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Spinal direct current stimulation modulate[s] gracile nucleus and primary somatosensory cortex in anaesthetized rats

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Non-technical summary Stimulation of the human brain with direct current is a simple but effective neuromodulation technique that is becoming increasingly popular due to its potentiality for non-invasively treating a variety of neurological and neuropsychiatric disorders. Recently, this neuromodulation technique has been extended to the stimulation of the human spinal cord. Here we investigated the mechanisms of action of spinal direct current stimulation (sDCS) in anaesthetized rats. We found that sDCS can selectively modulate the spontaneous activity entering the brain through the spinal cord via the somatosensory system, consequently modulating both the internal state of the brain and its responsiveness to external somatosensory stimuli. These findings have at least two levels of significance: from a physiological perspective, they remark on the importance of the spinal cord in regulating the state of the brain; from a clinical perspective, they offer a mechanistic rationale for the development of sDCS as an effective bottom-up neuromodulation technique.

Abstract Afferent somatosensory activity from the spinal cord has a profound impact on the activity of the brain. Here we investigated the effects of spinal stimulation using direct current, delivered at the thoracic level, on the spontaneous activity and on the somatosensory evoked potentials of the gracile nucleus, which is the main entry point for hindpaw somatosensory signals reaching the brain from the dorsal columns, and of the primary somatosensory cortex in anaesthetized rats. Anodal spinal direct current stimulation (sDCS) increased the spontaneous activity and decreased the amplitude of evoked responses in the gracile nucleus, whereas cathodal sDCS produced the opposite effects. At the level of the primary somatosensory cortex, the changes in spontaneous activity induced by sDCS were consistent with the effects observed in the gracile nucleus, but the changes in cortical evoked responses were more variable and state dependent. Therefore, sDCS can modulate in a polarity-specific manner the supraspinal activity of the somatosensory system, offering a versatile bottom-up neuromodulation technique that could potentially be useful in a number of clinical applications.

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Abbreviations LFP, local field potential; MUA, multi-unit activity; rMUA, rectified multi-unit activity; sDCS, spinal direct current stimulation.

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Introduction

Afferent signals running through the dorsal columns and the spinothalamic tract continuously update the brain about the somatosensory interaction between the environment and the body (Wall & Dubner, 1972). At least in acute conditions, afferent signals from the spinal cord can also dramatically influence the ongoing activity of the brain (Manjarrez *et al.* 2002). For example, increasing muscle afferent activity produces cerebral arousal (Motokizawa & Fujimori, 1964; Mori *et al.* 1973; Oshima *et al.* 1981; Lanier *et al.* 1986), whereas decreasing muscle afferent activity reduces it (Forbes *et al.* 1979; Schwartz *et al.* 1992). Similarly, reversible somatosensory deafferentation due to spinal anaesthesia decreases the arousal level of the brain, thereby reducing the requirements for sedation and general anaesthesia (Inagaki *et al.* 1994; Tverskoy *et al.* 1994; Hodgson *et al.* 1999; Antognini *et al.* 2000). Consistently with these observations, we recently showed that complete spinal cord transection immediately slows down cortical EEG activity (Aguilar *et al.* 2010) and decreases anaesthetic requirements in rats (Foffani *et al.* 2011). Overall, these studies suggest that afferent signals from the spinal cord play a critical role in regulating the functional state of the brain.

High-frequency spinal cord stimulation has been used for decades as a minimally invasive neurosurgical therapy for the management of patients affected by pain syndromes (Shealy *et al.* 1967; Kumar *et al.* 2007) and has been explored, with variable degree of success, for other pathologies such as multiple sclerosis (Illis *et al.* 1980; Read *et al.* 1980; Davis & Emmonds, 1992), spasticity (Siegfried *et al.* 1981), and Parkinson's disease (Fuentes *et al.* 2009; Thevathasan *et al.* 2010). The gate theory of sensory flow at spinal and brain levels was the physiological base to start using high-frequency spinal cord stimulation. In fact, even though the exact mechanisms of its clinical efficacy remain unclear, high-frequency spinal cord stimulation changes the activity of cortical areas associated with nociception (Nagamachi *et al.* 2006; Stancák *et al.* 2008; Kishima *et al.* 2010). High-frequency spinal cord stimulation is thus likely to act at least in part by modulating the supraspinal activity of the somatosensory system.

Hitherto, when applying spinal cord stimulation with typical clinical parameters, it is not possible to change the stimulation settings in order to selectively obtain an activating or deactivating modulation (Polácek *et al.* 2007; Qin *et al.* 2009; de Andrade *et al.* 2010), which limits the spectrum of potentially treatable symptoms. A promising solution to this limitation could be provided by direct current stimulation (Fuortes, 1954; Eccles *et al.* 1962; Ahmed, 2011). Direct current stimulation has long been applied to the human cortex (Nias, 1976; Lolas, 1977; Elbert *et al.* 1981; Priori *et al.* 1998; Priori, 2003), where it is generally assumed that anodal stimulation increases cortical neural excitability whereas cathodal stimulation decreases it (Nitsche & Paulus, 2000; Nitsche *et al.* 2008; Merzagora *et al.* 2010), consistent with animal studies (Bindman *et al.* 1962; Creutzfeldt *et al.* 1962; Bindman *et al.* 1964; Landau *et al.* 1964; Purpura & McMurtry, 1965). Very recently, direct current stimulation has also been applied to the human spinal cord (Cogiamanian *et al.* 2008, 2011; Winkler *et al.* 2010; Truini *et al.* 2011). Spinal direct current stimulation (sDCS) has therefore the potential to become an important non-invasive neuromodulation technique to treat a number of neurological symptoms. However, the physiological basis of sDCS effects needs to be better understood.

In the present study, we tested the hypothesis that sDCS can modulate the supraspinal activity of the somatosensory system in a polarity-specific manner. Anodal or cathodal sDCS was epidurally delivered at thoracic level in anaesthetized rats. We specifically studied the effects of sDCS on the spontaneous activity and on the somatosensory evoked potentials of the gracile nucleus, which is the main entry point for hindpaw somatosensory signals reaching the brain from the dorsal columns, and of the primary somatosensory cortex.

Methods

Animals

Experiments were performed following the rules of International Council for Laboratory Animal Science, European Union regulation 86/609/EEC and were approved by the Ethical Committee for Animal Research of the Hospital Nacional de Parapléjicos (Toledo, Spain). A total of 44 male Wistar rats were used in this study: 16 rats received only anodal sDCS, 17 rats received only cathodal sDCS, 11 rats received both (separated by at least 2 h). Of these animals, eight rats were used for histology with no electrophysiological recordings (4 only anodal and 4 only cathodal), 28 rats had cortical recordings under deep anaesthesia (12 only anodal, 5 only cathodal, 11 both), eight rats had cortical recordings under lighter anaesthesia (only cathodal). Of the 28 rats with cortical recordings under deep anaesthesia, 11 also had stable gracile recordings (3 only anodal, 1 only cathodal, 7 both). The final dataset of electrophysiological data is the following: gracile nucleus, anodal $n = 10$, cathodal $n = 8$; cortex, anodal $n = 23$, cathodal $n = 16$; cortex lighter anaesthesia, cathodal $n = 8$.

Surgery

Animals were anaesthetized with urethane. The level of anaesthesia was monitored by the EEG and by the

absence of pinch tail withdrawal and corneal reflexes. Supplemental doses (1/4 of original dose) were applied if necessary. The majority of our experiments were performed under deep urethane anaesthesia (induction dose 1.5 g kg^{-1}) characterized by absence of reflexes and EEG in a state of slow-wave activity (<1 Hz). In a subset of experiments ($n = 8$), animals were maintained at a lower level of anaesthesia (induction dose 1 g kg^{-1}), characterized by absence of pinch-withdrawal reflexes and EEG in a state of faster oscillations (typically 1–4 Hz). The deeper anaesthesia corresponded to a stage III-4 and the lighter anaesthesia to a stage III-3 according to Friedberg *et al*. (1999). The body temperature of the animals was regulated to maintain 36.5°C using an automatically controlled heating pad. The animals were then placed in a stereotaxic frame (SR-6, Narishige Scientific Instruments, Tokyo, Japan). Lidocaine 2% was applied over the body surface in contact with the frame and over the areas for incisions. A laminectomy was performed at thoracic level (T9–T10) keeping the dura mater intact. Electrodes for sDCS (see below) were placed in a dorso-ventral axis, with a pole on the spinal cord over the laminectomy in contact with the dura mater and the second one on the skin under the abdomen. The skin was cut caudal to the skull base until the second cervical vertebra and muscles were carefully moved sideways to reach the first cervical vertebra (C1). C1 was removed and the brainstem was exposed to gain access to the gracile nucleus. The dura mater was broken at the same level to drain cerebrospinal fluid and to lower the pressure inside the skull. The skin of the head was removed and the skull was exposed. A craniotomy was performed on the right side of the midline, over the primary somatosensory cortex (AP: 2 to –2 mm; ML: 1 to 5 mm; atlas of Paxinos & Watson, 2007). Small incisions in the dura mater were performed to permit the placement of the recording electrodes into the cerebral cortex. We studied both the spontaneous activity and the responses evoked by electrical stimuli delivered to the hindpaw before, during and after sDCS. We never provided additional anaesthesia between the beginning of the first recording before sDCS and the end of the last recording after sDCS. An overall schematic description of the experimental protocol is provided in Fig. 1.

