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Pulsed and Continous Radiofrequency Current Adjacent to the Cervical Dorsal Root Ganglion of the Rat Induces Late Cellular Activity in the Dorsal Horn

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Background: **Pulsed radiofrequency treatment has recently been described as a non-neurodestructive or minimally neurodestructive alternative to radiofrequency heat lesions. In clinical practice long-lasting results of pulsed radiofrequency treatment adjacent to the cervical dorsal root ganglion for the management of chronic radicular spinal pain have been reported without neurologic complications. However, the mode of action is unclear. An early (3 h) effect of pulsed radiofrequency as measured by an increase of c-Fos in the pain-processing neurons of the dorsal horn of rats has been described in the literature. This effect was not mediated by tissue heating. The authors investigated a possible late or long-term effect of three different radiofrequency modalities.**

Methods: **Cervical laminectomy was performed in 19 male Wistar rats. The cervical dorsal root ganglion was randomly exposed to one of the four interventions: sham, continuous radiofrequency current at 67 centigrades, or pulsed radiofrequency current for 120 s or 8 min. The animals were sacrificed and the spinal cord was prepared for c-Fos labeling 7 days after the intervention.**

Results: **The number of c-Fos immunoreactive cells in the dorsal horn was significantly increased in the three different radiofrequency modalities as compared with sham. No significant difference was demonstrated between the three active intervention groups.**

Conclusions: **The authors demonstrated a late neuronal activity in the dorsal horn after exposure of the cervical dorsal root ganglion to different radiofrequency modalities, which was not temperature dependent.**

CERVICOBRACHIALGIA can be defined as a radicular pain originating from the cervical spine radiating from the neck beyond the elbow into the upper limb with referral to a particular spinal segment. It is most commonly caused by an irritation of the segmental nerve as a result of cervical disc protrusion or spondylosis resulting in narrowing of the intervertebral foramen.[1–3] Radiofrequency heat lesions have been described as treatment for chronic cervical radicular spinal pain that is refractory to conservative therapy (pharmacological treatment, physiotherapy, transcutaneous electrical nerve stimulation).[4–6] The use of continuous radiofrequency treatment at 67°C adjacent to the dorsal root ganglion (DRG) for the management of cervicobrachial pain has been investigated in two randomized controlled trials[5,6]. Van Kleef *et al.*[5] compared the efficacy of radiofrequency at 67°C with sham intervention and found a significant reduction in pain intensity 8 weeks after the intervention. Slappendel *et al.*[6] found no difference in outcome when radiofrequency treatment with an electrode tip temperature of 40°C was used as compared with radiofrequency at 67°C.

The mode of action of radiofrequency was initially attributed to the thermocoagulation of nerve fibers,[7] but contradictory findings, notably that only transient sensory loss in the relevant dermatome is observed although the pain relief may be of much longer duration, gave rise to the hypothesis that temperature is not the only mechanism responsible for changes in pain transmission.[4] Moreover, the use of radiofrequency heat lesions adjacent to the DRG for the management of nonmalignant pain is becoming more controversial because of its potential neurodestructive nature. For that reason a nondestructive or minimally destructive technique would be more attractive.

A modified technique, pulsed radiofrequency (PRF)[8] treatment is developed whereby in 1 s two bursts of 20 ms each of an alternating current are delivered. The oscillating frequency of the alternating current is 500,000 Hz. During one cycle the "active" phase of 20 ms is followed by a silent period of 480 ms to allow for washout of the generated heat. The output is usually set at 45 volts, but if the electrode tip temperature exceeds 42°C, it is decreased to prevent cell damage. This temperature is selected based on the findings that necrosis in various soft tissue cell lines could only be induced by heating to greater than 43°C.[9,10] In clinical practice long-lasting effects after PRF treatment for both 120 s and 8 min has been reported in different indications.[8,11–19] In

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a recent clinical audit PRF treatment (120 s) adjacent to the cervical DRG in patients with chronic cervicobrachialgia or cervicogenic headache showed a short-term clinical success in 72% of the patients and a mean duration of pain relief of 9.2 months. The median duration of action determined by the Kaplan Meier curve, however, is only 3 months, indicating a wide variability in clinical response.[14] Because of the variability in outcome and the use of different treatment protocols in clinical practice, further research on the mode of action is required.[20]

