Rapid ultrasonic stimulation of inflamed tissue with diagnostic intent

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(Received 13 June 2012; revised 6 February 2013; accepted 8 February 2013)

Previous studies have observed that individual pulses of intense focused ultrasound (iFU) applied to inflamed and normal tissue can generate sensations, where inflamed tissue responds at a lower intensity than normal tissue. It was hypothesized that successively applied iFU pulses will generate sensation in inflamed tissue at a lower intensity and dose than application of a single iFU pulse. This hypothesis was tested using an animal model of chronic inflammatory pain, created by injecting an irritant into the rat hind paw. Ultrasound pulses were applied in rapid succession or individually to rats' rear paws beginning at low peak intensities and progressing to higher peak intensities, until the rats withdrew their paws immediately after iFU application. Focused ultrasound protocols consisting of successively and rapidly applied pulses elicited inflamed paw withdrawal at lower intensity and estimated tissue displacement values than single pulse protocols. However, both successively applied pulses and single pulses produced comparable threshold acoustic dose values and estimates of temperature increases. This raises the possibility that temperature increase contributed to paw withdrawal after rapid iFU stimulation. While iFU-induction of temporal summation may also play a role, electrophysiological studies are necessary to tease out these potential contributors to iFU stimulation.

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PACS number(s): 43.80.Qf, 43.80.Jz [CCC]

Pages: 1521–1529

I. INTRODUCTION

Temporal summation occurs when a single stimulus applied to tissue in repetition causes a greater or even increasing sensation than a single application of that same stimulus. This heightened sensation correlates to a phenomenon known as “wind-up.” Wind-up describes a phenomenon observed via standard electrophysiological techniques applied at and near the dorsal root, where peripheral nerves

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enter the spinal cord. Specifically, wind-up describes the production of an increasing number of action potentials created by repeated application of a single stimulus in the frequency band of 1–20 Hz (Herrero *et al.*, 2000). A direct relationship between “temporal summation” and wind-up has been shown through their common inhibition when N-methyl-D-aspartate (NMDA) receptors are blocked in the dorsal horn (Andersen *et al.*, 1996; Arendt-Nielsen *et al.*, 1995; Arendt-Nielsen *et al.*, 1996; Dickenson and Sullivan, 1987; Price *et al.*, 1994). Enhanced experience of temporal summation after a given stimulation relative to that experienced by healthy individuals is most commonly observed in populations suffering from chronic pain (Staud *et al.*, 2003) due to the action of central sensitization (Woolf, 2011). Therefore successful detection and quantification of temporal summation could aid in the diagnosis and treatment of central sensitization’s contribution to chronic pain. (Woolf, 2011).

Most temporal summation studies to date use superficial or intra-operative sources of stimulation—typically in the form of electrical or thermal energy—to study this phenomenon (Arendt-Nielsen *et al.*, 1994; Arendt-Nielsen *et al.*, 2000; Katsarava *et al.*, 2006). Others use focal mechanical pressure applied at the skin to stimulate deep muscular structures (Nie *et al.*, 2005). This methodology is deemed contentious because when a stimulus is applied on the skin, it may affect underlying anatomic structures even with use of topical anesthetics (Staud *et al.*, 2003). Interpretation of the test will therefore be difficult, and confounding factors cannot be excluded.

Intense focused ultrasound (iFU) may offer another means of inducing temporal summation with either superficial or subcutaneous stimulation. iFU is a type of specialized ultrasound that focuses ultrasonic energy within a roughly cylindrical-shaped volume of tissue (measuring roughly a centimeter in length and a few millimeters in diameter) that lies deep within the body. High-energy iFU can cause rapid, transient, and localized heat and momentum deposition leading to a local increase in tissue temperature and local tissue displacement (ter Haar and Coussios, 2007; Mourad, 2012). In sufficient quantities, the heat and displacement can destroy tissue. This means that sufficiently intense focused ultrasound—known as HIFU for “high intensity focused ultrasound”—is a promising therapeutic modality (Illing *et al.*, 2005; Klinger *et al.*, 2008; Park *et al.*, 2009). However, in lesser amounts, iFU has induced discernable sensations in human subjects (Gavrilov *et al.*, 1977a,b; Gavrilov *et al.*, 1996; Dalecki *et al.*, 1995; Wright *et al.*, 2002; Dickey *et al.*, 2012).