Electrophysiological recordings

Electrophysiological extracellular recordings were obtained using tungsten electrodes of 4–5 MΩ impedance at 1000 Hz (TM31C40KT and TM31A50KT of WPI, Inc., Sarasota, FL, USA). To obtain gracile recordings, an electrode was placed in the gracile nucleus following stereotactic coordinates (AP: –13.6 to 14.6; ML: 0.2 to 1 mm; Nuñez *et al.* 2000) and with the help of anatomical landmarks such as the obex and veins. To obtain cortical recordings, two electrodes were placed in the infragranular somatosensory cortex: one in the forepaw area (AP: 0.5 to 1; ML: 4 to 4.5) and the other one in the hindpaw area (AP: –0.5 to –1; ML: 2 to 2.5), following the coordinates of Chapin & Lin (1984). Beside the stereotactic coordinates, the antero-posterior and medio-lateral locations of the electrodes were adjusted with multiple penetrations (up to 5, typically 3 penetrations per electrode) in order to optimize the physiological responses to the corresponding peripheral stimuli (maximum amplitude, minimal latency). To adjust the recording depth, we first identified layer 4, where response latencies are the shortest, and then lowered the electrodes to the infragranular layers (depth: 1.1 to 1.8 mm). All recordings were amplified and filtered (1 Hz to 3 kHz) using a modular system of preamplifier, filter and amplifier (Neurolog; Digitimer Ltd, Welwyn Garden City, UK). Analog signals were converted into digital data at a 20 kHz sampling rate using a CED power 1401 (Cambridge Electronics Design, Cambridge, UK) controlled by Spike2 software (v6, Cambridge Electronics Design). Signals were stored in a hard disk of a PC for subsequent analysis.

Spinal direct current stimulation (sDCS)

Spinal direct current stimulation (sDCS) was delivered with a constant current electrical stimulator (Master8, A.M.P.I, Jerusalem, Israel) connected to a pair of electrodes: one placed on the thoracic spinal cord over the exposed dura mater, and the second under the skin of the anterior abdominal area. The rationale for this orthogonal arrangement was to maximize the current focus in the spinal cord below the dorsal electrode, and thus maximize the ability to detect supraspinal effects. This arrangement is similar to the one adopted by Eccles *et al*. (1962) in anaesthetized cats. However, in that work both the dorsal electrode and the ventral electrode were in contact with the spinal cord, simultaneously inducing anodal stimulation of the dorsal spinal cord and cathodal stimulation of the ventral spinal cord (or vice versa, depending on the polarity). In order to induce a stimulation that was as homogeneous as possible throughout the spinal cord, we moved the ventral electrode away from the spinal cord on the same orthogonal axis and placed it on the abdomen. Note that with this arrangement our stimulation presumably affected both ascending and descending pathways. Stimulating electrodes were silver–silver chloride circular electrodes with a radius of 5 mm (total contact surface: 0.785 cm^2): the electrodes were covered with a thin piece of saline-soaked synthetic gauze in order to maximize electrical contact and avoid tissue damage. In order to be able to observe relatively large effects at supraspinal levels, sDCS was delivered with a strength of 1 mA (which was the maximum strength

used in cats by Eccles *et al.* 1962) for a duration of 15 min (which was the duration used in humans by Cogiamanian *et al.* 2008, 2011 and by Winkler *et al.* 2010). Silver–silver chloride electrodes with total contact surface similar to our electrodes have previously been shown to be able to deliver 2 mA of cathodal or anodal direct current stimulation for at least 22 min (Minhas *et al.* 2010).

It is important to clarify that our stimulation was conservative with the available safety criteria to avoid damage of neural tissue (Nitsche *et al.* 2003; Liebetanz *et al.* 2009), both in terms of current density (stimulation strength (A)/electrode size (cm^2)) and charge density (stimulation strength (A) × total stimulation duration (s)/electrode size (cm^2)). Cathodal direct current stimulation was shown to damage cerebral neural tissue through the intact cranial bone in rats for current densities above 14.29 mA cm^{-2} and charge densities above 5.24 C cm^{-2} (Liebetanz *et al.* 2009). In our protocols we stimulated with a current density of 1.27 mA cm^{-2} (12.7 A m^{-2}) and a charge density of 1.15 C cm^{-2}. To verify the absence of sDCS-induced damage, we performed control experiments without electrophysiological recordings, in which animals were perfused 30 min after anodal ($n = 4$) or cathodal ($n = 4$)