Sluijter *et al.*,[8] suggested that the electric field rather than temperature induced changes in the nerve cells that were responsible for the pain relief observed in clinical practice. Exposure of cultured cells to an electric field is associated with up-regulation of the nonspecific intermediate-early gene marker, c-Fos, and also transcription of other, as yet unidentified, genes.[21] C-fos based functional anatomic mapping has been validated as a powerful technique to detect activated neurons.[22] Evidence is accumulating to suggest that changes in early gene expression within the nervous system signal long-term adaptation within particular neural pathways.[23]

In rats, exposure of the cervical DRG to PRF current showed an early significant increase in c-Fos-immunoreactive neurons in the superficial laminae I and II of the dorsal horn, 3 h after the procedure.[24] The late effect on the dorsal horn of PRF has not previously been studied. Also, although used routinely in the clinical setting, the effects of continuous radiofrequency (67°C) have not been investigated.

The aim of this study was to examine the late effect of different modes of radiofrequency current application (conventional radiofrequency 67°C, PRF during 120 s, and PRF during 8 min) adjacent to the cervical DRG in rats on the neuronal activity of the dorsal horn by identifying the inducible gene expression (c-Fos) 7 days after the intervention, a duration far beyond the normal window of c-Fos expression after an acute event.[22,23] This may enhance the understanding of the underlying mechanism of the reported clinical effect.

Materials and Methods

General Conditions

All studies were conducted following the ethical guidelines of the International Association for the Study of Pain[25] and approved by the Local Animal Care Ethics Committee, Maastricht, The Netherlands. Three-month-old male Wistar rats bred by Charles River Laboratories (Sulzfeld, Germany) (weight, 275–335 g) were used for the experiments. All animals were housed at the University of Maastricht facility for experimental animals in individual standard rodent cages with sawdust bedding and food and water *ad libitum*. The housing room was air conditioned with a 12 h:12 h light:dark cycle (lights on at 7:00 AM). Animals were checked daily for general condition and complications.

Surgical Procedure

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (Sanofi, Maassluis, The Netherlands) solved at 60 mg/ml and administered to the rats in a dose of 60 mg/kg.

The abdomen and cervical regions were shaved and the rat placed on an induction pad connected to a radiofrequency generator (Radionics, Burlington, MA). After identifying the cervical vertebra prominence as a landmark, a cervical hemilaminectomy was performed at the C5 and C6 levels. The corresponding DRG on the right side was identified and a 100 mm 30-gauge Levin cordotomy electrode (Radionics, Burlington, MA) with a 2 mm uninsulated tip was introduced adjacent to the DRG using a micro-manipulator (Leitz, Wetzlar, Germany).

After verifying the impedance, an indicator of the type of tissue surrounding the electrode tip, electrical stimulation was started at a rate of 50 Hz and 2 Hz. The rat was observed for withdrawal reaction of the right forelimb (50 Hz threshold). If muscle contractions were observed in the corresponding levels the stimulation threshold (2 Hz threshold) was noted.

All animals were unilaterally treated at one DRG on the right side with one of the four treatment modalities listed below. The treatment options were selected at random.

Treatment Groups

In group 1 (n = 4), the sham intervention group, the electrode was maintained in contact with the ganglion for 120 s, but no current was passed through the electrode. In group 2 (n = 5), the continuous radiofrequency group, the dorsal root ganglion was heated by continuous radiofrequency current and maintained at a temperature of 67°C for 60 s. In group 3 (n = 5), the pulsed radiofrequency 120 s group, the dorsal root ganglion was exposed to PRF for 120 s with a maximum temperature of 42°C. In group 4 (n = 5), the pulsed radiofrequency 8 min group, the dorsal root ganglion was exposed to PRF for 8 min with a maximum temperature of 42°C.

For all procedures impedance, temperature, ampere, voltage, and wattage were recorded at the start and the end of the intervention, if available. At the end of the treatment, the skin incision was closed with 3–0 silk sutures, and the animals were allowed to recover from anesthesia.

