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Electrical stimulation of dorsal root entry zone attenuates wide-dynamic range neuronal activity in rats

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Abstract

Objectives—Recent clinical studies suggest that neurostimulation at the dorsal root entry zone (DREZ) may alleviate neuropathic pain. However, the mechanisms of action for this therapeutic effect are unclear. Here, we examined whether DREZ stimulation inhibits spinal wide-dynamic-range (WDR) neuronal activity in nerve-injured rats.

Materials and Methods—We conducted in vivo extracellular single-unit recordings of WDR neurons in rats after an L5 spinal nerve ligation (SNL) or sham surgery. We set bipolar electrical stimulation (50 Hz, 0.2 ms, 5 min) of the DREZ at the intensity that activated only A α / β -fibers by measuring the lowest current at which DREZ stimulation evoked a peak antidromic sciatic A α / β -compound action potential without inducing an A δ /C-compound action potential (i.e., Ab1).

Results—The elevated spontaneous activity rate of WDR neurons in SNL rats [n=25; data combined from day 14–16 (n = 15) and day 45–75 post-SNL groups (n=10)] was significantly

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Authorship Statement:

Drs. Yang, Zhang, Xu, Tiwari and He performed animal surgery, electrophysiological recording and data analysis. Drs. Wang, Dong, Vera-Portocarrero, Wacnik and Dong contributed to the experimental design and the interpretation of the results. Drs. Raja and Guan designed the experiments and oversaw the overall execution of the project. Drs. Yang and Guan prepared the manuscript.

Conflict of Interest Disclosures:

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decreased from the pre-stimulation level ($p<0.01$) at 0–15 min and 30–45 min post-stimulation. In both sham-operated ($n=8$) and nerve-injured rats, DREZ stimulation attenuated the C-component, but not A-component, of the WDR neuronal response to graded intracutaneous electrical stimuli (0.1–10 mA, 2 ms) applied to the skin receptive field. Further, DREZ stimulation blocked windup (a short form of neuronal sensitization) to repetitive noxious stimuli (0.5 Hz) at 0–15 min in all groups ($p<0.05$).

Conclusions—Attenuation of WDR neuronal activity may contribute to DREZ stimulation-induced analgesia. This finding supports the notion that DREZ may be a useful target for neuromodulatory control of pain.

Keywords

Neurostimulation; dorsal root entry zone; wide-dynamic-range neurons; neuropathic pain; rats

INTRODUCTION

In patients with nerve injury, damaged sensory afferents enter the central nervous system (CNS) at the posterolateral aspect of the spinal cord through the dorsal root entry zone (DREZ). Accordingly, lesions of the DREZ (i.e., DREZotomy), such as by thermal coagulation or laser, have been used clinically to treat deafferentation pain, including pain caused by traumatic plexus avulsions (1, 2). DREZotomy may block the peripheral noxious inputs from reaching the CNS and ablate hyperactive spontaneously firing neurons in the superficial dorsal horn and thereby alleviate severe, chronic pain syndromes that are refractory to pharmacotherapy (1–3). Although DREZotomy is effective, the procedure is highly invasive, and the risk of complications is significant (e.g., cerebrospinal fluid leak, inadvertent motor and sensory deficits). The lesion is also irreversible, and some patients experience a relapse of pain after surgery. Compared to neuroablation, electrical neurostimulation therapies, such as spinal cord stimulation (SCS), are nondestructive, less invasive, adjustable, reversible, and hence more advantageous for chronic pain treatment (4, 5). In particular, SCS provides an important alternative strategy for treating neuropathic pain when other therapies have failed or when side effects associated with these therapies substantially impair a patient's quality of life (6).

During conventional SCS, fibers that mediate primarily non-noxious inputs ($A\beta$ -fibers) are most likely activated. Therefore, the treatment is associated with only mild and tolerable paresthesia in patients. Traditional SCS produces pain inhibition primarily by activating the dorsal column (5, 7). Intriguingly, emerging evidence suggests that the DREZ may also represent a useful target for electrical neurostimulation to treat pain. For example, DREZ stimulation alleviated chronic deafferentation pain in 50% of patients (8). Additionally, a case report showed that epidural stimulation of the DREZ of the affected dermatome reduced demyelinating lesion-induced central pain (9). Nevertheless, due to the small sample size in these studies, the clinical efficacy of DREZ stimulation to inhibit neuropathic pain remains to be established. Moreover, the mechanism for DREZ stimulation-induced pain inhibition is unclear.