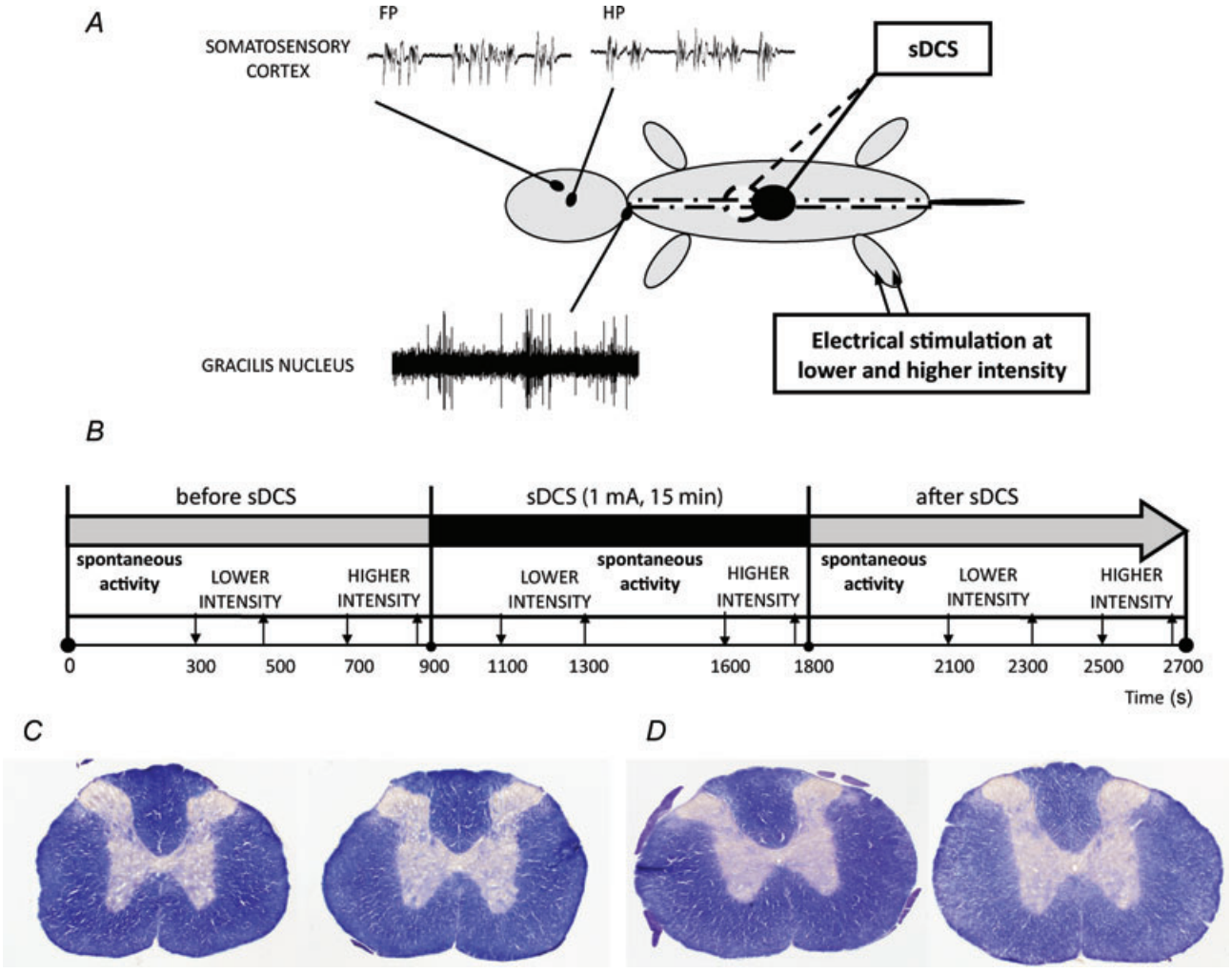


Figure 1. Experimental protocol
A, extracellular recordings were made in the gracile nucleus and in the forepaw (FP) and hindpaw (HP) representations of the infragranular somatosensory cortex in urethane-anaesthetized rats. Spinal direct current stimulation (sDCS) was applied using an active electrode located at the thoracic level (T9–T10) on the spinal cord and a reference under the abdomen. *B*, we studied both the spontaneous activity and the responses evoked by electrical stimuli delivered to the hindpaw at lower intensity (0.3 mA or 0.6 mA) and higher intensity (0.6 mA or 6 mA), before, during and after sDCS. *C* and *D*, representative coronal sections of the spinal cord (eriochrome cyanine staining) after anodal (*C*) and cathodal sDCS (*D*) (1 mA, 15 min). The left plots in *C* and *D* show spinal cord sections below the stimulating electrode (T9–T10), whereas the right plots show, as a control, spinal cord sections rostral to the stimulating electrode (T3–T4). sDCS did not produce any noticeable morphological damage to the spinal cord.

sDCS. Histology of the spinal cord was performed similarly to our previous study (Yague *et al.* 2011). We used Nissl staining (anodal $n = 2$, cathodal $n = 2$) to test possible morphological changes on cell bodies, and eriochrome cyanine staining (anodal $n = 2$, cathodal $n = 2$) to test possible morphological changes on fibres. For Nissl staining, animals were transcardially perfused with heparinized saline followed by neutral buffered formalin. Then, the spinal cord was removed and post-fixed for 4 h in the same fixative, and then immersed in phosphate-buffered saline (PBS) for 1 day. Sections of the spinal cord were obtained with a vibrating blade microtome (Microm HM 650 V (Micron International GmbH, Walldorf, Germany)) at 50 μm and Nissl stained. For eriochrome cyanine staining, animals were deeply anaesthetized and perfused transcardially with heparinized PBS followed by 4% paraformaldehyde solution (in PBS). Spinal cords were removed and post-fixed in the same fixative solution for 4 h. After post-fixation the spinal cords were stored at 4°C in sucrose solutions with increasing concentrations (10–20% and 30% sucrose in 0.1 M phosphate buffer) for 3–4 days and frozen with OCT medium. Frozen sections of 20 μm were cut on the Cryostat, immediately mounted onto slides and stored at −20°C. All sections were further processed for eriochrome cyanine staining. Both visual inspection and histological analysis confirmed that the spinal cord below the stimulating electrode remained morphologically intact (Fig. 1*C* and *D*).

In all experiments, the sDCS polarity (anodal or cathodal) refers to the dorsal electrode over the spinal cord.

Peripheral stimulation

Electrical pulses were applied using bipolar needle electrodes located subcutaneously in the ankle of the hindpaw, one pole on each side. The rationale for this stimulation was to activate all types of somatosensory fibres originating within the hindpaw, including tactile, proprioceptive and nociceptive fibres. Stimuli were delivered to the hindpaw ipsilateral to the gracile recordings and contralateral to the cortical recordings. The protocol consisted of a total of 100 pulse stimuli with duration of 0.5 ms and frequency of 0.5 Hz. Different intensities were applied: for the gracile recordings we used a very low intensity (0.3 mA) and a low intensity (0.6 mA); for the cortical recordings we used the low intensity (0.6 mA) and a high intensity (6 mA). Very low intensity and low intensity stimuli were intended to activate only a fraction of the available fibres, mainly low-threshold primary fibres running through the dorsal columns to the brainstem (Lilja *et al.* 2006; Yague *et al.* 2011). High-intensity stimuli were intended to activate the maximum number of fibres, including high-threshold primary fibres that make synapse in the dorsal horns of the spinal cord, in turn activating the spinothalamic tract (Lilja *et al.* 2006; Yague *et al.* 2011).

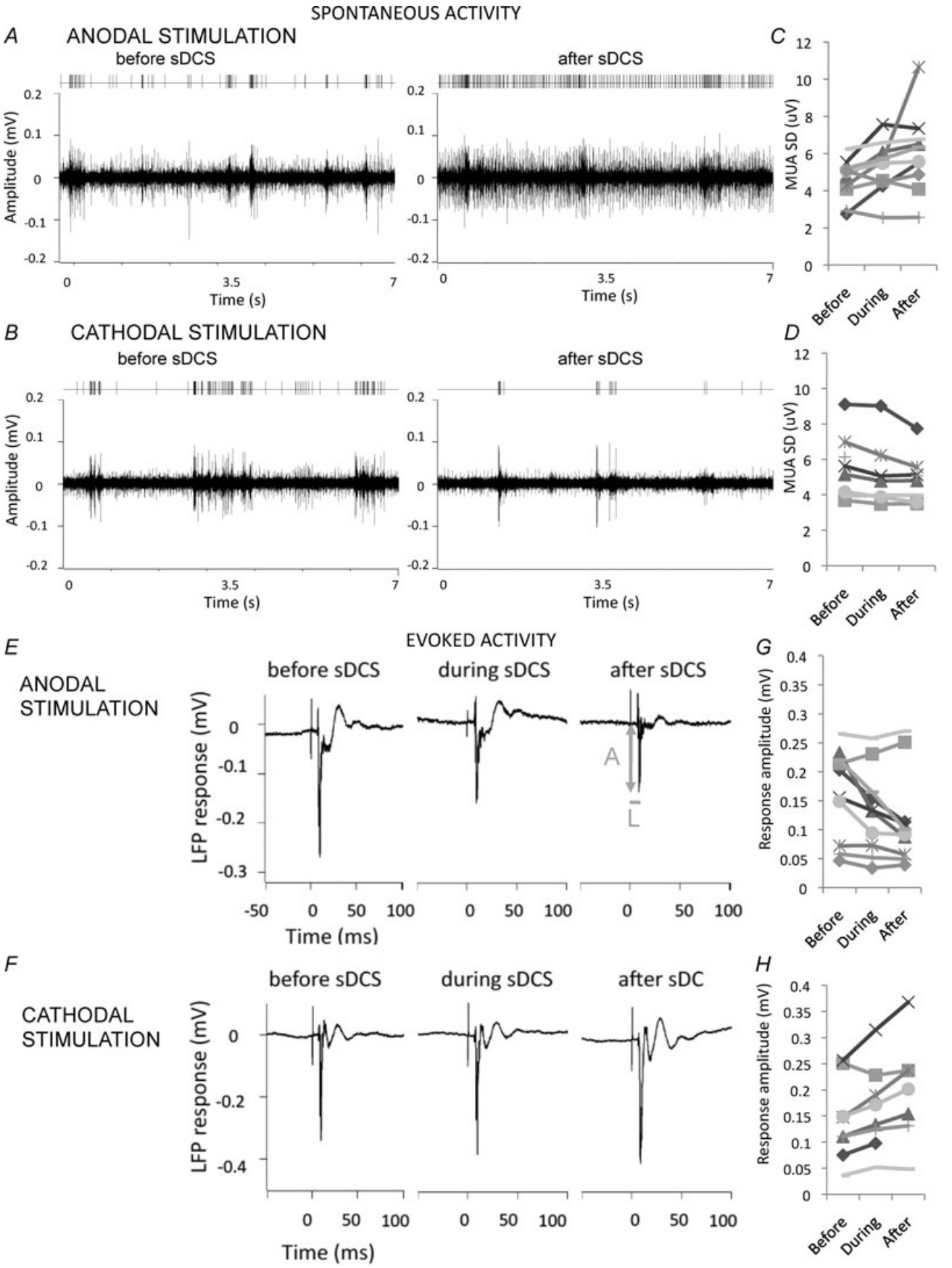
Data analysis

Gracile and cortical spontaneous activity. Spontaneous activity was studied in 300 s-long recordings performed before, during and immediately after sDCS (Fig. 1). To quantitatively evaluate the level of cortical spontaneous activity, we extracted the multi-unit activity (MUA) by band-pass filtering the raw signals at high frequencies (300–3000 Hz). We then used two main measures: (1) the standard deviation of the MUA and (2) the corresponding firing rate, which was obtained by detecting all spikes that exceeded the background noise by at least 5 standard deviations. In cortical recordings we also assessed the spectrum of the rectified MUA (rMUA): the rMUA signal was low-pass filtered, down-sampled to 1000 Hz and mean-detrended with a moving average of 10 s, and the rMUA spectrum was estimated with Welch's method, by averaging the periodogram of non-overlapping Hanning-tapered windows of 8192 samples. The main measure we extracted from the rMUA spectrum was the peak frequency, which quantifies the average alternation between active and silent states.

