Acute Differential Modulation of Synaptic Transmission and Cell Survival During Exposure to Pulsed and Continuous Radiofrequency Energy

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Abstract: Pulsed radiofrequency, in which short bursts of radiofrequency energy are applied to nervous tissue, has been recently described as an alternative technique devoid of nerve injury, a subsequent side effect of thermal lesions created by continuous radiofrequency lesioning. Yet the mechanism of this effect remains unclear. In this study we compared the acute effects of pulsed versus continuous radiofrequency energy on impulse propagation and synaptic transmission in hippocampal slice cultures and on cell survival in cortical cultures. A differential effect was observed on both systems, with pulsed radiofrequency producing a transient and continuous radiofrequency a lasting inhibition of evoked synaptic activity. In addition, although both continuous radiofrequency and pulsed radiofrequency treatments induced a distance-dependent tissue destruction under the stimulating needle, the effect was more pronounced in the continuous radiofrequency group. These findings suggest that the acute effects of pulsed radiofrequency are more reversible and less destructive in nature than the classic continuous radiofrequency mode, even in normothermal conditions. This model might help elucidate the importance of various parameters for the clinical application of radiofrequency lesioning and might open new horizons for the role of pulsed radiofrequency lesioning in cases of neuropathic pain.

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Key words: Radiofrequency, neuropathic pain, organotypic cultures, cortex, hippocampus.

R adiofrequency treatments are offered for a variety of pain syndromes: cervicogenic headaches,[27] occipital neuralgia,[5] whiplash injury,[11] intercostal neuralgia,[25] mechanical low back pain,[26] discogenic pain,[10] and pain associated with the sacroiliac joint.[6]

The rationale for the application of radiofrequency denervation is the assumption that selectively heating nervous structures can impede nociceptive input. Practically this is achieved by percutaneous application of small size electrodes at target neural tissues, resulting in size-controlled lesions at different anatomic positions, which depend on patients' symptoms.

However, other results[8,18] have questioned the utility of thermal lesioning, which is essentially neuroablative, in the presence of neuropathic pain. Accordingly, pulsed radiofrequency has been recently described as an alternative technique to apply a relatively high voltage near a nerve without nerve injury, a subsequent side effect of thermal lesions.[3,13,21] It is known that the application of pulsed radiofrequency energy on cell cultures induces immediate early gene expression[1] not mediated by tissue heating.[9]

Although preliminary clinical investigations have shown that pulsed radiofrequency can be used safely as an alternative interventional treatment in patients with refractory pain syndromes or with intolerable side effects resulting from their conservative treatment,[19] it is still not clear what are the differences or advantages between pulsed radiofrequency and continuous radiofrequency, in terms of both clinical outcome and biologic mechanisms involved.

The objective of our study was to investigate whether the acute application of radiofrequency energy, either on the pulsed or continuous mode, resulted in similar or different effects on functional parameters such as impulse propagation, synaptic transmission, and cell survival. To this end, we used in vitro preparations (hippocampal organotypic slice cultures and dissociated cortical neurons) that are ideally suited for such analyses.

Materials and Methods

All animal procedures were performed after obtaining approval from the Animal Care Committee of the University Medical Center in accordance with the Swiss Federal Animal Welfare Act.

Electrophysiologic Studies

Hippocampal organotypic slice cultures (n = 6 to 7) were prepared as previously described[24] from 7- to 14-

day-old Sprague-Dawley rats. Briefly, hippocampi were dissected in an ice-cold dissection medium consisting of 50% minimal essential medium supplemented with 25 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; 300- to 400-μmol/L thick slices were cut on a McIll-wain tissue chopper (Mickle Laboratory Engineering Co., Ltd., Gomshall, England) and then placed at the interface on a porous, translucid membrane (Millicell-CM; Mili-pore, Bedford, Mass) in a CO_2 incubator. The culture medium contained 25% horse serum, 25% Hank's solution buffered by 5 mmol/L Tris and 4 mmol/L $NaHCO_3$ at a pH of 7.2, with penicillin and streptomycin. Cultures were allowed to recover for 4 days at 36°C and then transferred to a 33°C incubator.