Peripheral nerve injury leads to dynamic changes in dorsal horn neuronal excitability, which may partially underlie the chronic disabling pain. Wide-dynamic-range (WDR) neurons are important to nociceptive processing and exhibit increased spontaneous firing and enhanced response to peripheral stimuli after nerve injury. Some high-threshold, nociceptive-specific dorsal horn neurons may show phenotypic changes and display WDR neuronal properties under pathological pain conditions (10). WDR neurons also display a phenomenon known as “windup,” a progressive increase in responses to repeated noxious inputs of the same stimulus intensity (11, 12). Windup represents a useful cellular model and an important experimental tool to study mechanisms that might trigger the development of a persistent pain and hyperalgesic states (13–15). In this study, our goal was to determine whether DREZ stimulation using SCS-like parameters inhibits WDR neuronal activity in rats after an L5 spinal nerve ligation (SNL). We further compared the spontaneous activity and evoked responses to graded electrical stimuli and windup-inducing stimulation in WDR neurons before and after DREZ stimulation.

MATERIAL AND METHODS

The Johns Hopkins University Animal Care and Use Committee approved all procedures as consistent with the National Institutes of Health Guide for the Use of Experimental Animals. All animals were euthanized at the end of the experiment by an intraperitoneal (i.p) injection of sodium pentobarbital (100–300 mg). To minimize experimenter bias, the investigator who performed the behavioral tests was blinded to the treatment conditions.

L5 Spinal Nerve Ligation

The L5 spinal nerve of adult male Sprague-Dawley rats (300–400 g, Harlan Bioproducts for Science, Indianapolis, IN) was ligated as described previously (16). Briefly, the animals were anesthetized with isoflurane (2%, Abbott Laboratories, North Chicago, IL). The left L5 spinal nerve was tightly ligated with a 6-0 silk suture and cut distally, with care being taken not to pull the nerve or touch the L4 spinal nerve. In a control, sham-operated group, the surgical procedure was identical, except that the left L5 spinal nerve was not ligated or transected. Animals were administered prophylactic antibiotics (Bicillin, 30,000 units/kg, Wyeth Laboratories, PA) by subcutaneous injection before the procedure, and the wound site was covered with antibiotic ointment after surgery. Antibiotics were not routinely administered after surgery. The animals were monitored after surgery for signs of wound infection, inadequate food and water intake, or weight loss until the surgical site was healed. Animals were not administered analgesia after surgery because analgesic agents may influence natural physiological pain processing and the development of neuronal sensitization after injury.

In light of possible differences in WDR neuronal excitability changes associated with “allodynic” and “non-allodynic” animals, and because SCS only normalizes the WDR neuronal hyperexcitability in allodynic rats after nerve injury (17), we excluded SNL rats from the electrophysiological studies if they did not show mechanical hypersensitivity (paw withdrawal threshold decrease of >50% from the pre-injury level at day 5 post-injury and 2–

3 days before the electrophysiological recording dates) as shown in previous studies (16, 18, 19).

Tracheotomy and mechanical ventilation

Animals were placed under general anesthesia for nonsurvival neurophysiological studies. The rats were initially anesthetized with pentobarbital (45–50 mg/kg, i.p.). Then a tracheotomy was performed and mechanical ventilation (Kent Scientific Corporation, Litchfield, CT) at 50–70 cycles/min with inspiratory pressures of 10–14 cm H₂O was initiated. During the neurophysiological experiments, the anesthesia was maintained with 1.5% isoflurane, and animals were paralyzed with pancuronium bromide (1–2 mg/kg, i.p., Elkins-Sinn Inc., Cherry Hill, NJ) via intermittent injections given as needed (1 mg/kg/h, i.p.). Sufficient depth of anesthesia was judged from areflexia to sensory stimuli (e.g., no withdrawal reflexes, corneal reflex) when rats were in the unparalyzed state and by the absence of gross fluctuations of heart rate (300–350 beats per min) during paralysis. Core body temperature was kept in the normal range (36.0–37.0°C).