Cortical and gracile evoked responses. Local field potential (LFP) responses were obtained by averaging across stimuli the raw signals recorded from the electrodes. LFP response amplitude was evaluated as the absolute value of the negative peak in the average response. LFP response latency was evaluated as the latency of the negative peak.

Statistical analyses

Comparisons between control conditions (i.e. before sDCS) were performed using Student's t test for unpaired data. Changes in gracile spontaneous activity were evaluated with mixed two-way ANOVA. The first factor was the POLARITY of the stimulation, with two independent-measures levels: anodal and cathodal. The second factor was TIME, with three repeated-measures levels: before, during and after sDCS. For the interaction between POLARITY and TIME, we performed follow-up one-way repeated-measure ANOVA to analyse the effects in TIME separately for anodal and cathodal sDCS. Changes in gracile evoked responses were evaluated with a three-way mixed ANOVA. The first two factors were POLARITY and TIME, as above. The third factor was the INTENSITY of the stimuli, with two repeated-measures levels: lower and higher. For the interaction between POLARITY and TIME, we performed follow-up two-way



repeated-measure ANOVAs to analyse the effects in TIME and the possible interactions between TIME and INTENSITY for anodal and cathodal sDCS.

In the great majority of cases, cortical data failed to respect the normality assumption of the ANOVAs, according to both the Lilliefors and Shapiro–Wilk tests ($P < 0.05$). Log-transformation was thus used to normalize all cortical data for statistical purposes. After log-transformation, deviations from normality (Lilliefors and Shapiro–Wilk tests $P < 0.05$) were negligible (0% of cases with Bonferroni correction, less than 6% of cases without Bonferroni correction). Changes in cortical spontaneous activity were evaluated with mixed two-way ANOVAs. The first factor was the POLARITY of the stimulation, with two independent-measures levels: anodal and cathodal. The second factor was TIME, with three repeated-measures levels: before, during and after sDCS. For the interaction between POLARITY and TIME, we performed follow-up one-way repeated-measure ANOVAs to analyse the effects in TIME separately for anodal and cathodal sDCS. In the analysis of the rMUA spectrum and in the experiments in which we applied cathodal stimulation under a lower level of anaesthesia, changes in cortical spontaneous activity were evaluated with one-way repeated-measures ANOVAs, with TIME as main factor (before, during and after sDCS). Changes in cortical evoked responses were evaluated with two-way repeated-measures ANOVAs, with TIME as first factor (before, during and after sDCS), and INTENSITY of the stimuli as second factor (lower, higher). Dunnett's test was used for all *post hoc* comparisons. All results were considered significant at $P < 0.05$.

Results

Effects of sDCS on gracile activity

To test the hypothesis that sDCS can modulate the supraspinal activity of the somatosensory system, we recorded the spontaneous activity of the gracile nucleus, which is where dorsal column fibres conveying hindpaw somatosensory signals make their first synapse in the brain (Fig. 2*A*-*D*). Gracile spontaneous activity was assessed before, during and immediately after sDCS delivered at thoracic level (T9–T10) for 15 min, with two different polarities: anodal ($n = 10$) or cathodal ($n = 8$). We preliminarily verified that there were no differences between the anodal and cathodal groups in gracile spontaneous activity before sDCS (t tests, $P > 0.17$). Overall, sDCS induced significant changes in the level of spontaneous activity of the gracile nucleus, as measured by both the standard deviation of the MUA (two-way ANOVA, interaction TIME × POLARITY: $F_{(2,30)} = 5.65$, $P = 0.0082$) and the corresponding firing rate (two-way ANOVA, interaction TIME × POLARITY: $F_{(2,30)} = 12.24$, $P = 0.0001$). Specifically, anodal sDCS significantly increased the level of spontaneous activity of the gracile nucleus while cathodal sDCS significantly decreased it (one-way follow-up ANOVAs; MUA standard deviation: anodal $F_{(2,18)} = 4.24$, $P = 0.0310$, cathodal $F_{(2,12)} = 6.27$, $P = 0.0137$; firing rate: anodal $F_{(2,18)} = 11.91$, $P = 0.0005$, cathodal $F_{(2,12)} = 4.56$, $P = 0.0336$). All values, except firing rate with anodal sDCS, reached significance only after the stimulation (Table 1).

We then investigated whether sDCS also modulated the responses evoked in the gracile nucleus by electrical stimuli delivered to the hindpaw at low intensity (0.6 mA) or very-low intensity (0.3 mA), (Fig. 2*E*-*H*). Both intensities supposedly activate mainly the dorsal columns (Lilja *et al.* 2006; Yague *et al.* 2011). We preliminarily verified that there were no differences between the anodal and cathodal groups in the amplitude of gracile LFP responses before sDCS (t tests, $P > 0.60$). Overall, sDCS induced significant changes in the amplitude of gracile LFP responses (three-way ANOVA, interaction TIME × POLARITY: $F_{(2,24)} = 11.64$, $P = 0.0003$). Specifically, anodal sDCS significantly decreased the amplitude of gracile LFP responses, while cathodal sDCS significantly increased it (two-way follow-up ANOVAs, TIME: anodal $F_{(2,14)} = 5.32$, $P = 0.0191$, cathodal $F_{(2,10)} = 7.84$, $P = 0.0090$). For anodal sDCS, the effects were significant both during and after sDCS, whereas for cathodal sDCS they reached significance only after sDCS (Table 1). Despite these effects on amplitudes, sDCS did not affect the latency of gracile LFP responses (three-way ANOVA, $P > 0.16$; Table 1).

Figure 2. Effects of sDCS on gracile spontaneous activity and evoked responses
A and *B*, representative examples of spontaneous activity recorded in the gracile nucleus before and after anodal (*A*) and cathodal (*B*) sDCS. In each panel, the lower trace represents the raw multi-unit activity (MUA), and the upper trace is the event channel of the spikes exceeding the background noise by at least 5 standard deviations. *C* and *D*, all data representing the spontaneous activity of the gracile nucleus (measured by the MUA standard deviation) before, during and after anodal (*C*) and cathodal (*D*) sDCS. *E* and *F*, representative examples of LFP responses of the gracile nucleus to hindpaw stimuli delivered at low-intensity (0.6 mA), before, during and after anodal (*E*) and cathodal (*F*) sDCS (average of 100 stimuli, stimulus onset is indicated as time 0 on the *x*-axis.). We measured amplitude and latencies of the responses (grey lines, A and L in panel *E*). *G* and *H*, all data representing the amplitude of gracile LFP responses to low-intensity stimuli before, during and after anodal (*G*) and cathodal (*H*) sDCS. Anodal sDCS increased gracile spontaneous activity while decreasing LFP responses. Cathodal sDCS decreased gracile spontaneous activity while increasing LFP response.