For electrophysiologic measurements, 1- to 2-week-old cultures were placed in an interface-type recording chamber continuously perfused with 126 mmol/L NaCl, 3 mmol/L KCl, 24 mmol/L $NaHCO_3$, 1.25 mmol/L NaH_2PO_4, 1.5 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$ at a pH of 7.4. Excitatory postsynaptic potentials were elicited with a stimulation electrode placed on CA3 neurons to activate the Schaffer collateral pathway and were recorded in the stratum pyramidale of area CA1. Stimulation intensities were adjusted so as to evoke below threshold excitatory postsynaptic potentials. The excitatory postsynaptic potentials slope and amplitude were continuously monitored and digitized on-line.

Slices were exposed to radiofrequency energy (RF3C plus; Radionics, Burlington, MA) by using 22-gauge SMK 54-mm, 5-mm active tip needle (COTOP BV, Amsterdam, The Netherlands). Cultures were divided into 3 treatment groups. Group I was exposed to pulsed radiofrequency energy at 500 kHz at a pulse rate of 2 Hz for a period of 120 seconds, with an adjusted voltage of 35 to 45 V, to permit maximal temperature of 38°C. Group II was exposed to pulsed radiofrequency at an adjusted voltage to allow maximal temperature of 42°C. Group III was exposed to 42°C thermal lesion by continuous radiofrequency energy for 120 seconds.

Morphologic Studies

Primary cultures of newborn Sprague-Dawley rat cerebral cortices were prepared as described previously.[28] Briefly, after removal of meningeal tissue, the cerebral cortex was mechanically dissociated in Hank's calcium- and magnesium-free medium and centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and plated on polylysine-coated coverslips in 35-mm Petri dishes with a seeding density of 10^6 cells per Petri dish. Under these conditions, cultures became confluent at the sixth day, and at this time, the serum-containing medium was replaced by serum-free medium (DMEM, Gibco, Basel, Switzerland; 15 μg/mL insulin, 20 μg/mL transferrin, 20 nmol/L progesterone, 100 mmol/L putrescine, and 30 nmol/L sodium selenite) in the presence of Ara-C (2.5 mmol/L). Under these conditions, neurons remained viable, assessed by morphologic criteria such as arborization pattern and cell shrinkage throughout the experimental period.

The effect of continuous and pulsed radiofrequency energy was tested on cultures maintained 2 days in a serum-free medium. A 22-gauge SMK 54-mm, 5-mm active tip needle was placed perpendicularly to the monolayer in contact with the serum-free culture medium to allow energy exposure. The distance between the monolayer and the needle tip was set to 2000, 1000, 500, and 250 μm, respectively, by changing the amount of medium in 3 treatment groups. Group I was exposed to pulsed radiofrequency energy at 500 kHz at a pulse rate of 2 Hz for a period of 120 seconds, with an adjusted voltage of 35 to 45 V, to permit maximal temperature of 38°C. Group II was exposed to pulsed radiofrequency at an adjusted temperature of 42°C. Group III was exposed to a 42°C continuous radiofrequency energy for 120 seconds. Electronic impedance, voltage, current output, and the tip temperature were recorded every 30 seconds. After stimulation, cultures were washed and cells were kept in culture for an additional 24 hours in serum-free medium before fixation.

Cultures were fixed in cold (4°C) 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 60 minutes and then washed several times in a phosphate-buffered saline (PBS) solution. Cultures were incubated with primary antibodies at room temperature for 2 hours and at 4°C overnight. The following primary antibodies were used: (1) rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP) (1:400 dilution) (Dakopatts, Copenhagen, Denmark) to identify astrocytes; (2) mouse monoclonal antibodies to isotype III β-tubulin (1:400 dilution) (Sigma, St Louis, MO) to identify neurons. These antibodies were diluted in PBS/0.5% bovine serum albumin/0.3% Triton X-100 solution. Bound antibodies were revealed with rhodamine-conjugated sheep anti-mouse immunoglobulin G (dilution 1:40) (Boehringer Mannheim Biochemicals, Rotkreuz, Switzerland) or fluorescein-conjugated sheep anti-rabbit immunoglobulin G (dilution 1:80) secondary antibodies (Boehringer Mannheim Biochemicals) diluted in PBS/0.5% bovine serum albumin solution.

