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Randomized Controlled Trial

Pulsed Radiofrequency Modulates Pain Regulatory Gene Expression Along the Nociceptive Pathway

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Background: Pulsed radiofrequency (PRF) therapy is a clinical treatment utilizing electromagnetic energy aimed to relieve neuropathic pain. This is the first study examining the modulated expression of pain regulatory genes following the induction of the spared nerve injury (SNI) pain model and subsequently treated with PRF therapy.

Objectives: The present study investigated the behavioral efficacy of PRF therapy in rats exhibiting sciatic nerve injury and examined gene expression changes in the sciatic nerve, ipsilateral L5 dorsal root ganglia (DRG), and spinal cord.

Study Design: A randomized, experimental trial.

Setting: Department of Biological Sciences, Illinois State University and Department of Psychology, Illinois Wesleyan University.

Methods: An SNI model was used in male Sprague-Dawley rats (weight 260-310 g). A sham surgery was also performed as a control group. After 3 days development of the SNI model, an RF electrode was applied to the sciatic nerve proximal to the site of injury and stimulated for 3 minutes. The response to mechanical stimuli was assessed throughout the duration of the study. Furthermore, changes in gene expression along the nociceptive tract (sciatic nerve, DRG, and spinal cord) were assessed 24 hours post-PRF therapy.

Results: It was observed that the mechanical allodynia, induced by SNI model, was reversed to control values within 24 hours post-PRF therapy. Additionally, modulated expression of pain regulatory genes was observed after induction of the SNI model. Following PRF therapy, expression of many of these genes returned to control values (sham) in each of the tissues tested. Increased proinflammatory gene expression, such as TNF-α and IL-6, observed in the sciatic nerve (site of injury) in the SNI group was returned to baseline values following PRF therapy. Up-regulation of GABAB-R1, Na/K ATPase, and 5-HT3r as well as down regulation of TNF-α and IL-6 were also observed in the DRG in the SNI-PRF group relative to the SNI group. Up-regulation of Na/K ATPase and c-Fos was found in the spinal cord following PRF treatment relative to the SNI group.

Limitations: Immediate changes in gene expression were observed at 24 hours to better determine the mechanism with no long-term data at this time. Protein expression was not assessed in addition to gene expression changes.

Conclusion: These results indicate that the electromagnetic energy applied via PRF therapy influences the reversal of behavioral and molecular effects of hypersensitivity developed from a peripheral nerve injury.

Key words: Pulsed radiofrequency, PRF; spared nerve injury, SNI; electromagnetic stimulation; Sprague-Dawley, rat; withdrawal threshold; mechanical allodynia, Von Frey; gene expression; nociceptive pathway; electroneuromodulation; cytokines

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Pulse radiofrequency (PRF) is a form of electromagnetic stimulation that has clinically been used to treat patients who suffer from neuropathic pain conditions (1). Neuropathic pain often exhibits symptoms of allodynia or hyperalgesia to mechanical and thermal stimuli resulting from sensitization of nociceptors after exposure to inflammatory mediators (2). Following nerve injury, immune cells in the central and peripheral nervous system release inflammatory mediators that promote nociception. Furthermore, neuroinflammation in the absence of obvious nerve injury is enough to produce nociception (3,4), altered spinal neuron excitability (5,6), and changes in phenotypic expression in afferent neurons (7,8).

The role of the immune system, specifically the role of glia, in the development and maintenance of neuropathic pain has been studied for its involvement in the expression of proinflammatory genes such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) within the central and peripheral nervous system following nerve injury (9-13). Recent studies have shown that selectively targeting key proinflammatory genes and their receptors yields significant pain relief for patients suffering from peripheral pain conditions (14-16). Alternatively, regulation of receptors such as gamma-aminobutyric acid B receptor 1 (GABAB-R1) and 5-hydroxytryptamine (serotonin) receptor 3a (5-HT3r) are known to inhibit pain signaling and thus decrease inflammation upon binding their respective ligands (17,18).

Pharmacological treatment of neuropathic pain with opioid medications and non-opioid medications (such as gabapentin, pregabalin, tricyclic anti-depressants, and dual serotonin-norepinephrine reuptake inhibitors) only provide relief for a limited group of patients (19,20). Furthermore, concerns with serious, life-threatening, adverse events associated with the use of anti-cytokine agents limit their clinical use, further demonstrating that an alternative to target these pathways with minimal side effects would be ideal for the patients suffering from chronic neuropathic pain. An alternative treatment for neuropathic pain gaining scientific support is PRF therapy (21-23). PRF applies high frequency electromagnetic energy to a target tissue in short bursts to allow heat dissipation (24). The theory behind the effects of PRF is that the electromagnetic field disrupts or somehow modulates neuronal transmission. However, the exact mechanism of PRF as a therapeutic modality is poorly understood. Although literature has shown the long-term beneficiary effect of PRF therapy both clinically (1,25-28) and in animal models (29,30), the main focus in this study was to assess early molecular events occurring after PRF therapy.

In vitro studies revealed short periods of reduced excitatory post-synaptic potentials in neurons after PRF application (31). Two similar studies examined c-Fos expression following continuous radiofrequency (RF) and PRF, both keeping tip temperatures below 42°C. While one study observed increased expression of c-Fos following PRF but not after continuous RF (22), the second failed to show a difference (32). More recently, a study demonstrated that PRF, but not continuous RF at the same temperature (42°C), decreased pain hypersensitivity in an adjuvant induced peripheral hyperalgesia model via modulation of the adrenergic and serotonergic descending pathways (21). This evidence of molecular changes following PRF therapy warrants further investigation.