More generally, the creation of an unusual or even painful sensation by iFU of a given dose at a given anatomic site relative to other sites might allow the user to localize a source of pain, thereby confirming the association between anatomic abnormality and perceived pain. For example, Gavrilov and colleagues suggested that iFU applied to individuals with neuropathic disorders could elicit abnormal sensations discernibly different from those elicited by focused ultrasound applied to normal tissue (Gavrilov *et al.*, 1977a,b; Gavrilov, 1984; Gavrilov *et al.*, 1996). Tych *et al.* (2013)

offer data consistent with this concept, demonstrating in an animal model that diffusely neuropathic tissue required less intense iFU (in the form of individual bursts) to generate withdrawal by rats from iFU stimulus as compared with iFU’s application to contralateral tissue. Also, McClintic *et al.* (2013) used individual pulses of iFU to differentiate a focal neuropathic tissue (a neuroma) from surrounding uninjured tissue *in vivo*. Finally, Garcia *et al.* (2013) extended this concept to inflamed tissue *in vivo* by demonstrating diurnal variation in stimulation threshold values due to individual pulses of iFU.

Gavrilov *et al.* (Gavrilov *et al.*, 1977a,b; Gavrilov, 1984) established that individual pulses of duration less than 100 ms likely generated sensations in healthy volunteers through the action of the radiation force. Also, Dalecki *et al.* (1995) showed that repeated application of short pulses (less than 100 ms) of ultrasound into peripheral tissue of healthy volunteers could induce discernable sensations. Through judicious use of an acoustic baffle between the ultrasound source and the tissue, Dalecki *et al.* showed that for their experiments that at least the radiation force, hence tissue palpation due to ultrasound, caused those sensations.

The study by Wright *et al.* (2002) investigated possible temporal summation induction caused by repetitive application of short pulses of iFU. They determined a tactile and/or pain threshold in terms of acoustic intensity for a variety of iFU stimulation protocols applied to the skin, muscle, and joints of healthy volunteers. They showed that as the pulse-train frequency increased above 1 Hz, the minimum intensity of iFU necessary to create a sensation decreased. They also showed that a given pulse train of iFU required less intense ultrasound to generate a sensation than a single pulse of iFU. These results are consistent with the hypothesis that iFU may induce temporal summation in normal human tissue through induction of rapid palpation of tissue.

Intensity is a reasonable measure of iFU to choose when studying temporal summation. This is because focal tissue displacement induced by the acoustic radiation force, proportional to intensity (e.g., Myers, 2006), represents at least one likely source of stimulation by individual, short pulses (less than 100 ms) of iFU as discussed in the preceding text. However, instead of or in addition to tissue displacement, one can in principle induce a sensation with iFU through its ability to produce heat within tissue (as Gavrilov and colleagues recognized for long individual pulses), a quantity proportional to the acoustic dose (intensity multiplied by the time during which the ultrasound is on—e.g., ter Haar and Coussios, 2007). Because tissue is sensitive to heat induction as well as palpation, one should consider both dose and intensity and corresponding estimates of increased temperature and displacement of tissue, especially when studying the ability of repeated pulses of iFU over an appreciable period of time.

Given anticipated application of iFU stimulation to the assay of chronic inflammatory diseases and central sensitization, we sought to test two hypotheses: (1) iFU applied to diffusely inflamed tissue in rapid succession could induce a withdrawal of the tissue by the animal (2) in a manner consistent with temporal summation. Here we tested these

hypotheses by applying iFU in individual or rapidly successive pulses to the inflamed paws of rats using an established animal model of inflamed tissue responsive to individual pulses of iFU (Garcia *et al.*, 2013).

II. MATERIALS AND METHODS

All animal procedures were approved by the University of Washington Animal Care Committee and conformed to relevant national guidelines. Briefly here, with more detail to follow: We induced inflammation in the hind paws of rats, used a standard metric for pain threshold (the Hargreaves heat lamp test – Hargreaves *et al.*, 1988) to assess the sensitivity of the paw before iFU application, then applied iFU to the paws in successive or individual pulses to assess the withdrawal threshold, and then performed the Hargreaves test again.

A. Inflammation induction

Inflammation was induced in the right hind paws of 29 adult male Fischer rats (approximately 180 g, Charles River). The rats were deeply anesthetized using a mixture of oxygen and 4% Isoflurane (Pitman-Moore, Mundelein, IL) via nose cone. The anesthetic plane was maintained with a mixture of oxygen and 2% Isoflurane. Inflammation was then induced using methods adapted from Nagakura *et al.* (2003). Briefly, 0.2 ml of complete Freund's adjuvant (CFA, Sigma Aldrich) was injected subcutaneously over 45 s into the plantar surface of the right hind paw at the base of the toes using a 25 gauge 5/8 in. needle. This produced significant inflammation in the right hind paw relative to the left, with maximum inflammation observed 5–7 days after injection.