Table 1. Gracile nucleus

	Anodal ($n = 10$)			Cathodal ($n = 8$)		
	Pre	DC	Post	Pre	DC	Post
Spontaneous activity						
MUA SD (μV)	4.6 ± 1.1	5.3 ± 1.4	**6.0 ± 2.1**	5.6 ± 1.8	5.2 ± 1.9	**4.9 ± 1.4**
		$P = 0.29$	$P = \mathbf{0.0174}$		$P = 0.17$	$P = \mathbf{0.0076}$
Firing rate (Hz)	9.7 ± 8.2	**20.2 ± 14.1**	**28.1 ± 16.7**	12.4 ± 6.2	10.5 ± 6.6	**6.8 ± 5.0**
		$P = \mathbf{0.0238}$	$P = \mathbf{0.0003}$		$P = 0.57$	$P = \mathbf{0.0222}$
Amplitude of LFP responses (mV)						
Very low intensity stimuli	0.13 ± 0.08	**0.10 ± 0.08**	**0.10 ± 0.08**	0.12 ± 0.07	0.13 ± 0.08	**0.16 ± 0.10**
Low-intensity stimuli	0.16 ± 0.08	**0.13 ± 0.07**	**0.12 ± 0.08**	0.14 ± 0.08	0.16 ± 0.08	**0.20 ± 0.10**
		$P = \mathbf{0.0471}$	$P = \mathbf{0.0154}$		$P = 0.18$	$P = \mathbf{0.0050}$
Latency of LFP responses (ms)						
Very low intensity stimuli	8.6 ± 0.7	8.8 ± 0.9	8.6 ± 0.8	8.8 ± 0.9	9.0 ± 0.7	8.9 ± 1.0
Low intensity stimuli	8.3 ± 0.7	8.3 ± 0.7	8.3 ± 0.7	9.1 ± 0.7	9.1 ± 0.7	9.1 ± 0.7

The table reports means ± standard deviations across animals of the indicated measures. *P* values indicate Dunnett's *post hoc* tests (DC and Post compared to Pre) when the corresponding ANOVA factor is significant, as indicated in the text. Significant post hoc tests are marked in bold.

Table 2. Hindpaw somatosensory cortex – spontaneous activity

	Anodal ($n = 23$)			Cathodal ($n = 16$)			Cathodal lighter anaesth. ($n = 8$)		
	Pre	DC	Post	Pre	DC	Post	Pre	DC	Post
MUA SD (uV)	4.7 ± 2.3	**6.0 ± 3.1**	5.5 ± 3.4	5.6 ± 2.3	5.6 ± 2.3	5.2 ± 2.3	8.6 ± 2.2	**6.1 ± 0.7**	**5.4 ± 1.0**
		$P = \mathbf{0.0080}$	$P = 0.0998$					$P = \mathbf{0.0034}$	$P = \mathbf{0.0004}$
Firing rate (Hz)	21.0 ± 23.9	**40.8 ± 53.5**	32.8 ± 44.9	18.4 ± 17.4	16.9 ± 17.6	**10.1 ± 9.4**	37.1 ± 23.1	**11.4 ± 8.7**	**10.7 ± 8.4**
		$P = \mathbf{0.0229}$	$P = 0.21$		$P = 0.32$	$P = \mathbf{0.0076}$		$P = \mathbf{0.0021}$	$P = \mathbf{0.0017}$
Peak of rMUA spectrum (Hz)	0.49 ± 0.12 ($n = 16$)	**0.66 ± 0.29**	0.53 ± 0.20	0.54 ± 0.16	**0.43 ± 0.19**	0.42 ± 0.22	1.35 ± 0.89	**0.42 ± 0.13**	**0.51 ± 0.14**
		$P = \mathbf{0.0222}$	$P = 0.86$		$P = \mathbf{0.0396}$	$P = 0.0510$		$P = \mathbf{0.0035}$	$P = \mathbf{0.0077}$

Details same as Table 1.

Effects of sDCS on cortical activity

In order to investigate whether sDCS also affected the state of upstream thalamo-cortical networks, we also studied changes of spontaneous activity in the hindpaw representation of the primary somatosensory cortex. As above, cortical spontaneous activity was recorded before, during and immediately after sDCS delivered at thoracic level (T9–T10) for 15 min, with two different polarities: anodal ($n = 23$) or cathodal ($n = 16$). First, we verified that there were no differences between the anodal and cathodal groups in cortical spontaneous activity before sDCS (t tests, $P > 0.24$). Overall, sDCS induced significant changes in the level of cortical spontaneous activity, as measured by both the standard deviation of the MUA (two-way ANOVA, interaction TIME × POLARITY: $F_{(2,72)} = 3.73$, $P = 0.0287$) and the corresponding firing rate (two-way ANOVA, interaction TIME × POLARITY: $F_{(2,72)} = 8.40$, $P = 0.0005$). Consistently with what we observed in the gracile nucleus, anodal sDCS significantly increased the level of cortical spontaneous activity while cathodal sDCS significantly decreased it (one-way follow-up ANOVAs; MUA standard deviation: anodal $F_{(2,44)} = 5.94$, $P = 0.0052$; firing rate: anodal $F_{(2,44)} = 4.80$, $P = 0.0130$, cathodal $F_{(2,28)} = 6.40$, $P = 0.0051$). For anodal sDCS, the effects were significant during the stimulation and lost significance after the stimulation (Table 2). For cathodal sDCS, the effects reached significance only after the stimulation (Table 2).

In our anaesthetized animals, in control conditions the hindpaw somatosensory cortex was in slow-wave activity at approximately 0.5 Hz. To further characterize the effects of sDCS on cortical spontaneous activity, we assessed the spectrum of the rectified MUA (rMUA). We specifically investigated whether the increased/decreased level of cortical spontaneous activity induced by anodal/cathodal sDCS corresponded to slightly faster/slower slow-wave oscillations (as measured by increased/decreased peak frequency of the rMUA spectrum), or to a complete change in the state of cortical activity. Indeed, with anodal sDCS in some animals ($n = 7$) the increase of cortical spontaneous activity was so dramatic to disrupt the synchronous slow-wave activity, substituting it with a more desynchronized activated state (Fig. 3). In the

remaining animals ($n = 16$), even without inducing a complete state change, anodal sDCS still speeded up the slow-wave oscillations, as measured by a significant increase in the peak frequency of the rMUA spectrum (one-way ANOVA: $F_{(2,30)} = 4.12$, $P = 0.0262$; Table 2). Conversely, cathodal sDCS ($n = 16$) slowed down the slow-wave oscillations, as measured by a significant decrease in the peak frequency of the rMUA spectrum (one-way ANOVA: $F_{(2,28)} = 3.69$, $P = 0.0378$; Table 2). The spectral analysis thus confirmed the activating effects of anodal sDCS and the deactivating effects of cathodal sDCS on spontaneous activity at cortical level.

The cortical effects of cathodal sDCS were more subtle compared to the effects of anodal sDCS. This was likely to have been due to fact that in our anaesthetized conditions the cortex was already in a deactivated state of slow-wave activity, so that a further decrease of tonic activity from the periphery could not