Results

Effects on Impulse Propagation and Synaptic Transmission

Hippocampal organotypic slice cultures are well-suited for quantitative analyses of the effects of various treatments on synaptic transmission. In these experiments, pulsed radiofrequency energy was applied through a 0.5-mm needle tip placed on the Schaffer collateral fibers, while continuously monitoring synaptic responses generated in CA1 cells and evoked by generating an action potential in CA3 neurons (Fig 1A). The amplitude of evoked synaptic responses thus correlated with the number of activated fibers or the efficacy of synaptic transmission.

Cultures were exposed to 3 types of radiofrequency energy: 38°C pulsed radiofrequency, 42°C pulsed radiofrequency, and 42°C continuous radiofrequency. Temperature profiles for all groups are shown in Fig 2A. Tissue temperatures increased from 28°C (temperature in
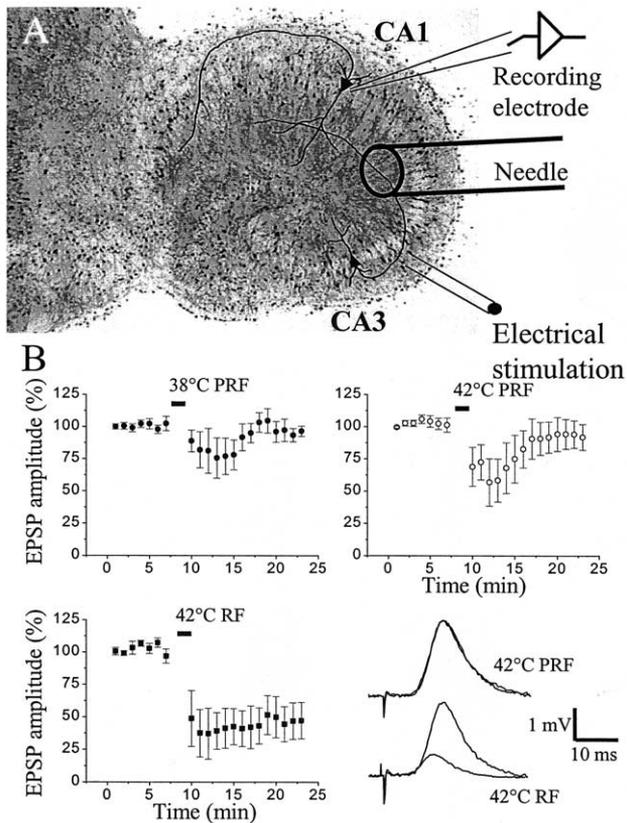
Figure 1. Modulation of evoked synaptic transmission by pulsed or continuous radiofrequency (RF). (A) Experimental design illustrating the placement of the stimulating and recording electrodes as well as the needle for pulsed or continuous RF on top of an organotypic hippocampal slice culture. RF was applied to Schaffer collaterals originating from CA3 neurons and making synaptic contacts with CA1 pyramidal cells. (B) Changes in amplitudes of excitatory postsynaptic potentials (EPSPs) evoked in CA3 and recorded in CA1 before and after application of pulsed and continuous RF at the indicated temperature. Results are mean ± standard error of mean of 6-7 different experiments per condition. The traces are examples of field potentials recorded before and 15 minutes after application of pulsed (top traces) or continuous (bottom traces) RF in a representative experiment.

Figure 2. Temporal course of temperature (A), impedance (B), and current (C) after exposure of an organotypic hippocampal slice culture to pulsed or continuous radiofrequency (RF). Temperature and impedance were stable and did not differ between the treatment groups (A, B). Note that values of impedance were due to the characteristics of the electrical circuit. As expected, peak current in the pulsed mode was significantly higher than in the continuous mode (C).

the perfusion chamber) to 38°C, 42°C, and 42°C, respectively, within 15 seconds from stimulation and returned to baseline after the conclusion of radiofrequency application. In all groups, tissue impedance decreased progressively over time, but the changes were not significantly different between the conditions tested (Fig 2B). As could be expected, the peak radiofrequency current was markedly higher in the pulsed radiofrequency groups, averaging 50 ± 19 versus 1.5 ± 0.6 mA in the continuous radiofrequency group (Fig 2C).