B. Behavioral testing

1. Heat lamp test

Each rat underwent a Hargreaves heat-lamp test followed by an iFU test, followed by another Hargreaves test, each described in the following text.

For the Hargreaves test (Hargreaves *et al.*, 1988), sets of three rats were placed in and habituated to separate compartments in a Plexiglas cage with a glass floor. Light from an intense heat lamp (model 33 Tail Flick Analgesia Meter, IITC Life Science Instruments Inc.) was then beamed onto one of their paws from 20 cm below. The lamp was turned on, and the time for the rat to withdraw its paw from the light source was recorded. If the rat did not withdraw its paw after 20 s, the lamp turned off. This was repeated five times for each paw.

2. iFU test

After the Hargreaves test and a brief rest period, the rats were again habituated to the Plexiglas cages for at least 10 min, but this time with a metal floor containing circular cutouts 1.5 cm in diameter and 0.5 cm apart in it to allow the tip of the cone of the iFU transducer to reach through the holes onto the plantar surface of the rats' paws with a limited

range of angle (approximately $\pm 25^\circ$) between the beam axis and the foot. We allowed the rats to become habituated to the feeling of the iFU device and gel on their feet as well as the sound made by the device when firing.

After habituating a set of three rats, we observed whether or not the rat would withdraw its hind paw from application a given dose and intensity of iFU. Specifically, starting at an acoustic intensity (I_{SATA} —the spatially averaged, temporally averaged intensity, within a single burst of ultrasound, defined in the following text) of approximately 100 W/cm², we applied ultrasound to the plantar surface of each hind paw of each rat in series. During and immediately after iFU application, we observed the animals, looking for any immediate hind paw withdrawal response, before returning to the other hind paw in each of the three rats. No rats were tested more frequently than every 30 s, and ultrasound gel was used to ensure adequate physical coupling of the transducer to the plantar surface of the rats' paws.

If the rat did not withdraw either hind paw after iFU application, we increased the intensity of ultrasound and performed the experiment again. We increased the intensity in increments of approximately 10%–30% until we observed a withdrawal response. However, we never increased the acoustic intensity above 1622 W/cm² because we observed cavitation of the ultrasound gel on top of the device at this intensity.

If we observed a paw withdrawal, we applied the same iFU protocol again to that paw after waiting 30 s. If the rat withdrew its paw again, we recorded the associated acoustic parameters— I_{SATA} and the duration of the iFU application, in seconds—as the iFU threshold intensity for that paw (if the rat did not withdraw its paw a second time, we increased the intensity again for both paws). We then applied iFU at the threshold value to each of the rat's hind paws for an additional six times looking for paw withdrawal each time to gather data for sensitivity and specificity calculations (described in the following text). We refer to this complete test of determining an iFU threshold value followed by the six additional iFU withdrawal tests as the “iFU test” for the remainder of the paper.

We performed the iFU test on both hind paws on each rat for the acoustic protocols (testing one protocol per rat per day) on days 5, 7, 12, and 14 post-CFA injection. We performed this sequence of tests on 19 rats, obtaining a total usable data set containing 49 points. All testing was performed between the hours of 9 a.m. and 5 p.m.

C. Ultrasound delivery system for the behavioral studies

Ultrasound was generated using the inner element (22.6 mm inner diameter, 48.5 mm outer diameter) of a two-element, 2 MHz annular array transducer (H-106 S/N-01, Sonic Concepts, Inc., Woodinville, WA), placed within a brass housing that facilitated hand-held deployment of the device. This device has a geometric focus at 62.6 mm away from the transducer, measuring approximately 1.2 mm in diameter and 15 mm in length (measured at the half pressure maximum contour) along the axis of acoustic propagation

FIG. 1. (Color online) Intensity field map at the focus of the iFU transducer, showing the axial map (left) and the XY lateral map (right) where the units are relative for each Watt SATA applied.

(see Fig. 1). We determined these parameters through numerical simulations using MATLAB (The MathWorks, Natlck, MA), confirmed via measurement of the focus with a needle hydrophone (NTR, Seattle WA) in a water-filled tank. A plastic cone filled with degassed water was used to transmit the ultrasound to the focus for our rat studies. The base of the cone was sealed to the transducer housing, and the truncated apex of the cone was sealed with a thin sheet of stretched latex. The height of the cone put the center of the ultrasound focus 6.9 mm beyond the proximal surface of our device as measured in degassed water.