Recording of sciatic compound action potentials evoked by DREZ stimulation

To standardize the DREZ stimulus intensity and selectively activate A α / β -fibers, we applied graded electrical stimulation (0.01 mA–1.0 mA, 0.2 ms) to the DREZ and recorded compound action potentials evoked at the sciatic nerve. Similar techniques have been used in previous studies (16, 20, 21). The left sciatic nerve and its branches were exposed and dissected from surrounding tissue. A monopolar silver hook electrode was placed on the sciatic nerve at mid-thigh level for recording compound action potentials (16). The reference electrode was placed in the nearby muscle. DREZ stimulation was applied through two tungsten needle electrodes (insulated except for the most distal 0.3 mm) inserted shallowly into the left DREZ at L4 spinal level (~200 μ m below the surface). The DREZ and the respective dorsal root were identified visually under the surgical microscope. Both stimulating and recording areas were covered with mineral oil.

Spinal dorsal horn recordings

The experimental setup and procedure are illustrated as a schematic diagram in Fig. 1A. Extracellular recordings of single dorsal horn neuron activity were obtained with microelectrodes as described previously (16). Briefly, analog data were collected with a real-time, computer-based data acquisition and processing system (DAPSYS 6; Brian Turnquist, the Johns Hopkins University, Baltimore, MD). WDR cells were identified by their characteristic responses (11, 22). Mechanical search stimuli ranged from mild to noxious and consisted of stroking the plantar skin with a cotton swab and mild pinching of the skin with fingers. The experimenter applied a series of mechanical test stimuli: brushing with a small camel-hair brush and indentation of the plantar skin with von Frey monofilaments (0.09–12.0 g, Stoelting Co., Wood Dale, IL) in ascending order to the cutaneous receptive fields of the neuron. Neurons that responded in a graded fashion with increasing firing rates to increasing stimulus intensity from non-noxious to noxious texture were classified as WDR cells. In addition, the WDR neuronal response to a suprathreshold electrical stimulus consists of an early A-fiber component (0–75 ms) and a later C-fiber component (75–500 ms) (16). To avoid confounding variables in data interpretation from

different neurophysiological properties of WDR cells in superficial vs. deep dorsal horn and in injured vs. uninjured spinal segments (11, 23), we examined WDR neurons located at deep laminae (III–V, 400–1200 μm below the dorsal surface) in the ipsilateral uninjured L4 spinal segment (i.e., the same segment receiving DREZ stimulation). The spinal segment was identified by the respective dorsal root and DREZ.

Experimental design

Using a protocol similar to that of previous studies (16, 20, 24), we stimulated the DREZ using SCS-like parameters (50 Hz, 5 min, 0.2 ms). Then we examined the effects of DREZ stimulation on spontaneous activity, on stimulus-response (S-R) functions of A- and C-fiber components to graded intracutaneous electrical stimuli (0.1–10 mA, 2.0 ms, 15-s interval, delivered to the receptive field), and on windup response to a train of 16 electrical pulses (0.5-Hz, supra-C-fiber threshold) at 0–15 min and 30–45 min. In the windup test, 12 pulses at 0.1 Hz (which does not induce windup) were delivered at 30 s after 0.5-Hz stimulation. Because 0.1 Hz stimulation rarely induces windup under physiological conditions, it was used as a negative control for 0.5 Hz stimulation.

The spontaneous activity of WDR neurons was recorded for 1 min. After each recording of spontaneous activity, we examined the S-R functions and then performed a windup test (10-min interval). The same test module was repeated at 0–15 min and 30–45 min after the DREZ stimulation. The electrical thresholds for activation of the A- and C-components were defined as the lowest stimulus current (mA) to evoke an action potential firing within the range of the A- and C-fiber latencies, respectively. If the threshold after DREZ stimulation was greater than the maximum stimulator power (10 mA), the value of 15 mA was assigned as the cut-off threshold. Studies were conducted in sham-operated rats (n=8) and in rats at day 14–16 (the maintenance phase, n=15) and day 45–75 (the recovery phase, n=10) after SNL (16, 25). Sham-operated rats at different postoperative time points (day 14–16: n=4, day 45–75: n=4) were combined for analysis.