The purpose of this study was to determine if electromagnetic fields generated by current delivered during PRF may inhibit neuropathic pain behavior and whether this correlates with modulation of pain regulatory gene expression locally and along the nociceptive pathway. Based on a comprehensive literature search, 15 genes known to modulate the development, maintenance, and/or potentiation of neuropathic pain were selected for quantitative analysis performed via real-time polymerase chain reaction (qPCR). A rat spared nerve injury (SNI) pain model was used to induce hyperalgesia which was assessed by mechanical allodynia testing while subsequent gene expression in the sciatic nerve, ipsilateral L5 dorsal root ganglia (DRG), and spinal cord were assessed using qPCR.

Methods

Animals

Studies were performed using 24 male Sprague-Dawley rats (260-350g; Harlan Laboratories, IN). Animals were individually housed at 20-23° Celsius and subject to 12-hour light and dark cycles. Standard food pellets and water were available *ad libitum*. Animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (Illinois State University and Illinois Wesleyan University, Bloomington-Normal, Illinois) ethical standards for animal research.

Treatment Groups and Design

A total of 24 animals were randomly assigned into one of the 4 treatment groups: (1) Sham-Sham, (2) Sham-PRF (3) SNI-Sham, and (4) SNI-PRF (Table 1). As there are 2 surgeries performed, the first term in the treatment group applies to potential implementation of the injury and the second applies to potential PRF therapy. Von Frey behavioral testing for mechanical allodynia was performed on Day 1, Day 3, and Day 4 of the study (done prior to PRF electrode placement on Day 3). Fig. 1 illustrates the timeline of the implemented procedures.

Rat Model of Neuropathic Pain

Animals underwent SNI surgery on Day 0 of the study to induce neuropathic pain in the animals. Prior to surgery, isoflurane gas was initially administered at 0.04 L/min then reduced to 0.03-0.035 L/min after the animal became unconscious. The skin over the left hind leg was shaved and disinfected using betadine. A small incision was made over the gluteus superficialis and biceps femoris muscles (Fig. 1A). These 2 muscles were then laterally separated to expose the sciatic nerve at the trifurcation into the tibial, common peroneal, and sural nerve (Fig. 1B). The tibial and common peroneal

Table 1. *Experimental assigned animal groups.*

Animal Groups	SNI Procedures	PRF Stimulation
Sham-Sham	No nerve transection	Exposure of sciatic nerve to PRF probe, with no stimulation
Sham-PRF	No nerve transection	PRF stimulation
SNI-Sham	Transection of the common peroneal and tibial nerves	Exposure of sciatic nerve to PRF probe, with no stimulation
SNI-PRF	Transection of the common peroneal and tibial nerves	PRF stimulation