Fig 1B illustrates the changes in excitatory postsynaptic potentials slope amplitudes observed before and after application of the 3 radiofrequency energy modes. For each experiment, results were expressed as percent of baseline values and then averaged across experiments (n = 6 to 7). As illustrated, application of radiofrequency energy resulted in a reduction of excitatory postsynaptic potential amplitude in all groups. However, whereas
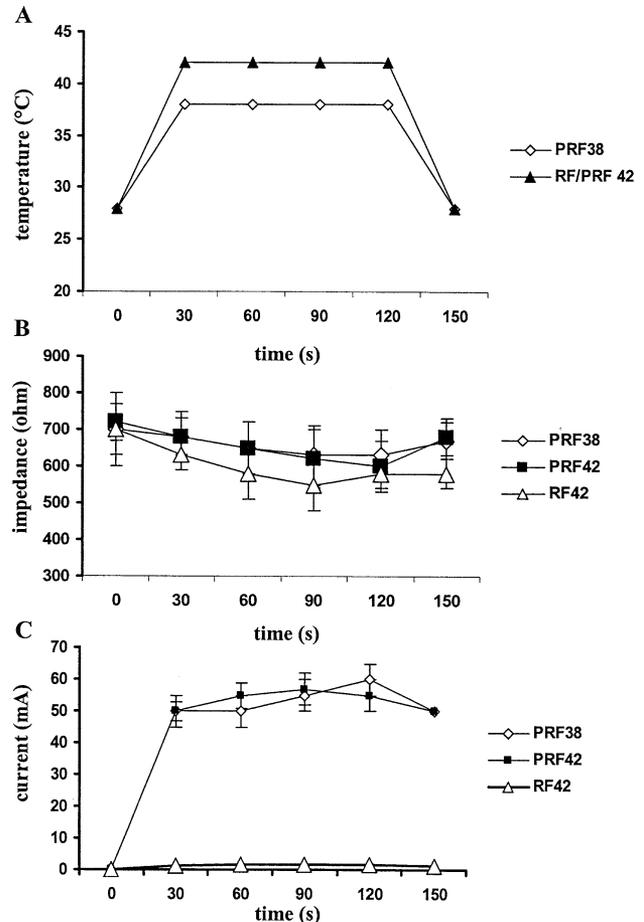
pulsed radiofrequency produced only a transient change followed by a fast and complete recovery, the reduction observed in the continuous radiofrequency group was pronounced and lasting, without signs of recovery for at least 15 minutes (Fig 1B) but up to 40 minutes in some longer experiments. These results thus suggested that continuous radiofrequency, but not pulsed radiofrequency, had produced prolonged alteration of synaptic transmission.

Morphologic Studies

To test this possibility, we then investigated how pulsed radiofrequency and continuous radiofrequency affected neuronal survival when applied to dissociated cortical monolayers, in which cell fate can be easily monitored over a few days. To evaluate and compare the possible damaging effects of pulsed radiofrequency and continuous radiofrequency, different distances between cell cultures and the tip of the stimulating needle were tested. Exposure to pulsed radiofrequency (38°C and
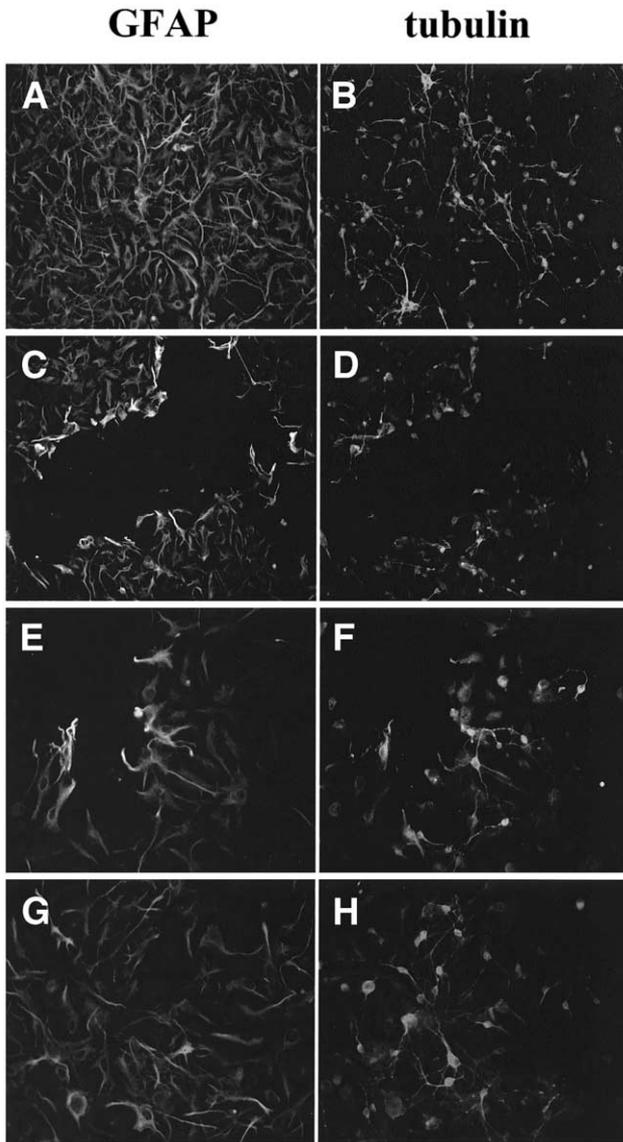
GFAP   **tubulin**