Observation of the Animals

All animals were tested on the "grid walk" by an experienced observer who was unaware of the treatment (W.H.) within 48 h postoperatively and on the seventh day after intervention to observe potential treatment influences on gait. In the grid walk test, a general evaluation of the sensory motor function of the forelimbs and hindlimbs, animals have to cross a 100-cm wire grid containing 40 × 40 mm holes. Footfalls (the inability to grasp a grid with one of the limbs resulting in a drop of the foot below the plane of the grid) were counted.[26]

Tissue Preparation

At postoperative day 7, the rats were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Sanofi, Maassluis, The Netherlands 60 mg/kg) and perfused transcardially with a flush of Tyrode solution (pH 7.4) followed by phosphate-buffered 4% paraformaldehyde (pH 7.4). The total cervical spinal cord was dissected and postfixed in phosphate-buffered 4% paraformaldehyde overnight at 4°C. The spinal cord was then divided into parts comprising one segment each and embedded in 10% porcine gelatin. The gelatin cups were allowed to harden on ice for 1 h and postfixed in phosphate-buffered 4% paraformaldehyde for 2 h. The tissue was cryoprotected in 15% sucrose in Tris-buffered saline overnight at 4°C and frozen in a liquid nitrogen cooled bath with 2-methylbutane 99%. Subsequently, cryostat sections (30 μm) were cut (Leica CM3050; Cryostat, Wetzlar, Germany) and collected in 0.1M phosphate-buffered saline (PBS).

Immunocytochemistry

Sections were rinsed in 0.1 M PBS for 10 min, using the free-floating method. The following incubations have been carried out at room temperature. The sections were incubated with 0.3% hydrogen peroxide (against endogenous peroxidase) for 30 min in 0.1 M PBS and subsequently washed 3 times in 0.1M PBS during 20 min. A preincubation in 0.1 M PBS with 0.1% bovine serum albumin and 0.2% Triton-X-100 (PBS-BT) was carried out for 30 min before the overnight incubation with rabbit-anti-c-Fos (1:20 000) (Santa Cruz Biotechnology Inc. SC-052, Santa Cruz, CA) in 0.1 M PBS-BT. After rinsing 3 times with 0.1M PBS for 20 min, the sections were incubated with donkey-antirabbit immunoglobulin G biotin conjugated 1:1500 in 0.1M PBS-BT (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) for 90 min. Then after three washes in 0.1 M PBS, sections were incubated with Vector ABC-Elite (Vector Laboratories, Ltd., Burlingame, CA) (1:800 M in 0.1 M PBS-BT) for 90 min followed by a preincubation with Di-amino-benzidine-Ni solution without perhydrol for 10 min. Then sections were incubated with Di-amino-benzidine-Ni solution with perhydrol for exactly 10 min. Finally, sections were rinsed three times for 20 min in 0.1 M PBS and then mounted on gelatin-coated object glasses (0.5% gelatin + 0.05% potassium chrome (III) sulfate), dried overnight in the oven at 37°C. Slices are dehydrated in alcohol series and cleared in Xylol before mounting them in Entellan. Omission of the primary antibody was used for negative controls. The slices received a code allowing blinded counting of c-Fos immunoreactive cells.

Analysis

Quantification of the number of c-Fos immunoreactive cells in the dorsal horn was performed for each animal in one section (30 μm) of the C5 and C6 levels, both ipsilateral and contralateral. Counting was done by a blinded and experienced investigator (W.H.). A second investigator (E.J.), also blinded to the origin of the slices, performed control counting of randomly selected preparations.

To describe the distribution of the c-Fos immunoreactive in the dorsal horn, the median was calculated for the sum of C5 and C6 at the ipsilateral and contralateral side for the four treatment groups. The sum of C5 and C6 were used for further analysis. Differences between the four study groups were compared using the Kruskal-Wallis test, and if this test was significant the sham group was pairwise compared with the active groups using the Mann–Whitney U test. Ipsilateral *versus* contralateral comparison of the number of c-Fos immunoreactive cells in the dorsal horn was analyzed by a Wilcoxon signed rank test. For all analyses significance was reached if $P < 0.05$.

Results

A total of 19 rats were investigated, four in the sham and five in each active intervention group (radiofrequency at 67°C, PRF 120 s, and PRF 8 min). The sections of two rats could not be analyzed because they were not anatomically intact, thus not allowing the determination of the level and a correct count of the c-Fos immunoreactive cells. The results presented refer to the c-Fos immunoreactive counted cells in the sections of 17 animals. All data are shown in table 1. The preintervention details are not significantly different between the groups: weight ($P = 0.99$), stimulation threshold at 50 Hz ($P = 0.09$), at 2 Hz ($P = 0.2$), and start impedance ($P = 0.07$).

The animals did not show postoperative complications during the 7-day observation period.

All animals performed well on the grid walk, when tested within 48 h and 7 days post-intervention, indicating that the surgical intervention did not result in major sensory motor impairment of the forelimbs or hindlimbs. No aberrations in gait (footfalls), even with special attention to the right forelimb, were observed.