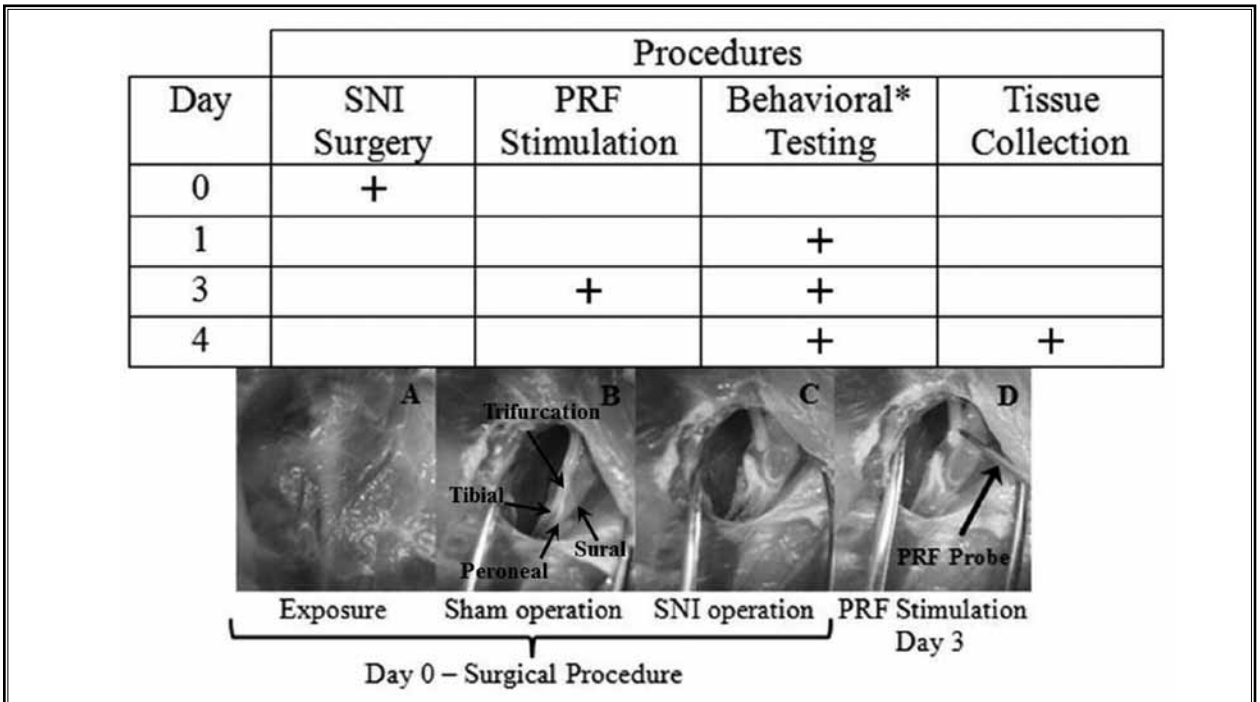

Fig. 1. *Timeline of animal surgeries and behavioral testing. Top portion represents timeline of animal procedures. Bottom are images of spared nerve injury (SNI) surgery performed on Day 0 (A-C) and pulsed radiofrequency surgery on Day 3 (D). A) Exposure of gluteus superficialis and biceps femoris muscles. B) Sham surgery group. C) SNI surgery group. D) Shows the probe in position for PRF stimulation.*
**All behavioral testing done prior to PRF stimulation on Day 3 and prior to tissue collection on Day 4.*

nerves were transected, leaving the sural nerve intact (Fig. 1C). In the Sham operation groups, the procedures were performed in the same manner without transecting the nerves. The muscles were massaged back into place and the incision was closed using stainless steel clips in all animal groups.

Behavioral Testing

Mechanical allodynia was assessed post-surgery (Day 1, Day 3, and Day 4) using an Electronic Von Frey Anesthesiometer (IITC Inc., California, USA). Each animal was placed in an elevated cage with wire mesh floors that allowed for testing withdrawal thresholds via mechanical stimulation. Probes were applied in ascending force order to the medial plantar aspect of the left and right hind paws to measure sensitivity to the mechanical stimulus. Each hind paw was tested 6 times per each filament in ascending deformation force values (1.5, 4.0, and 20 gram filaments) (IITC Inc., California, USA), with at least 2-3 minute intervals between same paw probing. Therefore, each paw was tested a total of 18 times per testing session. Maximal tip pressure (force applied in grams) occurring either at time of paw withdrawal or tip bending was automatically recorded in grams by the recording device. This data was used to measure the degree of mechanical allodynia, or neuropathic pain, induced and maintained by the SNI pain model and the degree to which PRF relieved hypersensitivity to mechanical stimulation.

Pulsed Radio Frequency (PRF) Therapy

Animals underwent PRF treatment on Day 3 of the study. As was previously done for the SNI surgery, anesthesia was administered at 0.04 L/min and then maintained at 0.03-0.035 L/min isoflurane gas. Staples from the initial surgery were removed and the original surgical incision was re-opened and the gluteus superficialis and biceps femoris were again parted to expose the trifurcation of the sciatic nerve. A SMK-5 RF electrode with a 5mm exposed tip and built-in thermocouple for temperature monitoring (Cosman Medical Inc.), anchored to a stereotaxic frame, was positioned perpendicular to the sciatic nerve and proximal to the site of trifurcation (Fig. 1D). PRF treatment was administered to Sham-PRF and SNI-PRF groups using a Radionics RFG-3c Plus radiofrequency generator with the same stimulation as it is used clinically: 500,000Hz, 45V, 20ms pulse burst within a 500ms pulse interval and applied 3mm proximal from the site of trifurcation for 3 minutes to stimulate the sciatic nerve trunk. The tip temperature was monitored to keep the tissue temperature ≤ 42°C by reducing the voltage as necessary. Sham-Sham and SNI-Sham groups underwent the same surgery and positioning of the electrode with no voltage applied. Following the PRF treatment, the RF probe was removed and the muscles were massaged back into place and the incision was closed with stainless steel clips.

Gene Expression Analysis

Animals were euthanized via CO_2 inhalation 24 hours following PRF treatment. Sections of the sciatic nerve, DRG, and spinal cord were removed. A 5mm section of sciatic nerve tissue was removed at the site of stimulation; 0.3-0.8mm from site of trifurcation. The intact L5 DRG tissue was removed. The L5 nerve root was traced to where it synapsed with the spinal cord and a 3-4mm section of spinal cord tissue was removed. Tissues were immediately stored in one mL TRIzol (Fisher Scientific, Massachusetts, USA) and kept on ice to preserve RNA. The tissues then underwent homogenization in TRIzol and were stored at -20°C for later RNA extraction.

RNA from tissue homogenate was isolated from TRIzol according to manufacturer's instructions and quantified using NanoDrop ND-2000 spectrophotometer at 260 nm (Eppendorf BioPhotometer, Hamburg, Germany). RNA was reverse transcribed into cDNA using High Capacity RNA to cDNA kit (Applied Biosystems, Carlsbad, CA) also according to manufacturer's instructions. Real-time PCR was carried out on individual tissues using a Model 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA). The mRNA sequences for the 15 pain related target genes were obtained through the NCBI database and designed using IDT PrimerQuest. The used primers, designed to amplify mid-range of the mRNA and be exon spanning, are shown in Table 2. Real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) based on the manufacturer's instruction. The data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels to account for differences in tissue size. All RNA quantification values are expressed relative to GAPDH.

Standards were prepared for each gene that was tested to quantify expression. The cycle threshold (Ct) value for each sample was used to calculate molar concentration based on standard curves. The molar concentration was then normalized to each tissue's GAPDH value and then averaged with the rest of the samples in the same treatment group.

