effects of distances between the epidural electrodes and the stimulated fibers in the dorsal columns. *A*: relationship between the degree of the indentation of the dura (*x*-axis) by the stimulating epidural electrode and the size of nerve volleys recorded in peripheral nerves. Means and SE of areas of nerve volleys; pooled data for 8 series of records from peroneal ($n = 4$) and sural ($n = 4$) nerves in 3 rats. The surface of the dorsal columns was estimated to be ~500 μm below the dura before its indentation. *B*: relationship between the distance between the 2 epidural electrodes and the degree of DC evoked facilitation of nerve volleys (*y*-axis) evoked by the stationary caudal electrode when the DC was applied at decreasing distances (*x*-axis). Symbols indicate cumulative effects of DC application for 2 min (filled circles) separated by 2 min of between polarization periods (gray diamonds). Means and SE from 11 series of measurements in 3 rats. Horizontal dotted lines indicate control level; dashed lines indicate nerve volleys twice larger than control volleys and likely to induce substantially stronger synaptic actions even if statistically significant increases in these volleys were only indicated by ANOVA for 400-μm indentation in *A* [$^{**}F(4,28) = 5.9249$, $P = 0.0139$, Tukey's HSD post hoc comparison); *t*-test (paired) indicating statistically significant differences between nerve volleys evoked by both 300- and 400-μm indentations ($P = 0.05$ and $P = 0.01$, respectively)]. In *B* there was a trend toward larger nerve volleys with decreasing distances between electrodes [$F(12, 84) = 1.95$, $P = 0.039$, 1-way repeated-measures ANOVA]; however, Tukey's HSD post hoc comparisons did not reveal distinct differences.

was used for applying DC and the other exclusively for stimulation 100–200 μm more caudally. Effects of epidurally applied DC were evoked after both long and short periods of DC application and were analyzed both during and after its application. Furthermore, we verified that similarly strong effects of epidurally applied DC were consistently evoked at different locations of the reference electrode (at positions just rostral to the level of the laminectomy as well as left, right, or caudal to it).

The decrease in efficacy of DC with increasing distances from its source indicated that the high DC density at the site of the stimulation of the fibers may be a critical factor. We therefore compared effects of DC evoked via silver ball or tungsten wire electrodes, both with ~0.5-mm^2 contact area, with the effects evoked by needle electrodes in the main series of the experiments. As the similar intensity of DC (1.1 μA) ($n = 6$) increased antidromically evoked volleys during DC application by <130%, and no facilitation outlasted DC, they indicate that current with the same intensity but a lower density may not suffice to induce the postpolarization effects.

DISCUSSION

The results of this study reveal that epidurally applied DC may potently increase the excitability of fibers within the dorsal columns. These DC-evoked changes are activity independent, develop within seconds, and are long-lasting, extending beyond the period of DC application by tenths of minutes. They are thus in contrast to the short-lasting increases in the excitability of peripheral nerve fibers that were related to effects of a relatively short electrotonus and persisted for milliseconds or seconds (Bostock et al. 2005; Burke et al. 2009; Nodera and Kaji 2006; Shu et al. 2006). As late as in 2004, Weragoda et al. (2004) stated that ". . . we are unaware of any reports of adaptive, long-term enhancement of excitability in intact axons... even if... they can display persistent hyperexcitability at sites of nerve injury and inflammation." Subsequently, long-lasting (24 h) increases in the excitability of unmyelinated nerve fibers were found by these authors in *Aplysia*, though under conditions when the fibers were depolarized by nerve crush, by a high concentration of K^+, or by strong depolarizing current pulses inducing repetitive discharges reminiscent of injury discharges. Transcutaneous polarization of human peripheral nerves was found to evoke a postpolarization increase in the excitability of motor nerve fibers when examined 2 min after DC offset, but may possibly have been longer-lasting (Ardolino et al. 2005), and similar effects were found by F. Bolzoni, R. Esposti, C. Bruttini, G. Zenoni, E. Jankowska, and P. Cavallari (submitted for publication). Effects of DC on mouse peripheral nerve fibers in vivo reported by Ahmed (2014) provide another example of long-lasting postpolarization changes in the excitability of nerve fibers. However, the interpretation of the changes reported by Ahmed is less straightforward, as they were complex, with a reduction of the excitability induced by cathodal DC but a significant shortening (by 0.4 ms) in the latency of action potentials that, as a rule, is associated with an increased excitability and, in addition, with unexpected opposite effects of DC during and after its application. The contribution of surround anodal or cathodal electrotonus or even block evoked by the fairly high DC intensity used in this study (10 μA) is likewise difficult to estimate.