Quantification of c-Fos immunoreactive cells in the dorsal horn was performed for each animal at the C5 and C6 levels. To avoid double counting of cells, only one section of 30 μm was used per level. The number of cells

Table 1. Animal pretreatment details, treatment parameters and number of c-Fos-IR cells

Rat	Weight (g)	Group	Stimulation threshold 1.0 ms		Impedance (Ω)		Temperature (°C)		mAmp		Volt		Watt		c-Fos-IR cells	
			50 Hz	2 Hz	start	end	start	end	start	end	start	end	start	end	ipsilateral	contralateral
RF 2	317	sham	0.5	0.6	1180	1180	32	32							0	3
RF 7	309	sham	0.3	0.4	1930	1930	30	30	–	–	–	–	–	–	6	6
RF 10	303	sham	0.85	0.9	1340	1340	32	32	–	–	–	–	–	–	5	8
RF 13	289	sham	0.45	0.5	1480	1480	33	33	–	–	–	–	–	–	3	3
RF 1	309	RF 67°C 60 s	0.15	0.19	628	530	32	67	18	18	10	10	0.2	0.2	9	9
RF 4	279	RF 67°C 60 s	0.3	0.33	1620	1090	31	67	0.8	0.8	14	14	0.1	0.1	16	10
RF 9	279	RF 67°C 60 s	0.4	0.45	950	798	33	67	27	21	18	15	0.5	0.4	n.c.	n.c.
RF 15	310	RF 67°C 60 s	0.3	0.4	1350	945	31	67	15	12	15	15	0.1	0.1	12	10
RF 19	335	RF 67°C 60 s	0.11	0.12	1350	1070	30	67	15	14	14	12	0.2	0.1	43	18
RF 3	310	PRF 120 s	0.5	0.6	1180	980	31	39	27	30	45	45	–	–	37	33
RF 6	308	PRF 120 s	0.3	0.35	694	580	29	42	62	69	45	45	–	–	34	30
RF 12	285	PRF 120 s	0.1	0.13	922	790	32	40	45	47	45	45	–	–	n.c.	n.c.
RF 14	275	PRF 120 s	0.4	0.43	1330	1150	32	42	23	27	45	45	–	–	18	21
RF 17	332	PRF 120 s	0.5	0.52	993	855	32	42	30	32	45	45	–	–	10	15
RF 5	289	PRF 8 min	0.24	0.3	740	641	31	42	48	51	45	43	–	–	7	9
RF 8	297	PRF 8 min	0.12	0.15	1070	987	31	40	25	34	45	45	–	–	46	12
RF 11	308	PRF 8 min	0.15	0.23	998	765	32	41	43	59	45	45	–	–	18	11
RF 16	318	PRF 8 min	0.35	0.35	1350	1110	33	41	21	30	45	45	–	–	44	4
RF 18	294	PRF 8 min	0.15	0.17	1100	882	33	42	22	30	45	45	–	–	32	39

The anatomical preparations of rat V9 and V12 could not be counted (n.c.).

c-Fos-IR = c-Fos immunoreactive; RF = radiofrequency; PRF = pulsed radiofrequency.

were added and presented per side in table 1. The number of c-Fos immunoreactive cells among the four study groups were significantly different for the ipsilateral side ($P = 0.03$) and the contralateral side ($P = 0.02$) (Kruskal-Wallis test). The animals that received a sham procedure showed a relatively low number of c-Fos immunoreactive cells. A significant increase in the number of c-Fos immunoreactive cells is observed in all sections of the animals that received active intervention as compared with sham operated animals ($P < 0.05$) (Mann–Whitney U test) (table 2). The typical nuclear stained c-Fos cells were mainly localized in the dorsal horn as shown in figure 1. No c-Fos immunoreactive cells were observed in the ventral or intermediate gray matter zones of the spinal cord.

A few c-Fos immunoreactive cells were observed in lamina X, surrounding the central canal. Control incubations (absence of the primary antibody and using 0.1 M PBS-BT only) were negative (fig. 1). Inspection of c-Fos expression at other spinal segments than C5–C6 did not show a particular increase at those levels.

No statistically significant difference in the number of c-Fos immunoreactive cells was noted between the active intervention groups. No significant difference was reached for the comparison between the number of c-Fos immunoreactive cells between the ipsilateral and the contralateral sides for the combined data of all animals ($P = 0.20$). Even for the c-Fos immunoreactive cell counts of only the animals that underwent active intervention, no significant difference was noted ($P = 0.12$). There was, however, a trend towards a higher number of c-Fos immunoreactive cells on the ipsilateral side.