Statistical Analysis

All data were analyzed using SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL). Behavioral data were analyzed using a 3 (1.5, 4.0, 20.0 gram filaments) x 3 (days 1, 3, and 4) x 4 (Sham-Sham, Sham-PRF, SNI-Sham, and SNI-PRF treatment groups) repeat measures ANOVA to determine overall significance. To further determine significance between individual groups, one way ANOVAs and independent t-tests were performed. Similarly, gene expression data were also analyzed using a 3 (sciatic nerve, DRG, and spinal cord tissues) x 15 (genes listed in Table 2) x 4 (Sham-Sham, Sham-PRF, SNI-Sham, and

Table 2. *Primers used for real-time polymerase chain reaction (qPCR)*

Description	NCBI ID	Sequence (5' to 3')*	Literature+
Glyceraldehyde 3-phosphate dehydrogenase	NM_017008	CTCATGACCACAGTCCATGC	
(GADPH)		TTCAGCTCTGGGATGACCTT	
Interleukin 1 beta	NM_031512	CTGTGACTCGTGGGATGATG	↑[46, 53]
(IL-1β)		GGGATTTTGTCGTTGCTTGT	
Gamma-aminobutyric acid B receptor 1	NM_031028.3	TACGAAACCAAGAGCGTGTCCACT	↓[54, 55]
(GABAB-R1)		ATCTTGGGCACAAAGAGCACAACC	
substance P	NM_012666.2	GTTTGCAGAGGAAATCGGTGCCAA	↓[55, 56]
(subP)		GCATCCCGTTTGCCCATTAATCCA	
Na+/K+ ATPase alpha 1 polypeptide	NM_012504.1	TTCACAAGAACCCAAACGCATCGG	↓[54, 55]
(Na/K ATPase)		AGAAACCTAGCACACGCTCTCCAA	
Osteosarcoma oncogene	NM_022197	TTTGCGCAGATCTGTCCGTCTCTA	↓[57, 58]
(c-Fos)		TCCTTTCCCTTCGGATTCTCCGTT	
5-hydroxytryptamine (serotonin) receptor 3a	NM_024394.2	TCAACATTTCCCTGTGGCGAACAC	↓[54, 55]
(5-HT3r)		AGCAAGAGGCTGACTGCGTAGAAT	
Tumor necrosis factor alpha	NM_012675	TGATCCGAGATGTGGAACTG	↑[46, 59]
(TNF-α)		TGGAACTGATGAGAGGGAGC	
Galanin	M18102.1	TGCCAACAAAGGAGAAGAGAGGCT	↑[55, 56]
(Gal)		CGGCCTCTTTAAGGTGCAAGAAAC	
Vasoactive intestinal peptide	NM_053991.1	TCTTGCAGAATGCCTTAGCGGAGA	↑[55, 56]
(VIP)		TCCGAGATGCTACTGCTGATTCGT	
Neuropeptide Y	NM_012614.1	TGCTCGTGTGTTTGGGCATTCT	↑[54, 55]
(NpY)		GGAAGGGTCTTCAAGCCTTGTTCT	
Interleukin 6	NM_012589	CCGGAGAGGAGACTTCACAG	↑[60, 61]
(IL-6)		ACAGTGCATCATCGCTGTTC	
Synaptosomal-associated protein 25	NM_030991.2	AATTCTGCGGGCTTTGTGTGTGTC	↓[54, 55]
(SNAP25)		TCTGGCGATTCTGGGTGTCAATCT	
Glial fibrillary acidic protein	NM_017009.2	CAACGTTAAGCTAGCCCTGG	↑[55, 62]
(GFAP)		TCCTTAATGACCTCGCCATC	
Integrin, alpha M	NM_012711	CATCACCGTGAGTTCCACAC	↑[62, 63]
(ITGAM)		GAGAACTGGTTCTGGCTTGC	
Brain-derived neurotrophic factor	NM_012513	GAGCTGAGCGTGTGTGACAG	↑[55, 64]
(BDNF)		CGCCAGCCAATTCTCTTTTTGC	

*Forward Primer listed first then Reverse Primer

+Literature demonstrating changes in gene expression resulting from an injury model.

SNI-PRF treatment groups) repeat measures ANOVA to determine overall changes in gene expression related to either induction of nerve injury and/or PRF therapy across all tissues. To further determine significance in gene expression changes between individual treatment groups, one way ANOVAs and independent t-tests were performed per individual tissues. In all cases, significance was determined when $P < 0.05$.

Results

Behavioral Testing

The average of the deformation force applied to the hind paw either ipsilateral or contralateral to the SNI lesion was computed for each day of testing. Although the overall repeated measures ANOVA revealed a significant main effect of filament and several significant interactions ($P < 0.009$), the main focus of the behavioral testing was to examine a potential day by filament by treatment group interaction to determine 1) whether SNI lesions led to hypersensitivity and, if so, 2) whether PRF stimulation could alleviate this hypersensitivity. A significant day by filament by treatment group interaction was observed, $F12,72 = 2.683$, $P = 0.005$. Subsequent one way ANOVAs revealed that the 1.5 and 4.0 gram filaments were not effective ($P > 0.05$) in elucidating the effects of either the SNI lesion or the subsequent PRF therapy. All of the following behavioral effects were observed using the 20.0 gram Von Frey filament, $F6,36 = 3.014$, $P = 0.017$.