Figure 3. Effects of pulsed and continuous radiofrequency on cortical monolayers 24 hours after stimulation. (A, B) In control groups, astrocytes (labeled with GFAP) form a confluent monolayer with neurons (labeled with tubulin) situated on the top. (C-F) Application of continuous radiofrequency at a distance of 1000 μm resulted in a complete destruction of astrocytes (C) and neurons (D) on the monolayer under the needle. (E) and (F) are higher magnifications illustrating that juxtaposed neurons remained preserved. (G, H) Application of pulsed radiofrequency at the same distance of 1000 μm resulted in no destruction of astrocytes (G) and neurons (H) on the monolayer under the needle. Bar: (A-D) 100 μm; (E-H) 55 μm.

42°C) and continuous radiofrequency (42°C) did not affect glial cell or neuronal morphology when needle was placed at a distance of 2000 μm or more from the cortical monolayer. However, at 1000 μm, neuronal architecture under the needle was destroyed with continuous radiofrequency but not with pulsed radiofrequency energy. At distances of 500 μm or less, we found a complete destruction of the monolayer in all treatment groups (Fig 3C to F).

The denuded area had a quasicircular morphology (Fig 3C, D) corresponding to the shape of the needle, and the border of the lesion was relatively sharp. Glial cells lying adjacent to the lesion site showed no morphologic signs of degeneration, but the intensity of the GFAP immunostaining was more intense than in other glial cells elsewhere in the monolayer (Fig 3E). Neurons, revealed by the specific β-tubulin III staining, also preserved their morphology next to the lesion site (Fig 3F).

Discussion

Neuropathic pain is usually considered a contraindication for the use of thermal lesioning by radiofrequency.[15] It makes little sense to perform a neurodestructive procedure in the presence of altered neural function, risking aggravating neural pathology (ie, deafferentation pain, neural damage). However, pulsed radiofrequency, in which short bursts of radiofrequency energy are applied to the nerve, is thought to be a safer alternative, because there is no clinical evidence of neural damage, with less postprocedure soreness as experienced after thermal radiofrequency lesioning.[22] The mechanism by which pulsed radiofrequency works remains unclear.

In this study we took advantage of in vitro models to compare more directly the acute effects of pulsed radiofrequency and continuous radiofrequency. The main finding of our present study is that exposure of neurons to pulsed radiofrequency resulted in a transient inhibition of evoked excitatory transmission with full recovery of synaptic activity within a few minutes, whereas continuous radiofrequency resulted in a lasting blockade that did not recover during the next 15 to 30 minutes. The amplitude of evoked synaptic responses in the hippocampus is known to correlate with the number of activated axonal fibers. The observation of a lasting reduction of synaptic transmission after continuous radiofrequency application strongly suggests therefore a reduced capacity of Schaffer collaterals to propagate action potentials, an effect that might very well result from damage to the tissue.

Temperature is unlikely to be the main factor responsible for this lasting inhibition because similar changes were reached during pulsed radiofrequency and continuous radiofrequency, even though the voltage applied during continuous radiofrequency was only a fraction of the voltage applied during the active phase of pulsed radiofrequency. It would seem that it is the mode of radiofrequency energy administration and not the temperature that accounts for the difference.

This study showed that both procedures could produce cellular damage when cells are very close to the tip of the needle (< 500 μm). At larger distances (> 2 mm), no effect of either pulsed radiofrequency or continuous radiofrequency was observed. This is thus consistent with the interpretation that radiofrequency energy application produces only very localized effects. However, between 500 and 2000 μm, our protocol clearly showed a differential alteration of cell survival by pulsed radiofrequency and continuous radiofrequency; continuous radiofrequency was more damaging than pulsed radiofrequency.