Data analysis

The number of action potentials in the C-component evoked by each stimulus in the train was used to plot windup curves/functions against the stimulation number of the train. Absolute windup = (total number of action potentials in C fiber-component evoked by the 0.5-Hz train – 16 $\times$ input). Input was defined as the number of action potentials evoked by the first stimulus of the 0.5-Hz train. A one-way repeated measures ANOVA was used to compare the spontaneous activity, the total responses to graded electrical stimuli, and absolute windup between the pre- and post-conditioning stimulation conditions. The Tukey honestly significant difference post-hoc test was used to compare specific data points. The C-thresholds to graded electrical stimulation are presented as medians and were analyzed by nonparametric ANOVA (Friedman and Kruskal-Wallis); the Wilcoxon Matched Pairs Test was used as the post-hoc test. STATISTICA 6.0 software (StatSoft, Inc., Tulsa, OK, USA) was used to conduct all statistical analyses. Two-tailed tests were performed, and data are expressed as mean $\pm$ SEM.

RESULTS

Characterization of the antidromic sciatic compound action potential evoked by stimulation of the DREZ

At supra-threshold intensity, the sciatic compound action potential evoked by DREZ stimulation often revealed two distinct groups of waves (Fig. 1B). Different compound action potential waveforms corresponding to A α / β - and A δ -fiber activation were distinguished on the basis of the activation threshold and the conduction velocity (CV). The fast component corresponds to the A α / β -fiber activation (CV: 14.3 ± 0.5 to 45.2 ± 2.7 m/s, mean $\pm$ SEM). The slower component, referred to as the A δ -compound action potential, usually had a smaller amplitude than the fast A α / β component and could be distinguished by a higher threshold and slower CV (8.6 ± 0.8 to 13.6 ± 0.4 m/s). These CVs are comparable to those reported previously (16, 26). We determined online the A α / β -plateau intensity (Ab1, approximately the lowest intensity to evoke a peak A α / β -compound action potential without inducing an A δ /C-fiber component, Fig. 1B), which was later used for the DREZ stimulus. The Ab1 values were comparable between different experimental groups (Fig. 1C).

Stimulation of DREZ at 50 Hz attenuated the elevated spontaneous discharges of WDR neurons in SNL rats

Before DREZ stimulation, the spontaneous activity rate (action potentials/min) of WDR neurons was significantly higher in SNL rats [data were combined from day 14–16 ($n = 15$) and day 45–75 post-SNL groups ($n=10$)] than in sham-operated rats ($n=8$, Fig. 2). At 0–15 min and 30–45 min after DREZ stimulation, the spontaneous activity rates were significantly decreased from the pre-stimulation baseline in SNL rats (Fig. 2A, B).

Stimulation of the DREZ decreased the C-component of the WDR neuronal response to graded intracutaneous electrical stimuli in both sham-operated and nerve-injured rats

DREZ stimulation did not affect the S-R functions of the A-component (Fig. 3A), but it depressed that of the C-component in both sham-operated and SNL rats (Fig. 3B). The total number of action potentials to graded intracutaneous test stimuli (0.1–10 mA, 2 ms) was significantly decreased in the C-component at 0–15 min post-stimulation in rats at day 14–16 ($n = 15$) and day 45–75 post-SNL ($n = 10$), and at 30–45 min post-stimulation in sham-operated rats ($n=8$), as compared to the respective pre-stimulation baseline (Fig. 3C). The C-component remained significantly decreased at 30–45 min post-stimulation on days 14–16 post-SNL (Fig. 3C). The median threshold intensity for activation of the C-component was significantly increased from the pre-stimulation value in all groups (Fig. 3D). The number of action potentials in the A-component was unchanged.

Stimulation of the DREZ inhibited windup

Before DREZ stimulation, windup was induced in both sham-operated and SNL rats by 0.5-Hz stimulation, but not by 0.1-Hz stimulation applied 30 s later. At 0–15 min post-stimulation, windup functions to 0.5 Hz stimulation were depressed in all groups (Fig. 4A–C). We further calculated the absolute windup [(total number of action potentials in C fiber-

component evoked by the 0.5-Hz train) – ($16 \times$ the number of action potentials evoked by the first stimulus of the 0.5-Hz train)], which was significantly decreased from the respective pre-stimulation value in each group at 0–15 min post-stimulation (Fig. 4D).

DISCUSSION

Inhibition of WDR neuronal activity by dorsal column stimulation may partially contribute to SCS-induced analgesia (16, 17, 20, 27). The current study shows that DREZ stimulation with SCS-like parameters also inhibits WDR neurons as dorsal column stimulation does. The findings suggest that DREZ may represent a useful target of spinal segmental neuromodulation for pain control.