Effect of SNI Lesion on Mechanical Allodynia

No significant changes were observed in the contralateral hind paw throughout the duration of the study ($P > 0.05$). The force required to elicit a withdrawal response in SNI-Sham animals dropped from the averaged Sham-Sham withdrawal threshold of 33.9 ± 3.8 g to 14.8 ± 4.6 g on Day 3 (a 56% decrease; $P = 0.010$). Similar results were obtained on Day 4 among the Sham-Sham (withdrawal threshold of 32.7 ± 4.0 g) and the SNI-Sham groups (15.1 ± 2.6 g) (a 54% decrease; $P = 0.009$). The behavioral effects of SNI lesioning are presented as a mean ± standard error in Table 3.

Effect of PRF Stimulation on Mechanical Allodynia

Comparing the SNI-Sham group to the SNI-PRF group revealed a significant attenuation of the pain response following PRF treatment on Day 4 ($P = 0.009$). Mechanical threshold from Day 3 to Day 4 increased from 15.2 ± 3.1 g to 25.1 ± 3.2 g in the SNI-PRF group ($P = 0.043$). The PRF therapy returned mechanical sensitivity levels back to control (Sham) values, as indicated by comparing the Sham-Sham and SNI-PRF groups ($P > 0.05$). The behavioral effects of PRF treatments are presented as a mean ± standard error in Table 3. In the absence of injury, Sham-PRF animals displayed normal sensitivity in both hind paws, relative to the Sham-Sham animals, throughout all phases of the study ($P > 0.05$).

Gene Expression

The genes examined were selected by a comprehensive literature search based on prior peripheral neuropathic pain models (Table 2). Gene expression changes in the various animal groups were studied using qPCR. All data for gene expression were normalized to GAPDH and displayed as percent fold expression changes. Although the overall repeated measures ANOVA revealed significant main effects of tissue and gene expression and several significant interactions ($P < 0.000$), the main focus of this study was to examine a potential tissue by gene by treatment group interaction to determine 1) whether SNI lesions and PRF therapy significantly altered gene expression and 2) whether these changes might differ depending on selected tissues. Based on our statistical analysis, a significant tissue

Table 3. *Behavioral data demonstrating pulsed radiofrequency (PRF) reversing injury induced mechanical allodynia. Average force applied (g) per animal group per day post-SNI surgery. By Day 3, all SNI animals displayed marked sensitivity. PRF therapy, applied after behavioral testing on Day 3, resulted in a significant increase in withdrawal thresholds relative to the SNI-Sham group ($P = 0.009$) by Day 4 to within control group values ($P > 0.05$). *Significantly different from Sham-Sham group ($P < 0.05$).*

	Force Applied per day of testing (g)		
Groups	**Day 1**	**Day 3**	**Day 4**
Sham-Sham	24.8 ± 3.6	33.9 ± 3.8	32.7 ± 4.0
Sham-PRF	24.8 ± 2.8	25.9 ± 2.2	25.7 ± 4.7
SNI-Sham	26.7 ± 3.7	14.8 ± 4.6*	15.1 ± 2.6*
SNI-PRF	16.7 ± 2.7	15.2 ± 3.1*	25.1 ± 3.2

by gene by treatment group interaction was observed ($F_{84,336} = 8.840$, $P < 0.000$). All subsequent one way ANOVAs demonstrating significant effects among the tissues are summarized in Table 4. Moreover, using independent t-test, significant effects observed between group comparisons within tissues are summarized in Table 5.

Sciatic Nerve

One way ANOVAs were performed to determine which genes in the sciatic nerve were modulated following the various treatment options. Changes in gene expression following the treatment conditions were observed in TNF-α, IL-6, BDNF, subP, and c-Fos genes. No other significant effects in the sciatic nerve were observed ($P > 0.05$). Subsequent independent t-tests failed to demonstrate any significant changes in c-Fos expression relating to SNI lesion or PRF treatment.

Sciatic Nerve: Effect of SNI Lesion on Gene Expression Tissue

Relative to the Sham-Sham group, 4 genes were modulated upon SNI induction in the SNI-Sham group, exhibiting significant up-regulation of TNF-α ($P = 0.006$), IL-6 ($P = 0.013$), and BDNF ($P = 0.006$). In addition, significant down-regulation of subP ($P = 0.032$) was also observed. Table 5 shows the modulation of these genes after peripheral nerve injury in the sciatic nerve.

Sciatic Nerve: Effect of PRF Stimulation on Gene Expression

PRF stimulation alone (without any prior SNI lesion) led to a significant up-regulation of BDNF ($P = 0.004$) when comparing the Sham-Sham and Sham-PRF groups. No other significant effects following PRF stimulation alone were observed. Following SNI lesions, PRF treatment significantly down-regulated TNF-α ($P = 0.032$) and IL-6 ($P = 0.035$), with gene expression levels returning back down to control (Sham-Sham) levels (Table 5).