This result was thus perfectly in line with the electrophysiologic data, which suggested that prolonged alteration of synaptic transmission observed with continuous radiofrequency could indeed have resulted from damage to axonal and dendritic fibers. It is interesting also that the effects were observed only in the area directly under the tip of the needle, with a sharp limit with the surrounding tissue that remained perfectly preserved. Thus radiofrequency energy application appeared to produce acute effects that decreased progressively as a function of distance and localization in relation to the tip of the needle, with pulsed radiofrequency being less damaging than continuous radiofrequency. These observations could be compatible with the assumption that pulsed radiofrequency results in a neuromodulatory rather than a neurodestructive effect when compared to continuous radiofrequency.

Other studies suggested indirectly this neuromodulatory effect. High frequency stimulation might induce long-term depression in the spinal cord[16,17], and exposure of dorsal root ganglion to pulsed radiofrequency currents activated c-fos protein expression in lamina I and II neurons, not seen with continuous radiofrequency.[9] Radiofrequency energy at subcytotoxic levels might induce dramatic metabolic changes without morphologic cell changes, and distinct "zones" can be identified.[7]

Hyperthermic neuromodulation might involve an increase in cell membrane permeability, affecting impulse propagation and inducing protein synthesis such as heat shock protein 70[12]; however, there was no indication that temperature played a role in the effect of pulsed radiofrequency.[20] Conversely, vanilloid receptors, which are heat gated ion channels, might be involved in the pain modulation as seen when exposing dorsal root ganglion to pulsed radiofrequency energy.[14] Another possibility suggested by experiments on fibroblast-like cells exposed to pulsed radiofrequency was an up-regulation of identified and unidentified genes (c-fos, c-myc, c-jun), all probably as a result of membrane perturbation without rupture, a phenomenon shown in dielectrophoretic manipulation through pulsed radiofrequency application in which functional modulation was associated without loss of cellular integrity.[1]

Presently, it is difficult to relate the above mentioned findings to clinical data. Lesion parameters (sensory and motor stimulation thresholds), electrode position, lesion duration, and local tissue properties might all be parameters that might influence clinical outcome. Furthermore, differences in tissue milieu (in vitro culture systems versus intact nervous system), mode of energy application (directly on neurons versus percutaneous on nerve fibers), developmental age of subjects (adult humans versus young rats), and the state of neural substrates (non-nociceptive versus nociceptive stimulus) render clinical appreciation difficult. However having said that, a differential biologic behavior does exist.

Our data indicate that pulsed radiofrequency is less damaging than continuous radiofrequency, even when adjusting voltages to reach similar temperatures. This suggests that probably we must abandon the traditional concept of thermal versus nonthermal radiofrequency lesioning and regard the mode of application as the main factor in lesion mechanism. If this is the case, we might conclude that in our experiments pulsed radiofrequency showed an acute neuromodulatory effect (ie, transient inhibition of evoked synaptic activity), whereas continuous radiofrequency exhibited rather a neurodestructive effect (ie, lasting inhibition associated with tissue disruption). This might be of clinical relevance because pulsed radiofrequency might be a "softer" version of radiofrequency lesioning, appropriate when alterations of functional neural pathways exists, such as in neuropathic pain states.

Conclusion

If pain chronifies through centralization, a neuroablative procedure with no peripheral input remains senseless.[20] Pulsed radiofrequency was advocated as a nondestructive pain therapy, on the basis of the fact that patients treated with pulsed radiofrequency did not show clinical signs of nervous tissue destruction.[22] However this evidence was indirect, and neural tissue injury might actually occur.[23] The fact that fluctuating hyperthermia by pulsed administration of radiofrequency energy instead of continuous radiofrequency might give rise to a neuromodulating and a nondestructing effect remains speculative.

Our study suggests that this hypothesis might be true, and that 38°C and 42°C pulsed radiofrequency treatments provide a "stunning" transitory modulation of neural transmission with less disruptive morphologic changes, as compared to continuous 42°C radiofrequency. The actual neuromodulatory mechanisms remain to be elucidated and will definitely continue to fuel an already ongoing heated debate.[2,4]

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