Spontaneous and ongoing pain are important components of neuropathic pain that are attenuated by SCS (28–30). The ectopic discharges generated from a neuroma and increased spontaneous discharges in peripheral and dorsal horn neurons may all contribute to central sensitization and partially underlie ongoing pain and prolongation of neuropathic pain (31–33). Further, the increased spontaneous firing in WDR neurons after nerve injury is temporally related to the development of mechanical allodynia in animal models of neuropathic pain. Our previous study showed that dorsal column stimulation with SCS-like parameters inhibited the increased spontaneous activity in WDR neurons of SNL rats (16). Here, we provide novel evidence that such an effect can also be achieved by stimulating the DREZ at an intensity that primarily activates large afferent fibers. The presumed rationale for DREZotomy is also to eliminate ectopic discharges that reach the spinal cord and induce hyperactivity of dorsal horn neurons. However, mechanisms of DREZ ablation are probably different from those of DREZ stimulation that uses SCS-like parameters, as the latter may involve spinal network mechanisms (e.g., activation of inhibitory dorsal horn neurons) (5, 7, 30). It is possible that high-frequency DREZ stimulation might inhibit pain by changing conduction properties or inducing conduction block of afferent fibers (34) and thereby partially mimic the effect of DREZ lesions. Yet, support for this hypothesis would require additional study.

In experimental animals, repetitive electrical activation of afferent C-fibers transiently enhances the excitability of WDR neurons, a phenomenon called windup (12, 15). Windup represents a form of short-term neuronal sensitization that may contribute to the development of persistent pain (11, 12, 15). Importantly, a windup-like phenomenon also occurs in humans during natural stimulation of C-fibers (35), such as by heat stimuli and repeated noxious mechanical stimuli (e.g., deep pressure or strong pinch) (36–38). The reduction of windup after DREZ stimulation suggests that it has an inhibitory effect on the progress of neuronal sensitization to repetitive noxious inputs.

Similar to previous observations with dorsal column stimulation (16, 17), the duration of WDR neuronal inhibition exceeded the short DREZ stimulation period. As in SCS-induced analgesia, this prolonged inhibition may result from the slow release and sustained actions of inhibitory neurotransmitters and changes in gene expression (19, 39). Also like SCS, effects of DREZ stimulation may be mediated by a complex set of interactions at several levels of the nervous system and could possibly trigger descending pain-controlling

pathways from supraspinal structures. Regardless, DREZ stimulation may have other neurophysiologic mechanisms of action that differ from those of conventional SCS. Whereas SCS primarily activates the dorsal column structure at several levels rostral to the affected spinal segment, DREZ stimulation may modulate both primary afferent fibers entering spinal cord and other segmental neuronal substrates in superficial dorsal horn that are important to nociceptive transmission, owing to their close proximity to the stimulating electrodes. For example, the electrical field from DREZ stimulation may directly affect tract of Lissauer functions and superficial dorsal horn neurons (e.g., projection neurons) (40). It will be important in the future to correlate the electrophysiological findings with the putative pain inhibitory effect of DREZ stimulation in animal behavioral studies. However, currently, no implantable DREZ electrode is suitable for behavioral tests in awake rats.

Minimally invasive electrical neurostimulation therapies have been used to treat chronic pain conditions. A small number of studies have shown that stimulation with standard SCS electrodes placed laterally over the DREZ may be effective for pain treatment. After extensive, but unsuccessful, trials of medical pharmacotherapy, half of patients with chronic pain syndromes obtained good long-term benefit after DREZ stimulation (8, 9). However, insights regarding site of action, neuronal circuitry, and cellular mechanisms through which DREZ stimulation induces pain inhibition may be difficult to obtain in patients. Our current findings suggest that WDR neurons may be an important component and effector within the neuronal circuitry that underlies the pain-relieving mechanisms of DREZ stimulation. Thus, our study provides some information about potential neuronal mechanisms and targets that may support clinical application of DREZ stimulation for pain treatment. Yet, applying DREZ stimulation in clinical pain management remains challenging. Although lateral placement of standard SCS leads over the DREZ was effective for alleviating certain pain conditions (8, 9), some problems are associated with this application. Because the DREZ is highly sensitive to electrical stimulation, changes in lead conductance/position associated with movements may cause unpleasant paresthesia. Accordingly, specific electrodes and stimulation paradigms would need to be developed to optimize DREZ stimulation. In addition, dorsal root ganglion stimulation, which may be more resistant than DREZ stimulation to movement artifacts, is another promising treatment modality for pain control (41, 42).