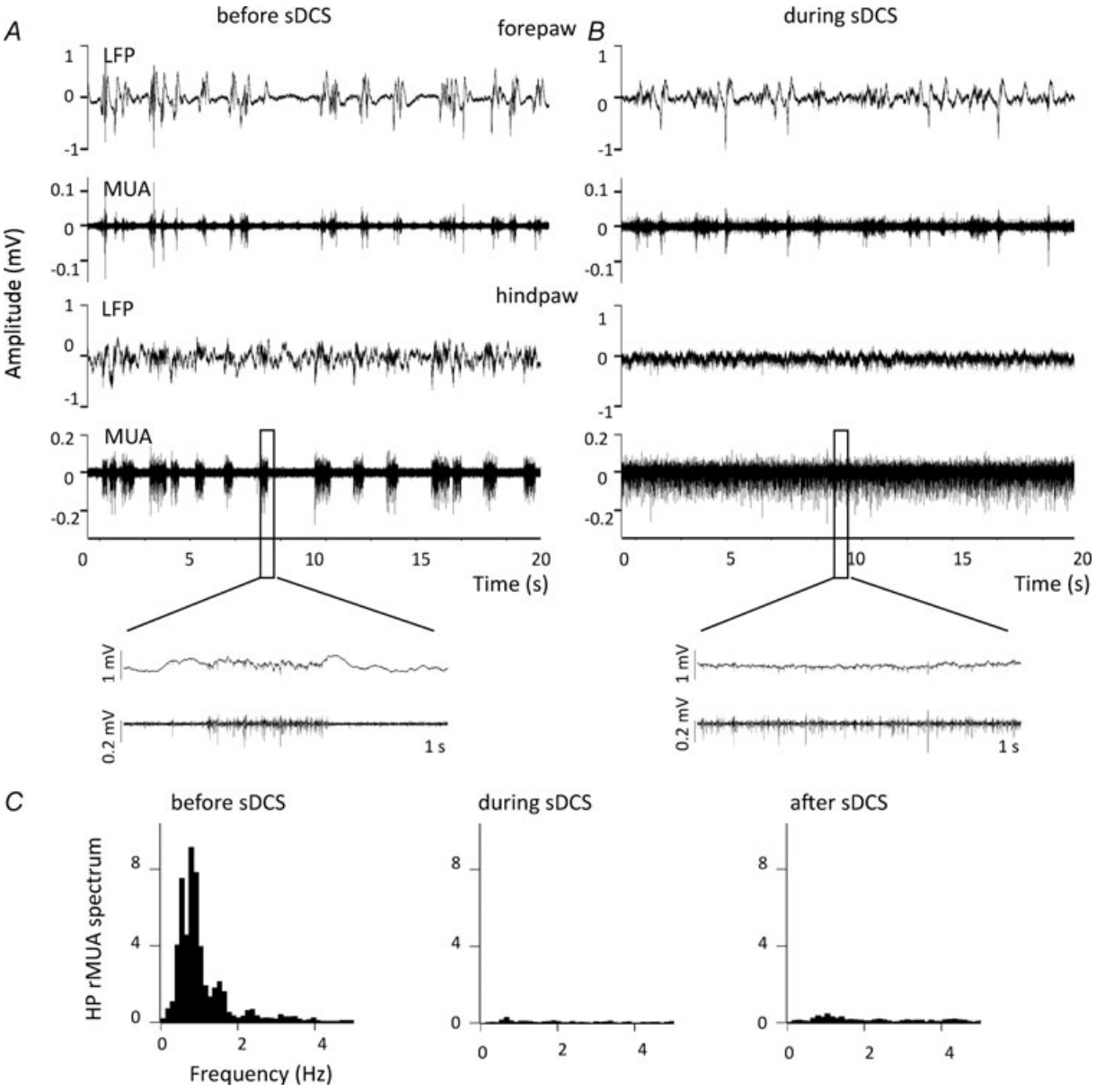


Figure 3. Effects of anodal sDCS on cortical spontaneous activity
A and *B*, example of spontaneous activity in a representative animal. Local field potential (LFP) and multi-unit activity (MUA) recorded from forepaw cortex (on the top) and hindpaw cortex (on the bottom) before (*A*) and during (*B*) sDCS. The inset of 1 s of spontaneous activity shows firing of neurons. *C*, power spectrum of the rectified MUA (rMUA) recording from the hindpaw cortex before, during and after sDCS, corresponding to the spontaneous activity shown in *A* and *B*. In this animal, anodal sDCS completely desynchronized the activity of the hindpaw somatosenory cortex.

induce much more deactivation. We thus performed additional experiments in a group of animals that were maintained at a lower level of anaesthesia ($n = 8$). In these animals, in control condition the peak of the rMUA spectrum was at 1.35 ± 0.88 Hz. As predicted, cathodal sDCS significantly decreased cortical spontaneous activity, as measured by the standard deviation of the MUA (one-way ANOVA: $F_{(2,14)} = 19.29$, $P < 0.0001$; Table 2), the corresponding firing rate (ANOVA: $F_{(2,14)} = 11.71$, $P = 0.0010$; Table 2) and the peak of the rMUA spectrum (ANOVA: $F_{(2,14)} = 22.21$, $P < 0.0001$; Table 2). In these animals the cortical deactivation induced by cathodal sDCS could be as dramatic as the cortical activation induced by anodal sDCS (Fig. 4).

Changes in cortical spontaneous activity can dramatically affect the cortical responses evoked by somatosensory stimuli (Petersen *et al.* 2003; Aguilar *et al.* 2010). We therefore investigated whether sDCS also affected the responses of the hindpaw representation of the primary somatosensory cortex to electrical stimuli delivered to the contralateral hindpaw. We employed two stimulus intensities: low intensity (0.6 mA) to activate mainly the dorsal columns (Lilja *et al.* 2006; Yague *et al.* 2011), and high intensity (6 mA) to activate also the spinothalamic tract (Lilja *et al.* 2006; Yague *et al.* 2011). Overall, anodal sDCS ($n = 23$) induced a small but significant increase in the amplitude of cortical LFP responses (two-way ANOVA, TIME: $F_{(2,44)} = 3.39$, $P = 0.0427$),

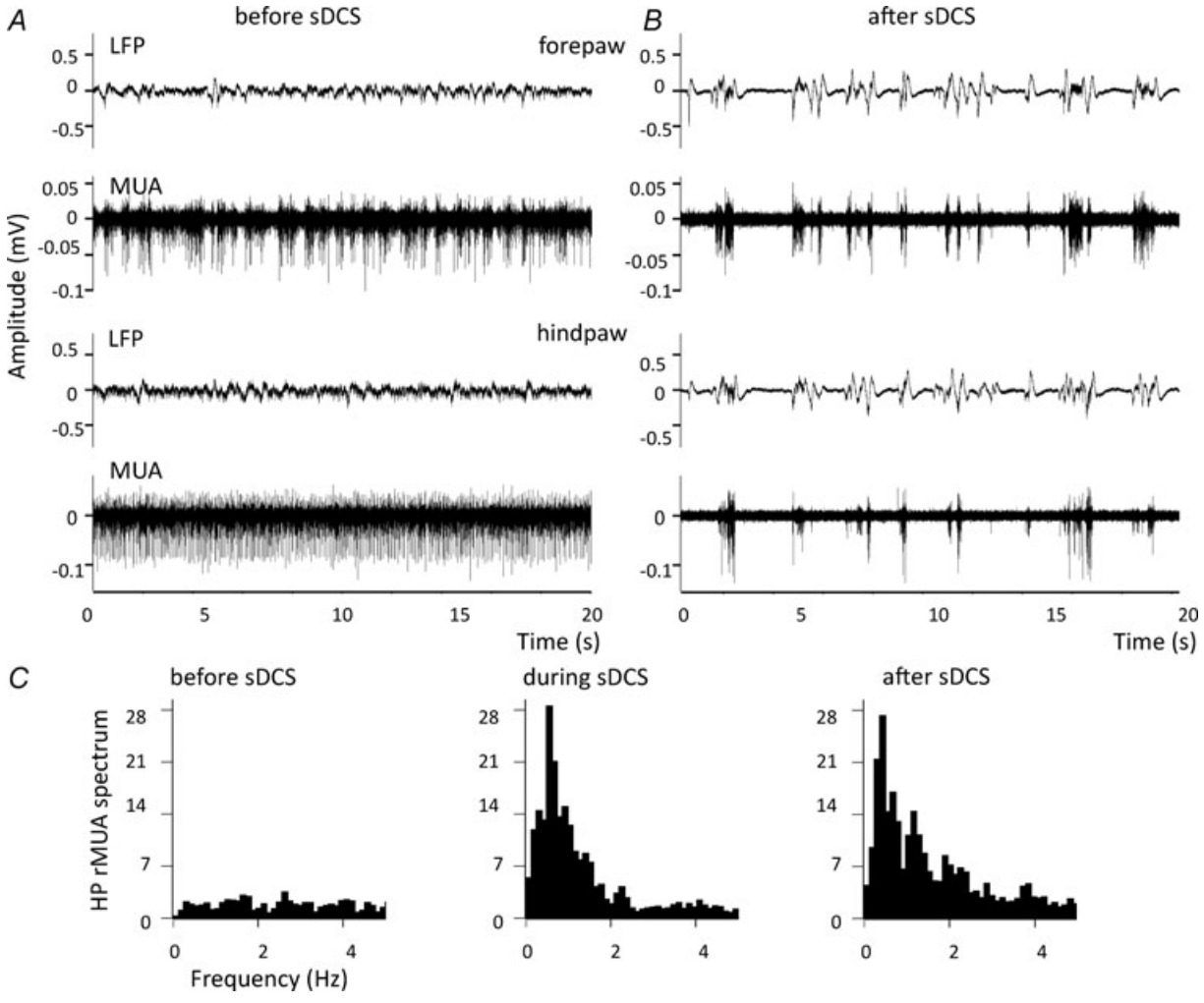


Figure 4. Effects of cathodal sDCS on cortical spontaneous activity
A and *B*, example of spontaneous activity in a representative animal that was initially kept at a low level of anaesthesia. Local field potential (LFP) and multi-unit activity (MUA) recorded from forepaw cortex (on the top) and hindpaw cortex (on the bottom) before (*A*) and after (*B*) sDCS. *C*, power spectrum of the rectified MUA (rMUA) recording from the hindpaw cortex before, during and after sDCS, corresponding to the spontaneous activity shown in *A* and *B*. In this animal, the effect of cathodal sDCS was the opposite of the effect of anodal sDCS shown in Fig. 3.

whereas cathodal sDCS did not produce any significant change independently of the level of anaesthesia (two-way ANOVA, TIME: $P > 0.30$; Table 3). These modest changes likely reflected a more complex dependence between spontaneous activity and evoked responses. This dependence was clearly evident with anodal sDCS (Fig. 5): in the animals that did not desynchronize ($n = 16$), the amplitudes of LFP responses increased (two-way ANOVA, TIME: $F_{(2,30)} = 13.26$, $P < 0.0001$; Table 3). Conversely, in the animals that did desynchronize ($n = 7$), the amplitudes of LFP responses decreased (two-way ANOVA, TIME: $F_{(2,12)} = 5.69$, $P = 0.0183$; Table 3).