The ultrasound transducer was driven by two arbitrary waveform generators (33120 A, Agilent Technologies Inc., Palo Alto, CA) and an A150 power amplifier (ENI, Rochester, NY). The first waveform generator controlled the pulse duration and pulse repetition frequency by gating the 2 MHz sinusoidal output of the second waveform generator. The output of the gated waveform generator was amplified by the power amplifier, which was connected to the transducer via an impedance matching network.

Prior to any studies, the acoustic output of the transducer as a function of input voltage was measured using a radiation force balance (Hill *et al.*, 1994; Poliachik *et al.* 2001). During studies, input voltage to the transducer was recorded using a Wave Runner LT 322 oscilloscope (LeCroy Corporation, Chestnut Ridge, NY). Using data from the numerically simulated beam pattern, the input voltage to the transducer and the total acoustic output measured by the radiation force balance allowed us to calculate the intensity at the focus. Results are reported in terms of I_{SATA} , the temporally averaged (averaged over 75 ms, the duration of each pulse of ultrasound), spatially averaged intensity (averaged over the area enclosed by the half-pressure-maximum contour in the focal plane assuming linear conditions). This is a standard measure of ultrasound intensity described and justified by Hill *et al.* (1994). We have also measured the peak

positive and peak negative pressures associated with representative intensity values (Table I) using a calibrated hydrophone (Onda, Sunnyvale CA), using linear extrapolation at large values of pressure and intensity. Additionally, we measured the acoustic output in terms of the acoustic dose—the product of I_{SATA} and the total iFU duration.

D. Ultrasound protocols for the behavioral studies

We created two acoustic protocols. The first acoustic protocol consists of a single pulse measuring 75 ms in duration (our “brief acoustic protocol” or BAP) of comparable duration to that chosen by Wright *et al.* The second acoustic protocol consists of a train of five such pulses, each measuring 75 ms separated by 75 ms during which the ultrasound is off (our “temporal summation acoustic protocol” or TAP). This puts the frequency of our iFU repetition at 6.66 Hz, comparable to one of the frequencies observed by Wright *et al.* to produce temporal summation in humans.

TABLE I. Relationship between intensity, peak positive pressure and peak negative pressure.

Intensity (W/cm ²)	Peak positive pressure (MPa)	Peak negative pressure (MPa)
250	0.96	−0.85
500	1.39	−1.18
750	1.76	−1.45
1000	2.06	−1.66
1250	2.31	−1.80
1500 ^a	2.61	−1.97
2000 ^a	3.27	−2.32
2500 ^a	3.87	−2.67
3000 ^a	4.47	−3.02

^aExtrapolated values.

E. Estimating iFU-induced tissue heating

Observations of iFU-induced temperature, proportional to the observed acoustic dose within tissue show that heat dissipation can take longer than 75 ms with, however, insufficient time for tissue perfusion to substantially affect the associated temperature rise (ter Haar and Coussios, 2007). We therefore calculated the temperature rise induced by iFU using the “bioheat” equation (ter Haar and Coussios, 2007) in a manner that accounted for the absorption of ultrasound by tissue as well as by the dissipation of heat through diffusion. Specifically, we evaluated the bioheat equation at the focus of our iFU source using MATLAB (Waltham, MA) for two circumstances. One simulated our TAP protocol of alternating patterns of 75 ms of iFU on then 75 ms of iFU off for a total of 375 ms iFU on spread over 750 ms. The other calculation represented heat induction assuming iFU was on continually for a total of 375 ms. For both calculations, we note that a rat’s paw consists of multiple tissue types complicating any effort to accurately model temperature change with iFU exposure. Therefore our intent here is to predict relative temperature changes using various tissue models where the tissue is assumed to be homogeneous, isotropic, and infinitely large.

To evaluate the model for ultrasound absorption requires the following parameters: α is the amplitude attenuation coefficient, the intensity at the focus (I_{SATA} here), ρ is the tissue density, and C is the specific heat (Parker, 1985) and the thermal conductivity coefficient K (Chapman, 1991). Values of the parameters necessary for evaluating the net temperature induction by ultrasound absorption for the four tissues modeled are shown in Table II (Duck, 1990; Hoque and Gandhi, 1988).

F. Estimating iFU-induced tissue displacement

In addition to heating tissue, ultrasound absorption and reflection pushes tissue via the acoustic radiation force. As with the heating estimates, the complexity of the rat’s paw limits the accuracy of any displacement estimate. Here we sought to give an estimate of relative maximum tissue displacement at the focus caused by each isolated pulse of iFU using the formula from Myers (2006). This is reasonable because most observations of iFU-induced displacements have a return to baseline that measures less than 20 ms in duration (Nightingale *et al.*, 2000; Nightingale *et al.*, 2001; Nightingale *et al.*, 2002), significantly less than the time between an iFU pulse (75 ms) in our protocol. To evaluate

TABLE II. Parameters to calculate iFU-induced tissue displacement and heating.