In previous experiments, effects of intraspinally applied DC were found to be both long lasting and activity independent (Bolzoni and Jankowska 2015; Jankowska et al. 2016) but might have involved preterminal compartments of the nerve fibers with properties different from those of myelinated fibers at a distance from the terminals (for review see Debanne et al. 2011). In addition, their timing differed from the timing of effects of epidural polarization found in this study. The conditions under which the long-lasting changes in the excitability

of epidurally activated nerve fibers appear and some of the implications of these changes are discussed in the first and second parts of this section, respectively.

Optimal Conditions for Long-Lasting Enhancement of Nerve Fiber Excitability Induced by DC

A high density of current in a radial electric field generated by needle electrodes might be one of the critical factors for inducing the reported potent effects of epidurally applied DC, and the dependence of the efficacy of tDCS on current density was stressed as soon as it was introduced (Nitsche et al. 2008; Nitsche and Paulus 2000). In the original study by Bindman (Bindman et al. 1962), long-lasting facilitation of cortical neuronal responses was found when DC was applied via a glass micropipette, i.e., with a current similarly applied locally and with most likely a similar or even higher current density around the electrode tip. Accordingly, when we reduced the current density by replacing needle tungsten electrodes by silver ball or tungsten wire electrodes with a larger contact area with the dura, DC applied at the same intensity evoked only negligible effects. We also found that the potentiation of effects of DC attributable to the increased current density within the stimulated area occurred when the distance between the DC delivering electrode and the fibers to be activated was decreased and that this was particularly effective when indenting the dura mater and reducing the layer of the cerebrospinal fluid and thereby the distance between the surface of the dura and the surface of the spinal cord. The distances between the tips of the DC-delivering and epidurally stimulating electrodes in the rostrocaudal plane were optimal in terms of facilitatory effects when they were separated by 100−200 μm or when DC was delivered by the same electrode. The possibility of evoking facilitation of epidurally evoked effects in human subjects may thus depend on the conditions under which epidural stimuli and DC are delivered. Epidural stimulation in humans is applied under conditions in which a considerable volume of the cerebrospinal fluid separates the dura mater from the surface of the spinal cord. Effective epidural stimuli are accordingly ~100 times stronger in humans than under our experimental conditions, falling within the milliampere range, e.g., ~5 mA (Schade et al. 2010) rather than $<$50 μA. Hence, much higher intensities of DC might likewise be needed. The parameters deemed to be the most effective might also differ depending on the types of epidural electrodes used (Coburn 1980; Holsheimer and Buitenweg 2015; Ramasubbu et al. 2013). Those commonly used in humans have a large surface area in contact with the dura mater and are often flanked by reference electrodes in different configurations restricting the spread of current but are also most likely modifying its relative distribution within the subdural space and the dorsal columns. Nevertheless, even a 2-fold rather than 5- or 10-fold increase in the number of fibers activated via epidural electrodes might make a substantial clinically relevant difference and increase the probability of pain relief. More effective and sustained activation of spinal neuronal networks by epidural stimulation could also increase the possibility of the return of lost motor functions, including locomotion, already demonstrated to be improved by electrical epidural stimulation after spinal cord injuries (Dimitrijevic et al. 1998; Gerasimenko et al. 2008; Harkema et al. 2011; Minassian et al. 2004) as well as by intraspinal stimulation (Holinski et al. 2016).