Discussion

The present study shows that anodal sDCS at thoracic level increases the spontaneous activity and decreases the amplitude of LFP responses in the gracile nucleus, whereas cathodal sDCS produces the opposite effects. At the level of the primary somatosensory cortex, the changes in spontaneous activity induced by sDCS are consistent with the effects observed in the gracile nucleus, but the changes in cortical evoked responses are state dependent. Therefore, sDCS can modulate – in a polarity-specific manner – the supraspinal activity of the somatosensory system.

Methodological considerations

In order to understand how much of our results can be conceptually translated to humans, it is important to initially discuss with some detail the advantages and possible limitations of our animal model.

First, we performed our experiments under urethane anaesthesia, which provides stable recording conditions for relatively long periods of time. The level of anaesthesia – and more in general the state of arousal – can dramatically affect the spontaneous activity in the entire somatosensory system, from the gracile nucleus (Fernández de Sevilla *et al.* 2006), to the thalamus (Friedberg *et al.* 1999; Aguilar and Castro-Alamancos, 2005; Bezdudnaya *et al.* 2006), up the cortex (Steriade, 1997; Erchova *et al.* 2002). We therefore expect the effects of sDCS to be critically dependent on – and to interact with – the level of arousal, particularly when monitored at cortical level. In our case, the majority of the changes induced by anodal sDCS reached significance during the stimulation, whereas the majority of the changes induced by cathodal stimulation reached significance only after the end of the stimulation. This was likely to be due to a floor effect, because in our deep anaesthetized conditions the somatosensory system was already in a deactivated state. From this deactivated state it was easier to produce activation (through anodal sDCS) than additional deactivation (through cathodal sDCS). In fact, in the experiments performed under lighter anaesthesia the changes induced by cathodal sDCS did reach significance during the stimulation. Further investigations on the supraspinal effects of sDCS should thus carefully consider this interdependence with the level of arousal.

Second, we did not systematically investigate the duration of sDCS after-effects, which would be important to establish the potential of the technique to induce long-term changes. Nonetheless, after sDCS we did report significant changes in the evoked responses to electrical stimuli that were delivered 5 min after the end of sDCS (lower intensity) and more than 10 min after the end of sDCS (higher intensity). Therefore, at least some effects of sDCS lasted more than 10 min after the end of the stimulation. Importantly, these after-effects were obtained with 15 min of sDCS, whereas

Table 3. Hindpaw somatosensory cortex – evoked activity

	Anodal desynch. ($n = 7$)			Anodal no desynch. ($n = 16$)		
	Pre	DC	Post	Pre	DC	Post
Low-intensity stimuli	0.21 ± 0.21	**0.15 ± 0.13**	**0.15 ± 0.13**	0.21 ± 0.16	**0.24 ± 0.18**	**0.27 ± 0.16**
High-intensity stimuli	0.29 ± 0.23	**0.23 ± 0.14** $P = 0.0303$	**0.24 ± 0.15** $P = 0.0187$	0.36 ± 0.27	**0.40 ± 0.27** $P = 0.0435$	**0.42 ± 0.24** $P < 0.0001$
	Cathodal ($n = 16$)			**Cathodal lighter anaesth.** ($n = 8$)		
	Pre	DC	**Post**	**Pre**	**DC**	**Post**
Low-intensity stimuli	0.16 ± 0.11	0.18 ± 0.13	0.15 ± 0.11	0.28 ± 0.20	0.29 ± 0.18	0.23 ± 0.20
High-intensity stimuli	0.36 ± 0.28	0.35 ± 0.31	0.37 ± 0.32	0.43 ± 0.38	0.41 ± 0.36	0.40 ± 0.39

Details same as in Table 1.

no after-effects were reported in a recent study with 3 min of sDCS with a different experimental protocol in mice (Ahmed, 2011). This dependence of the duration of sDCS after-effects on the duration of the stimulation is consistent with the results obtained with transcranial DCS in humans (Nitsche & Paulus, 2001). Nonetheless, further investigations will be required to characterize the exact relation between the duration of sDCS and the duration of its after-effects.

Third, we delivered sDCS at a relatively high intensity – within safety limits (Liebetanz *et al.* 2009; see Methods) – using an invasive orthogonal arrangement (stimulating electrodes placed epidurally on the dorsal spinal cord and referenced to the abdomen). The rationale for these choices was to maximize the current focus in the spinal cord, in order to be able to observe relatively large effects at supraspinal levels. Importantly, none of the choices made here (arrangement, intensity, invasivity) is implied to be optimal for clinical purposes, and safety issues should be properly considered in the translation to human applications. It is indeed unlikely that current and charge densities of the order of magnitude used here could be obtained in the spinal cord of human subjects with cutaneous stimulation.

Finally, the local effects of polarizing currents in the spinal cord are very complex, depending not only on the polarity, duration and intensity of the current, but also on the types of neural elements involved (cell bodies and fibres), on the distance from the stimulating electrodes, and on the geometry and anatomy of the tissues involved between the electrodes (Creutzfeldt *et al.* 1962; Eccles *et al.* 1962; Basser & Roth, 2000; Iles, 2005; Manola *et al.* 2007; Rattay & Wenger, 2010). For example, anodal sDCS seems to be able to both excite and inhibit spinal circuits depending upon the pathway assessed (Winkler *et al.* 2010; Cogiamanian *et al.* 2011; Ahmed, 2011). In our animal