In summary, our findings suggest that DREZ that receives input from a painful area may be an effective target for neuromodulatory control of neuropathic pain. Yet, the optimal parameters and the respective mechanisms for DREZ stimulation to inhibit different pathological pain conditions remain to be examined. Our *in vivo* cellular model and experimental approach may be applicable to future preclinical studies that examine the optimum configuration and biological basis for the therapeutic effects of DREZ stimulation.

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Figure 1.

(A) A schematic diagram shows the experimental paradigm used in the neurophysiologic studies. The antidromic compound action potentials evoked by bipolar electrical stimulation (0.2 ms, 0.01–3.0 mA) at the dorsal root entry zone (DREZ) at L4 spinal level were recorded at the sciatic nerve with a monopolar recording electrode. Extracellular recordings of dorsal horn neurons were obtained with a microelectrode inserted within the same spinal segment. Intracutaneous electrical test stimuli were applied to the skin receptive field of the dorsal horn neuron. (B) Sciatic compound action potentials evoked by high intensity DREZ stimulation usually revealed two distinct groups of waves corresponding to Aα/β and Aδ fiber activation. The Aδ component is often hard to differentiate or missing. Ab0 = Aα/β-threshold; Ab1 = Aα/β-plateau, the lowest stimulus intensity that evokes a peak Aα/β component without inducing an Aδ component; Ad0 = Aδ-threshold. (C) The Ab1 for each experimental group was plotted. Data are expressed as mean + SEM in C.

Figure 2.

Stimulation of the dorsal root entry zone (DREZ) inhibited the spontaneous activity of WDR neurons in nerve-injured rats. **(A)** Peri-stimulus time histogram shows the spontaneous WDR neuronal activity before and 0–45 min after DREZ stimulation (5 min, 0.2 ms, Ab1, bin size: 2.0 s) in a rat after an L5 spinal nerve ligation (SNL). **(B)** The spontaneous activity rates were significantly higher in SNL rats [groups at day 14–16 ($n=15$) and day 45–75 ($n=10$) were combined] than in sham-operated rats ($n=8$) before DREZ stimulation. The spontaneous activity rates in SNL rats were significantly reduced at 0–15 min and 30–45 min after DREZ stimulation. Data are expressed as mean + SEM.

Figure 3. Dorsal root entry zone (DREZ) stimulation inhibited C-component of WDR neuronal response to graded intracutaneous stimuli. **(A–B)** The stimulus intensity–response (S–R) functions of the A- and C-component of WDR neuronal responses to graded electrical stimulation (0.1–10 mA, 2 ms) applied to the skin receptive field were plotted for each group (sham-operated: n=8; day14–16 post-injury: n=15; day 45–75 post-injury: n=10). **(C)** Stimulation of the ipsilateral L4 DREZ significantly attenuated the total number of action potentials (APs) in the C-components (right), but not in the A-component (left), of nerve-injured and sham-operated rats. **(D)** The threshold intensities for activation of the C-component were significantly increased after DREZ stimulation. Data are expressed as mean + SEM in A and B. The box & whisker plot in D shows the median intensity for activation of the C-component.

Figure 4.

Dorsal root entry zone (DREZ) stimulation inhibited windup response in WDR neurons. (**A–C**) C-component responses of WDR neurons to successive electrical stimuli applied to the skin receptive field at a frequency of 0.5 Hz (16 pulses, 2.0 ms, 1.5 x C-th) and then at 0.1 Hz (12 pulses, applied at 30 s after the cessation of 0.5 Hz stimulation) were plotted against the stimulation sequence number of each trial (error bars are omitted to improve the clarity). Before DREZ stimulation, windup was produced in all three groups during the first 0.5 Hz stimulation, but not during the following 0.1 Hz stimulation (sham-operated: n=8; 14–16 days: n=15; 45–75 days: n=10). DREZ stimulation attenuated windup response to 0.5 Hz stimulation in all three groups; the peak effect occurred at 0–15 min post-stimulation. (**D**) The absolute windup values for 0.5 Hz stimulation (total response during 0.5 Hz stimulation – 16 x input) were significantly decreased from the respective pre-stimulation values at 0–15 min post-stimulation in all three groups. Data are expressed as mean + SEM.