Ipsilateral L5 Dorsal Root Ganglia (DRG) Tissue

One way ANOVAs were performed to determine which genes were modulated in the DRG following the various treatment options. Changes in gene expression

Table 4. *Summary of significant one way ANOVA gene expression results.*

	Sciatic Nerve				Ipsilateral-Dorsal Root Ganglia				Spinal Cord			
	F(x,y)		F-value	p-value	F(x,y)		F-value	p-value	F(x,y)		F-value	*P*-value
	x	y			x	y			x	y		
IL-1β												
GABAbr1					3	21	3.222	0.047	3	22	29.537	0.000
subP	3	21	4.562	0.015	3	21	3.386	0.041	3	22	12.799	0.000
Na/K					3	21	4.152	0.013	3	22	7.837	0.001
c-Fos	3	19	4.940	0.013	3	20	6.944	0.003	3	22	5.960	0.005
5-HT3r					3	21	3.964	0.025	3	21	14.772	0.000
TNF-α	3	21	3.208	0.048	3	19	6.110	0.004				
Gal					3	19	4.442	0.016				
VIP					3	19	6.863	0.003				
NpY												
IL-6	3	21	6.736	0.003	3	19	19.721	0.000				
SNAP25												
GFAP					3	21	4.893	0.006				
ITGAM												
BDNF	3	20	4.648	0.015								

x = numerator degrees of freedom
y = denominator degrees of freedom

Table 5. *Significant Percent Fold Changes in Gene Expression After Sciatic Nerve Injury and Pulse Radiofrequency (PRF) Therapy*

	SN				DRG				SC			
Gene	**Sham PRF**	**Injury**	**PRF therapy**	**Return to control**	**Sham PRF**	**Injury**	**PRF therapy**	**Return to control**	**Sham PRF**	**Injury**	**PRF therapy**	**Return to control**
GABAB-R1						↓32	↑57	Yes	↓19	↓58		No
subP		↓80		No		↓47		Yes		↓50		No
Na/K						↓45	↑96	Yes		↓56	↑48	No+
c-Fos					↓78				↑193	↓67	↑354	Yes
5-HT3r						↓50	↑213	Yes		↓60		No
TNF-α		↑121	↓29	Yes	↓38		↓44	No*				
Gal						↑184		No				
VIP						↑648		No				
NpY									↑73			
IL-6		↑176	↓53	Yes		↑338	↓58	Yes				
GFAP						↑49		Yes				
BDNF	↑487	↑343		No								

Sham PRF = Sham-PRF group relative to Sham-Sham (control)
Injury = SNI-Sham versus Sham-Sham; $p<0.05$
PRF therapy = SNI-PRF versus SNI-Sham; $p<0.05$
Return to control = SNI-PRF versus Sham-Sham (control); Yes: PRF treatment returned expression to control levels; No: PRF treatment failed to return expression to control levels
* = TNF-α in DRG was the only gene not to have a significant effect from SNI induction yet had a significant effect following PRF therapy
+ = Na/K in SC was only gene to be affected by both SNI surgery and PRF therapy with its expression NOT returning to control levels

following the treatment conditions were observed in GABAB-R1, subP, Na/K ATPase, c-Fos, 5-HT3r, TNF-α, Gal, VIP, IL-6, and GFAP genes. No other significant effects in the DRG were observed ($P > 0.05$).

DRG: Effect of SNI Lesion on Gene Expression

In the DRG, several genes were modulated in the SNI-Sham group relative to the Sham-Sham group. Induction of the SNI pain model led to up-regulation of Galanin ($P = 0.001$), VIP ($P < 0.001$), IL-6 ($P < 0.001$), and GFAP ($P = 0.015$) as well as down-regulation of GABAB-R1 ($P = 0.020$), subP ($P = 0.007$), Na/K ATPase ($P = 0.002$), and 5-HT3r ($P = 0.045$) (Table 5).

DRG: Effect of PRF Stimulation on Gene Expression

PRF stimulation alone (without any prior SNI lesion) led to a significant down-regulation of c-Fos ($P = 0.001$) and TNF-α ($P = 0.016$) when comparing the Sham-Sham and Sham-PRF groups. No other significant effects following PRF stimulation alone were observed. Following SNI lesions, PRF treatment significantly modulated the expression of multiple genes. Relative to the SNI-Sham group, SNI-PRF treated animals exhibited up-regulation of GABAB-R1 ($P = 0.042$), Na/K ATPase ($P = 0.010$), and 5-HT3r ($P = 0.015$) (Table 5). The PRF treatment appeared to return these genes to control levels, as evidenced by comparing SNI-PRF and the Sham-Sham groups ($P > 0.05$). Down-regulation of TNF-α ($P = 0.030$) and IL-6 ($P = 0.001$) was also observed (Table 5). However, only IL-6 returned back to control levels following PRF treatment (TNF-α $P = 0.003$; IL-6 $P = 0.155$).

Spinal Cord Tissue

One way ANOVAs were performed to determine which genes were modulated in the spinal cord following the various treatment options. Changes in gene expression following the treatment conditions were observed in GABAB-R1, subP, Na/K ATPase, c-Fos, and 5-HT3r genes. No other significant effects in the spinal cord were observed ($P > 0.05$).

Spinal Cord: Effect of SNI Lesion on Gene Expression

The induction of SNI surgery resulted in the down-

regulation of GABAB-R1 (P = 0.000), subP (P = 0.000), Na/K ATPase (P = 0.008), c-Fos (P = 0.002), and 5-HT3r (P = 0.000) relative to control (Sham-Sham) (Table 5).

Spinal Cord: Effect of PRF Stimulation on Gene Expression

PRF stimulation alone (without any prior SNI lesion) led to a significant up-regulation of c-Fos (P = 0.032) and down-regulation of GABAB-R1 (P = 0.019) when comparing the Sham-Sham and Sham-PRF groups. No other significant effects following PRF stimulation alone were observed. Following SNI lesions, the SNI-PRF treated animals showed up-regulation of Na/K ATPase (P = 0.012) and c-Fos (P = 0.020) relative to the SNI-Sham group (Table 5). Expression of c-Fos returned to control level following PRF treatment, with no significant differences observed between the Sham-Sham and SNI-PRF groups (P = 0.255). Expression of Na/K ATPase increased following PRF therapy; however, its expression level did not return to that of control (P = 0.049).