The fact that the DC polarization may evoke a long-lasting increase in the excitability of sensory fibers, even in the absence of concomitant activation of these fibers, opens even more promising possibilities in its use than the application of DC simultaneously with epidural stimuli. Provided the activity-independent effects of DC are reproduced in human subjects, a brief episode of polarization lasting only a few minutes could precede a session of epidural stimulation that might be technically easier than when DC and epidural stimuli are delivered via the same electrodes.

Comparison Between Long-Lasting Increases in Excitability of Dorsal Column Fibers by DC and Other DC Effects

DC-evoked increases in the excitability of fibers stimulated within the dorsal columns appear to differ in two major ways from those evoked intraspinally, intracortically, or transcortically, first and foremost because the postpolarization facilitation of responses evoked epidurally developed within a few seconds (see Fig. 3 and Fig. 5*C*), in contrast to the previously reported DC-evoked facilitation requiring much longer-lasting (at least a few minutes) and often repeated episodes of DC application. (For slowly building up effects of intraspinally applied DC, see, e.g., Bolzoni and Jankowska 2015 and Fig. 5*D*; for intracortically applied DC see Bindman et al. 1964; and for tDCS effects see Jackson et al. 2016; Jamil et al. 2017; Nitsche et al. 2012; Nitsche and Paulus 2000; Santarnecchi et al. 2014).This discrepancy could not be related to different kinds of electrodes or to technical aspects of DC delivery, at least when they were same for the intraspinal and the epidural DC application. Differences in the trajectory of the polarized fibers, with fibers running in tightly packed parallel bundles in the dorsal column compared with the more widespread and separated single fibers stimulated within the spinal cord or the cortex, might be a more essential factor, as they would allow a larger number of fibers in the dorsal column to be accessed by a high density of current within the DC target area. However, it might also be considered that the much more potent increases in the excitability of fibers stimulated within the dorsal column are related to different properties of the myelinated axons (in particular within the nodes of Ranvier) and of the partly unmyelinated preterminal axon collaterals or the initial segments of axons and axon hillock in the spinal gray matter or within the cortex. The differences between them are numerous, in gross morphology as well as at the molecular level, in immunocytochemistry and in physiology (for references see, e.g., Debanne et al. 2011; Kole and Stuart 2012; Larsen and Sjöström 2015).

A further major difference between effects of differently applied DC lies in the degree to which effects of DC during and after its application are coupled. For effects of epidural DC, they appear to be very closely coupled, as the postpolarization facilitation invariably followed the facilitation evoked during DC application, even after periods of polarization lasting only a few seconds, and to a very similar extent. In contrast, postpolarization facilitation following DC applied intraspinally, intracortically, or transcortically gradually developed over longer periods of time and with indications for mechanisms specific for the postpolarization effects. For instance,

Santarnecchi et al. (2014) found only minor effects of either anodal or cathodal tDCS during 15 min of DC application, with a transient enhancement of effects at the beginning of the polarization and with opposite effects of the anodal and cathodal tDCS developing only during the postpolarization period. Nitsche et al. (2003) found also that the induction of long-lasting effects of tDCS but not of changes in the excitability evoked during DC application is prevented by antagonizing NMDA receptors, i.e., that they are differently affected pharmacologically.

Despite these differences, epidurally and intraspinally DC-evoked long-lasting increases in fiber excitability share an important feature in that both were evoked when DC was applied without concomitant activation of the fibers and that both may thus be classified as activity independent. The activity-independent increases in epidurally stimulated fibers are striking in being consistently evoked after periods of DC application as brief as 15 s and being followed by as potent long-lasting postpolarization effects as after longer polarization periods. Whether activity-independent DC-evoked changes contribute to activity-dependent postpolarization effects of tDCS remains a largely unresolved question. Certain effects of tDCS, at least D waves involving direct activation of corticospinal neurons or their axons (Di Lazzaro et al. 2013) and most likely monosynaptic activation of finger motoneurons by transcranial magnetic stimulation (TMS) expressed in the shortest-latency motor-evoked potentials, were found when the test stimuli were applied in humans before and after but not during tDCS application (see Jamil et al. 2017; Lefaucheur et al. 2008; Nitsche et al. 2008; Nitsche and Paulus 2000). Under these conditions, they thus did not depend on activation of corticospinal neurons by TMS during tDCS application, which might classify them as activity independent. However, as stated by Jackson et al., "...unlike in brain slice and anesthetized animal models, the human cortex is constantly active such that tDCS is always applied in conjunction with ongoing synaptic input even if it is not explicitly paired with another intervention" (Jackson et al. 2016). It would therefore be of great interest to know to what extent mechanisms of long-lasting activity-independent effects of DC on nerve fibers are shared with, or differ from, mechanisms of activity-dependent tDCS actions.