Discussion

PRF has recently been introduced as a non-neurodestructive or minimally neurodestructive alternative to radiofrequency for the management of chronic pain.[8] The mode of action of radiofrequency is not yet clear. The selective effect of radiofrequency lesions on small A-δ and C fibers, followed by a delayed effect on fibers in

Table 2. Median number (range) of c-Fos-IR cells and P values

	Median number (range) of c-Fos-IR cells in C5/C6			
	Ipsilateral side	*P* versus Sham	Contralateral side	*P* versus Sham
Sham (n = 4)	5 (0–6)	–	4.5 (3–8)	–
RF 67°C (n = 4)	16 (9–43)	0.02	10 (9–18)	0.02
PRF 120 s (n = 4)	34 (10–37)	0.02	25.5 (15–33)	0.02
PRF 8 min (n = 5)	38 (7–46)	0.01	11 (4–39)	0.049

c-Fos-IR = c-Fos-immunoreactive; RF = radiofrequency; PRF = pulsed radiofrequency.

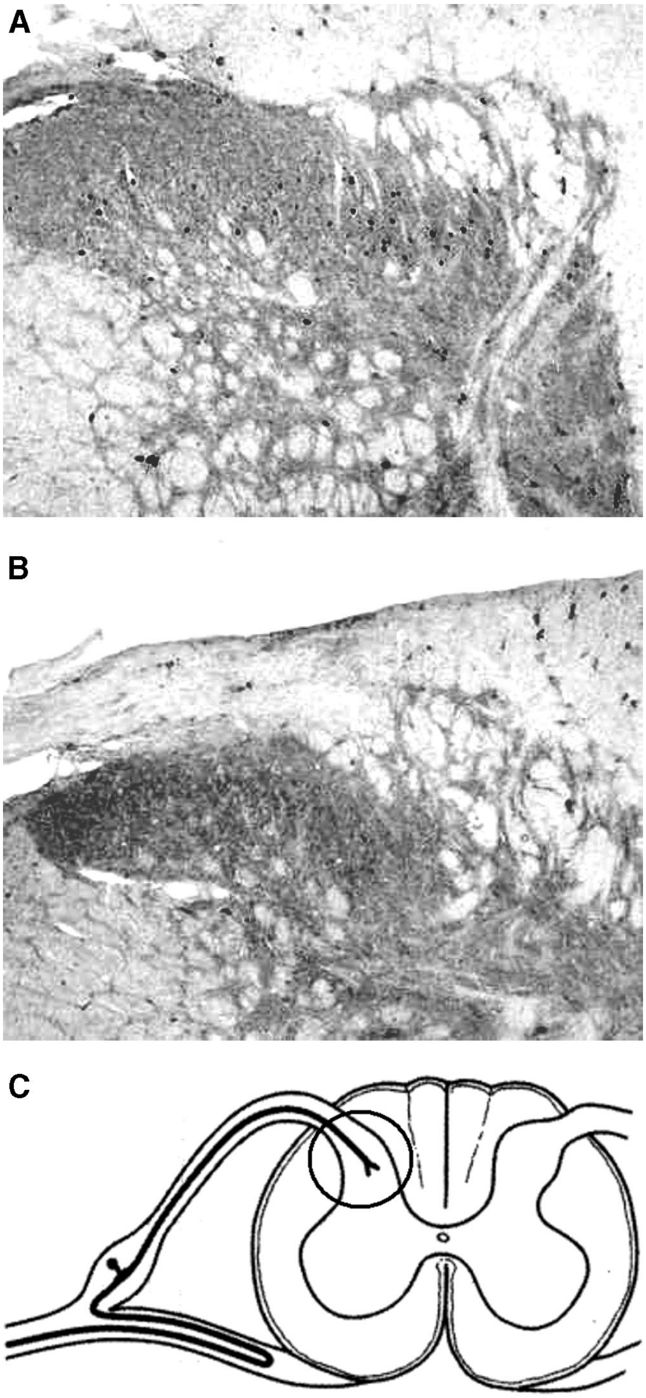

Fig. 1. Transverse sections of the cervical spinal cord at C5: nuclear c-Fos immunoreactive cells located in the dorsal horn of an animal treated with pulsed radiofrequency adjacent to the dorsal root ganglion (*A*) as compared with a sham operated animal (*B*). Schematic drawing of a transverse section of rat spinal cord at C5. The position of the area shown in *A* and *B* is indicated (*C*).

the A-α and β group, has been described in the cat.[27] Although a fiber-selective effect is suggested, other authors have described an "indiscriminate" effect on all types of peripheral fibers in the beagle dog.[7] Furthermore, radiofrequency at 67°C adjacent to the DRG of the goat produced proliferation of satellite cells and regeneration of damaged nerve fibers 2 weeks after the intervention, without microscopically observable signs of tissue damage (*e.g.*, necrosis).[28] Considering the contradictory findings regarding the selectivity in nerve fiber destruction it is assumed that mechanisms other than thermocoagulation are also involved in its mode of action.