Discussion

To study the effects of PRF in a pain state we chose to use the SNI model for its ability to induce long-lasting chronic pain that can be evaluated via mechanical hypersensitivity, its consistency, and reproducibility (33). Other more commonly used injury models, such as the chronic constriction injury (CCI) model, were considered but not applied due to variability in ligatures and resultant induced sensitivity (34). It was desired to have inflammation instigated by a single source, such as the SNI nerve transection, to reduce variability. Furthermore, the SNI model focuses on the rat sciatic nerve which is similar in size to the ilioinguinal and occipital nerves which are targets for the treatment of other neuropathic painful conditions, such as with PRF, making this an optimal model for translational research for clinical applications.

The SNI groups demonstrated a significant increase in paw hypersensitivity by 3 days post-injury that, when left untreated, was maintained through the end of Day 4. However, applying PRF therapy on Day 3 post-injury led to a significant reduction of hypersensitivity in the ipsilateral hind paw to control levels within 24 hours. This study demonstrates that PRF therapy rapidly results in a clear reduction of mechanical sensitization induced by the SNI pain model. Previous studies utilizing different pain models, such as nerve ligation by the dorsal root and adjuvant induced pain, have also shown that PRF significantly reverses allodynia (21,23,30,35). The studies that investigated immediate behavioral changes after PRF therapy demonstrated a significant decrease in allodynia within 24 hours post-PRF therapy following either nerve ligation or adjuvant induced hypersensitivity (21,23). Similar studies monitoring long-term analgesic effects of PRF have demonstrated pain relief persisting for longer than 28 days after treatment (30,35). Clinically, the duration of PRF-induced analgesia has been shown to be effective in a variety of neuropathic pain conditions such as lasting for greater than 5 months for ankle pain (27), 3 months for thoracic pain (36), 9 months for inguinal pain (37), and 1.5 years for pudendal neuralgia (38). This study further supports research demonstrating that PRF therapy attenuates/relieves neuropathic pain.

It is generally believed that application of electrical stimulation, and perhaps electromagnetic stimulation, generates an electrical disruption of sensory information to elicit its analgesic effect, potentially by similar mechanisms proposed in the gate control theory (39). In addition, evidence has accumulated in both animal and human studies demonstrating that modulation of inflammatory gene expression facilitates a wide range of analgesic effects in different pain systems (40-42). For the first time, this study has shown that the application of electromagnetic energy applied to axons of peripheral neurons not only provides pain relief but, more importantly, these changes are associated with modulation of pain regulatory gene expression (Fig. 2).

Increasing evidence suggests that the development and maintenance of pain states is mediated by molecular changes in neighboring uninjured neurons (43-45). Following a peripheral injury, both Kleinshnitz et al (46) and this study observed significant increases in proinflammatory cytokine expression close to the site of injury in the sciatic nerve relative to the control animals. Immunoregulatory factors such as TNF-α are known to be induced at the site of injury and undergo retrograde transport to the DRG (47-50). Studies by Shubayev and Myers (49,50) (2001, 2002) further demonstrated anterograde transport of this critical and important pain causing cytokine from DRG to the site of injury. This may provide a possible explanation of ascending/descending alterations in gene expression between the sciatic nerve and DRG as being due to transport of various transcription factors (51). In the sciatic nerve, PRF treatment regulated expression of the genes modulated upon peripheral nerve injury that could then facilitate or prevent ascending alterations in gene expression. In the SNI-PRF group, down-regulation of TNF-α