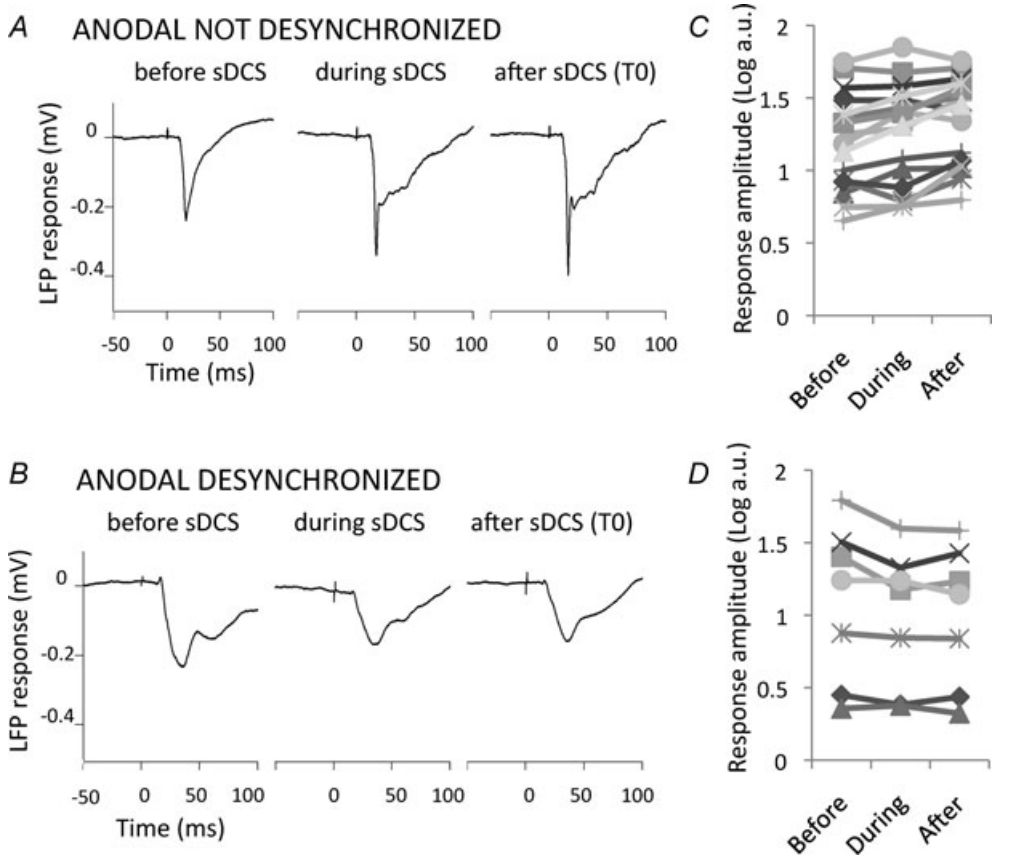


Figure 5. Effects of anodal sDCS on cortical evoked responses
A and *B*, LFP responses of the hindpaw somatosensory cortex to hindpaw stimuli delivered at low intensity (0.6 mA), before, during and after anodal sDCS (average of 100 stimuli, stimulus onset is indicated as time 0 on the *x*-axis.), in a representative animal that did not desynchronize (*A*) and in a representative animal that did desynchronize (*B*). *C* and *D*, all data representing the amplitude of cortical LFP responses to low intensity stimuli before, during and after anodal sDCS in animals that did not desynchonize (*C*) and in animals that did desynchonize (*D*). Log arbitrary units (a.u.) correspond to base-10 logarithms of tenths of microvolts (i.e. 2 log a.u. = 1 mV). If anodal sDCS did not desynchronized the cortical spontaneous activity, cortical evoked responses increased. In constrast, if anodal sDCS desynchronized the cortical spontaneous activity, cortical evoked responses decreased.

model, sDCS probably modulated both ascending and descending pathways, but the complexity of the possible spinal mechanisms of sDCS is beyond the scope of the present work. In the remaining discussion we will focus on the supraspinal effects of sDCS uncovered by our experiments.

Effects of sDCS on the activity of the gracile nucleus

Anodal sDCS increased the spontaneous activity of the gracile nucleus while decreasing its LFP responses to somatosensory stimuli, and cathodal sDCS did the opposite. These observations are consistent with the results obtained in humans by Cogiamanian *et al.* (2008), who reported that anodal sDCS decreased and cathodal sDCS tended to increase the amplitude of somatosensory evoked potentials at brainstem level (P30) – the equivalent of our gracile nucleus. Furthermore, our additional observation that decreased LFP responses were associated with increased spontaneous activity (and vice versa) are phenomenologically similar to the results recently obtained by Ahmed (2011), who showed that anodal trans-spinal DCS increased the spontaneous activity of the tibial nerve while decreasing the magnitude of cortically elicited muscle contractions in mice. In our experiments, several mechanisms could complementarily explain the inverse relationship between gracile spontaneous activity and evoked responses, including pre-synaptic inhibition (Willis, 2006), synaptic depression (Nuñez & Buño, 2001), and shunting inhibition (Mitchell & Silver, 2003). From a post-synaptic perspective, the simpler explanation for our results is that with anodal sDCS gracile neurons were probably responding to the stimuli from a more depolarized membrane potential, consequently displaying smaller EPSPs compared to the same response before sDCS; with cathodal sDCS, on the contrary, neurons were probably responding to the stimuli from a more hyperpolarized membrane potential, consequently displaying larger EPSPs compared to the same response before sDCS (Canedo & Aguilar, 2000; Nuñez & Buño, 2001). Overall, even though the spinal mechanisms of sDCS are likely to be complex, the postsynaptic supraspinal effects of sDCS in our animal model are fairly simple: anodal sDCS increases while cathodal sDCS decreases the ongoing activity of the gracile nucleus.

Effects of sDCS on somatosensory cortex

The main effects of sDCS measured at cortical level were consistent with what we observed in the gracile nucleus: anodal sDCS increased cortical spontaneous activity while cathodal decreased it. These changes in cortical spontaneous activity produced state-dependent changes in the cortical evoked responses. In particular, the effects induced by anodal sDCS suggest an intriguing threshold behaviour. On the one hand, if the increased spontaneous activity induced by sDCS was not sufficient to disrupt the state of cortical slow-wave activity, then cortical evoked responses increased. This direct relation between spontaneous activity and evoked responses is consistent with the stochastic resonance observed in the cat somatosensory system (Manjarrez *et al.* 2003) – with anodal sDCS being the source of 'noise'. On the other hand, if the increased spontaneous activity was sufficient to change the state of cortical slow-wave activity into a desynchronized state, then cortical evoked responses decreased. This observation is consistent with the smaller responses typically observed in active states compared to silent states in the rat somatosensory system (Petersen *et al.* 2003; Hirata & Castro-Alamancos, 2011). We did not observe an opposite threshold behaviour with cathodal sDCS, possibly because the increased evoked responses expected in the transition from the more activated/desynchronized state to slow-wave activity (Aguilar *et al.* 2010) were compensated by the greater hyperpolarization of gracile cells. In fact, when neurons respond from a more hyperpolarized membrane potential, EPSPs are larger but they also typically produce fewer action potentials. We cannot exclude other mechanisms related to possible effects of cathodal sDCS on spino-thalamic activity, which we did not observe. Besides these physiological implications, from a more practical perspective these results show the limitations of studying changes in cortical evoked responses without monitoring the corresponding changes in cortical spontaneous activity.

Overall, the supraspinal activation induced by anodal sDCS resembles the increased arousal induced by increasing muscle afferent activity (Motokisawa & Fujimori, 1964; Mori *et al.* 1973; Oshima *et al.* 1981; Lanier *et al.* 1986). Vice versa, the supraspinal deactivation induced by cathodal sDCS is conceptually similar to the decreased arousal induced by decreasing muscle afferent activity (Forbes *et al.* 1979; Schwartz *et al.* 1992), by spinal anaesthesia (Inagaki *et al.* 1994; Tverskoy *et al.* 1994; Hodgson *et al.* 1999; Antognini *et al.* 2000; Aguilar *et al.* 2010) or by spinal cord injury (Tran *et al.* 2004; Boord *et al.* 2008; Aguilar *et al.* 2010). The present work thus confirms that the spinal cord plays a critical role in regulating the state of the brain (Manjarrez *et al.* 2002; Aguilar *et al.* 2010).

Clinical significance

The possibility to modulate afferent somatosensory signals with sDCS is particularly appealing from a clinical perspective. The most promising application of sDCS is probably the treatment of pain, for which epidural spinal stimulation has been used for decades (Shealy *et al.* 1967;

Kumar *et al.* 2007). The potential advantages of sDCS over classical high-frequency spinal stimulation are twofold: its effects are polarity specific and it can be delivered transcutaneously (Cogiamanian *et al.* 2008; Winkler *et al.* 2010; Cogiamanian *et al.* 2011; Truini *et al.* 2011). Furthermore, sDCS could be easily combined with transcranial direct current stimulation (tDCS), which is being investigated for the treatment of neuropathic pain with encouraging results (Fregni *et al.* 2006; Soler *et al.* 2010), offering an attractive and cost-effective methodology to neuromodulate the sensorimotor system both top-down and bottom-up.

Besides pain, sDCS could also be useful for the treatment of other pathologies. For example, a recent study showed that high-frequency spinal stimulation restores locomotion in mouse and rat models of Parkinson's disease (Fuentes *et al.* 2009), inducing a supraspinal cortical activation that resembles the one we observed with anodal sDCS. Intriguingly, similar widespread cortical activation is one of the possible mechanisms by which deep brain stimulation (DBS) of the subthalamic nucleus (STN) might exert its well-known clinical effects (Jech *et al.* 2006; Li *et al.* 2007; Dejean *et al.* 2009; Kuriakose *et al.* 2010). The somewhat discouraging results obtained with high-frequency spinal stimulation in patients with Parkinson's disease (Thevathasan *et al.* 2010) might be due to the inability of this technique to efficiently activate supraspinal structures in humans compared to the animal models. Our study offers a rationale for testing whether sDCS might prove more efficient in activating supraspinal structures compared to classical high-frequency spinal stimulation.

We conclude that sDCS can modulate in a polarity-specific manner the supraspinal activity of the somatosensory system, offering a versatile bottom-up neuromodulation technique that could become useful in a number of clinical applications.

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Author contributions

J.A., A.O., A.P. & G.F. designed the study. F.P., R.D. & J.A. performed the experiments. F.P. & G.F. analyzed the data. G.F. wrote the paper. J.A. & G.F. supervised the study. All authors contributed to the scientific discussion, edited the paper and approved the final version for publication. The work was done in the Hospital Nacional de Parapléjicos, Toledo (Spain).

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