Because pain transmission is modulated in the dorsal gray matter, we investigated the late effect in the rat dorsal horn of continuous and pulsed (120 s and 8 min) radiofrequency current adjacent to the cervical DRG *versus* sham intervention. Our study results demonstrate a late or sustained effect of the three different radiofrequency current delivery modalities 7 days after the intervention. We observed a significant increase of c-Fos expression in the dorsal horn of animals that underwent active intervention compared with the sham-operated controls. An early increase in c-Fos immunoreactive cells in lamina I and II of the dorsal horn has been described after pulsed radiofrequency.[24] Here we demonstrated an additional late c-Fos activity with a bilateral response. Under basal conditions the detectable c-Fos concentrations are very low,[29] a finding that was confirmed in the sections of the sham-operated animals and also in sections from higher and lower segments of the spinal cord of animals from the active groups. After an acute challenge c-Fos proteins, nonspecific markers for cellular activity, are induced within a few minutes and the maximum concentrations occur between 1 and 3 h.[22,30–35] In addition another peak of c-Fos expression was seen within the deep layers, commencing at 8 h and peaking at 16 h. This second wave of labeling started ipsilaterally and spread to become bilateral.[23] For this reason we selected the time point of 7 days after the intervention to evaluate the late effect because it is far beyond the described window of c-Fos expression after an acute event. Our findings indicate no significant difference in neuronal activity in the dorsal horn of animals after radiofrequency and the modified technique PRF. These results further support our hypothesis that temperature is not an important factor for the obtained effect on c-Fos expression. In addition, Cahana *et al.*[36] demonstrated in a cell culture model that the acute effects of PRF are more reversible and less destructive than continuous radiofrequency even under nonthermal conditions.

It is well known that stimulation of central nervous system neurons with rectangular electrical pulses can induce immediate early gene expression, *e.g.*, the inducible transcription factor c-Fos. These immediate early gene products are involved in triggering the long-term changes in gene expression that underlie neuronal plasticity.[24] Alterations in the genetic program of neurons underlie the central nervous system changes responsible for long-term potentiation and other phenomena.[37] Randic *et al.*[38] reported that brief high-frequency electrical stimulation of primary afferent fibers produced a long-term potentiation or long-term depression of fast monosynaptic and polysynaptic excitatory postsynaptic potentials in a high proportion of dorsal horn neurons. A distinct long-lasting modulation of synaptic efficiency can be induced at primary afferent synapses with neurons in the superficial laminae of the spinal dorsal horn by high-frequency stimulation of dorsal root afferents. These changes may be physiologically relevant for transmission and integration of sensory information. Furthermore, Sandkühler *et al.*[39] identified a robust long-term depression of synaptic transmission in substantia gelatinosa neurons that can be induced by low-frequency stimulation of primary afferent A-δ fibers, which may be relevant for long-lasting segmental antinociception after afferent stimulation. This is in accordance with our results, which indicate that PRF, delivered at 2 Hz during 120 s and 8 min, produced late changes in the dorsal horn. These changes may also contribute to the mode of action of the different radiofrequency techniques. Based on our experiments, we were not able to explain the difference in neuronal effects between radiofrequency and PRF. Further controlled experimental studies on the efficacy and safety of pulsed radiofrequency should be performed to establish the differential characteristics observed in clinical practice. Our observations on late bilateral c-Fos expression in the rat suggest that bilateral effects might be obtained in clinical practice. To our knowledge this has not been investigated. Further clinical research is needed to evaluate this potential bilateral response.

Conclusions

Exposure of the cervical DRG to continuous radiofrequency (67°C) and PRF current induces increased cellular activity in the dorsal horn ipsilateral and contralateral 7 days after the intervention. The hereby demonstrated late and temperature-independent cellular activity in the rat spinal cord after application of different radiofrequency modalities is suggested to be part of the underlying mechanism during clinical use of pulsed radiofrequency.

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