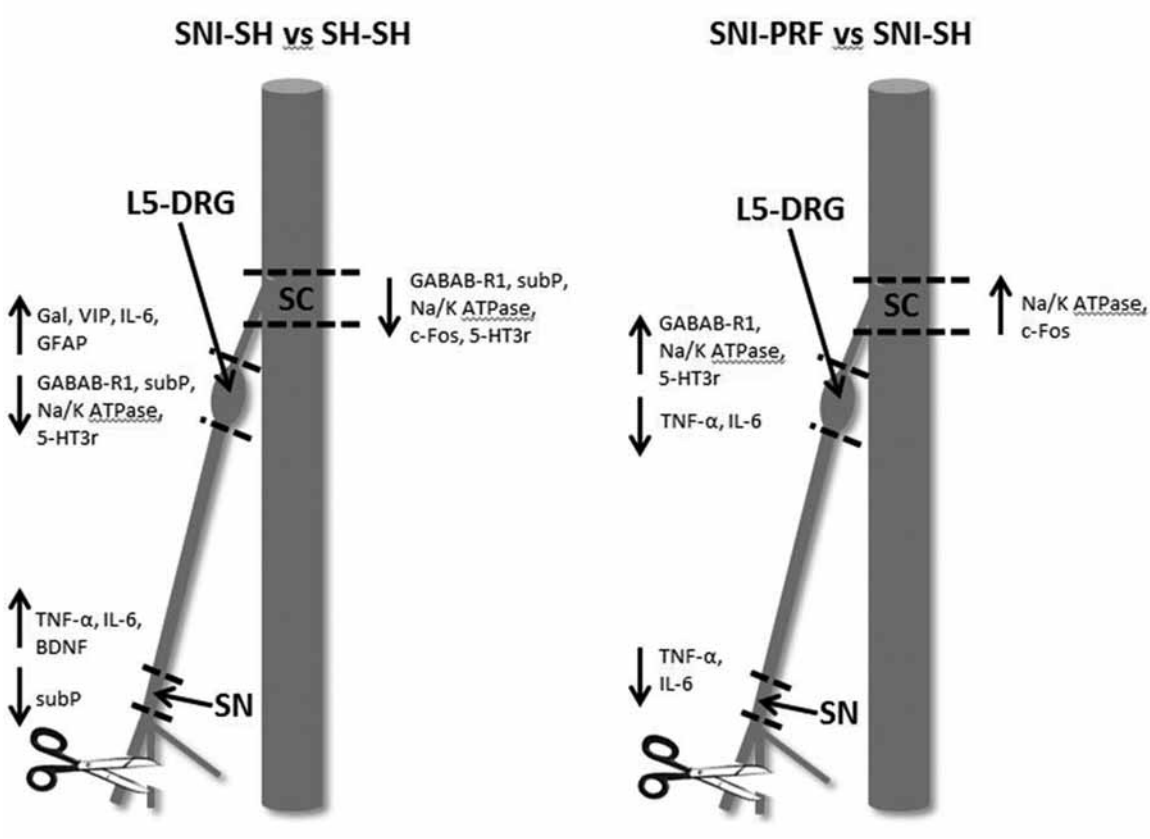

Fig. 2. *Schematic representation indicating tissues analyzed along with significant modulated gene expression within each tissue.*

and IL-6 in both the sciatic nerve and DRG demonstrate that PRF can reduce the expression of pro-inflammatory cytokines in adjacent tissues either directly or indirectly. Interestingly, TNF-α expression in the DRG was the only gene which showed an altered expression (decreased) following PRF therapy with no modulated expression seen as a result of the SNI model. As TNF-α is one of the major players in the development and maintenance of neuropathic pain (52), the decreased expression of TNF-α, and other cytokines modulated by it, indicates that PRF potentially alleviates neuropathic pain states by attenuating neuroinflammation at the molecular level.

In the DRG, the down-regulation of GABAB-R1, 5-HT3r receptors, and Na/K ATPase following the SNI surgery was reversed in the SNI-PRF treatment group. Both GABAB-R1 and 5-HT3r receptors are known to have a role in attenuating pain (17,18). This demonstrates another potential mechanism in which PRF treatment may relieve pain by returning expression of anti-pain mediators to control levels. The ability of PRF therapy to induce its analgesic effect, at least in part, through the serotonergic pathway has already been established (21). Here we demonstrate the ability of PRF therapy to attenuate a variety of pain related genes following induction of a peripheral injury model.

Further modulation along the nociceptive pathway was observed in the spinal cord in the SNI model with decreased expression of Na/K ATPase and c-Fos which then was reversed following PRF therapy. Similar to our study, it was observed that peripherally applied PRF therapy led to increased gene expression of c-Fos in the spinal cord (22). While the analgesic effects of c-Fos are unknown, it is primarily known as a marker for neuronal activity. This implies that upon PRF stimulation, the spinal cord neurons are significantly more active compared to injured animals. In contrast to c-Fos expression which returned to control levels following PRF stimu-

lation in the SNI group, Na/K ATPase failed to return to control values. The Na/K ATPase gene was the only gene to have undergone altered expression due to the SNI injury with a subsequent reversal following PRF that did not return to control levels. Although, in this study, PRF therapy revealed some significant effect on a small subset of genes, c-Fos and Na/K ATPase, this limited action could be due to the short duration of the study in which tissues were collected post PRF treatment. As pain signaling is known to occur along the nociceptive pathway, changes in gene expression within the spinal cord may not have fully developed after only 24 hours.

The overall results in this study revealed that the highest prevalence of gene modulation occurred in the DRG, either after SNI or PRF treatment. This clearly indicates that this tissue which contains the neuron's cell bodies, is very important and very active in pain management at the molecular level. Greater gene expression modulation could have been observed in the spinal cord for both the SNI injury and PRF therapy at later times after PRF treatment. However, the aim of this study was to assess early gene expression changes following PRF therapy. Concerns with serious, life-threatening, adverse events associated with the use of anti-cytokine agents limit their clinical use, further demonstrating that an alternative to target these pathways with minimal side effects would be ideal for the patients suffering from chronic neuropathic pain.

In conclusion, this is the first animal study to treat a peripheral nerve injury with PRF and observe not only behavioral changes but concurrent gene modulation in multiple neuronal tissues adjacent to the site of injury. The results indicate that changes in gene expression not only occur at the site of PRF treatment, but also along the nociceptive path and can also induce co-regulation between the peripheral and central nervous system. The evidence supporting that electromagnetic fields applied to central and peripheral nerve structures can modulate neuroinflammation may create new alternative therapies for the management of neuroinflammatory conditions. As more of the mechanism behind electromagnetic stimulation is understood, patient selection for treatment will become more optimized, thus making PRF a more viable clinical option. Future research must aim to not only identify the optimal parameters being applied for PRF treatment, but also to understand the mechanism of injury-induced gene expression and elucidate proper targets for pain management.

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