Over the years, the activity-dependent tDCS actions have been increasingly closely related to LTP and long-term depression (LTD) in the cortex and in the hippocampus. In earlier reports, effects of tDCS were described as inducing LTP- and LTD-like cortical excitability alterations (Nitsche and Paulus 2000) or sharing some features with the phenomena of LTP and LTD (Nitsche et al. 2008). The latest reviews are more decisive in this respect, referring to LTP as " a mechanism by which tDCS is thought to modulate brain function" (Giordano et al. 2017) or referring to LTP/LTD as "...induced by either tetanic stimulation or DC" (Jackson et al. 2016).

However, the relationships between tDCS and LTP/LTD have been considered almost exclusively with respect to synaptic plasticity, and the neuronal compartments listed as influenced by tDCS in the latest and the most comprehensive review by Jackson et al. (2016) include soma, dendrites, and synaptic terminals, but not axon hillock nor initial axonal segment, and axons only in terms of axonal grows and guidance or morphological reorientation of axon terminals. These relationships have thus been considered using "the somatic doctrine of tDCS." It is often accepted that tDCS may result in nonsynaptic effects when referring to aftereffects of polarization of peripheral nerve fibers as reported by Ardolino et al. (2005). It is also accepted that almost all nonneuronal tissues in the brain, in particular, glial cells, may be affected by tDCS (see Lefaucheur et al. 2017). However, these nonsynaptic and nonneuronal mechanisms have as yet not been incorporated in the discussions of the mechanisms underlying effects of tDCS, except by stressing that their better knowledge would be important. DC-evoked changes in the excitability of nerve fibers to electric stimuli have accordingly not been incorporated in the animal models of synaptic effects of transcranial DC stimulation (Giordano et al. 2017; Jackson et al. 2016). Nonsynaptic mechanisms might also have been considered as not sufficiently meaningful in the context of questions, methodological approaches, and/or technical aspects of studies on tDCS. However, even if not relevant for the analysis of synaptic effects of tDCS, DC-evoked changes in the excitability of nerve fibers should be relevant for theoretical aspects of epidural stimulation and not least for the joint use of the two techniques. Furthermore, as mutual facilitation of effects of epidural stimulation and of epidural polarization outlasts the period of their joint application, it adds to the repertory of DC effects. It would thus be particularly important to establish whether not only synaptic but also nonsynaptic effects of DC, in particular the long-lasting increases in fiber excitability, share some of their mechanisms with LTP/LTD, being combined with, e.g., increases in BDNF secretion, TrkB activation, and transcription of plasticity-related genes found to be associated with repeated periods of DC application in the rat cortex and hippocampus (see Fritsch et al. 2010; Jackson et al. 2016; Kim et al. 2017; Nitsche et al. 2012). For the analysis of mechanisms underlying the long-lasting increases in fiber excitability evoked by epidural polarization, or DC-evoked plastic changes in any other nerve fibers, the timing of these effects and the way DC affects the sequence of events within the nodes of Ranvier would be of particular importance. It would be also essential for future studies of this so-far hardly explored form of axonal plasticity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.J., D.K., F.B., and I.H. conceived and designed research; E.J., D.K., F.B., and I.H. performed experiments; E.J., D.K., F.B., and I.H. analyzed data; E.J., D.K., F.B., and I.H. interpreted results of experiments; E.J. and D.K. prepared figures; E.J. and I.H. drafted manuscript; E.J., D.K., F.B., and I.H. edited and revised manuscript; E.J., D.K., F.B., and I.H. approved final version of manuscript.

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