	Muscle	Fat
Attenuation, α (Np/cm/MHz)	0.150	0.070
Density, ρ (g/ml)	1.070	0.937
Specific heat, C (J/g/°C)	3.720	2.500
Thermal conductivity, K (W/cm/°C)	0.00470	0.00160
Shear modulus, $\square$ (kPa)	12	10
Speed of sound, c (m/s)	1566	1479

their formula requires the following parameters: The intensity at the focus, given by our experiments as I_{SATA} , the length of the propagation path of the ultrasound from the tissue surface to the focus (here, 0.69 cm), the frequency of the ultrasound (here, 2.0 MHz), the radius of the transducer (here 4.85 cm), as well as “ m ,” the shear modulus of tissue, and “ c ,” the speed of sound, all given in Table II (Duck, 1990; Hoque and Gandhi, 1988).

G. Data analysis

As previously described, the intensity and acoustic dose of each threshold value were calculated based on the threshold input voltage and acoustic output measured using a radiation force balance. All calculations were done using Microsoft EXCEL 2007 (Microsoft, Redmond WA) unless otherwise specified. For each acoustic protocol (BAP and TAP), we calculated the average threshold intensity and dose value, the standard deviation of that data, and the translation of that data into predictions of iFU-induced temperature rise and maximum tissue displacement. We then evaluated the statistical significance of those quantities for the different protocols and animal types using Excel. We consider any p value less than or equal to 0.05 to be a statistically significant difference. Box and whisker plots of each data set were created using SIGMAPLOT 11.0.

We used the results of the iFU threshold test to calculate the sensitivity and specificity of iFU’s ability to distinguish the inflamed from contralateral paw. To do so, we defined a true positive response to iFU as a withdrawal of the sensitized paw after iFU application to that paw, while a false positive response was a withdrawal of the contralateral control paw after iFU application to that paw. A true negative response to iFU was no withdrawal of the contralateral paw after iFU application to that paw, while a false negative response was no withdrawal of the sensitized paw after iFU application to that paw. We then calculated the sensitivity and specificity of BAP and of TAP. We define the sensitivity of a given iFU protocol as the proportion of subjects with a true positive test divided by the proportion with true positive plus the proportion with false negative tests. Similarly, the specificity of a given iFU protocol is defined as the true negative proportion divided by the proportion of true negative plus the proportion with false positive responses.

III. RESULTS

We observed that the average Hargreaves latency times for all protocols was 10.1 ± 5 s for the inflamed paw and 17.7 ± 3 s for the normal paw. These latency times did not vary in a significant way between the acoustic protocols nor did they vary before versus after the iFU test.

For the iFU tests, all rats withdrew their inflamed paws but not their normal paws at the threshold amount of iFU. After first identifying the threshold value of iFU for a given rat then applying iFU another six times at that same threshold value, we were able to calculate the sensitivity and specificity of iFU stimulation. The average sensitivity for the TAP (the temporal summation acoustic protocol) protocol was $93\% \pm 8\%$ while that for BAP (the brief acoustic protocol)

FIG. 2. Threshold results for each acoustic protocol in CFA injected rats in units of intensity and calculated equivalent for associated maximum tissue displacement in muscle (in units of microns).

was $75\% \pm 15\%$. The average specificity for TAP was $92\% \pm 12\%$ while the BAP specificity measured $97\% \pm 1\%$.

Identification of the threshold value of iFU allowed us to report the amount of ultrasound that, when applied to rats with an inflamed paw, caused the rats to reliably withdraw their paw. In terms of intensity (Fig. 2, Table III), BAP is significantly higher than TAP. With respect to acoustic dose (Fig. 3, Table IV), BAP is not statistically significantly different from TAP.

We then calculated the predicted maximum tissue displacement for two tissue types (muscle and fat) using our measured values of intensity (Table III, Fig. 2). The predicted BAP maximum tissue displacement is significantly higher than TAP.

With regard to the time course of temperature (Fig. 4), the temporal evolution of the temperature field associated with the TAP protocol shows a saw-tooth pattern. Each secondary maximum is associated with one of the five iFU pulses that comprise the TAP protocol, followed by a temporary and partial reduction from that local maximum in temperature until the arrival of the next pulse. The result is a net increase in temperature over the time course of iFU application during the TAP protocol via a series of ratcheting steps. This is in contrast to the predicted temperature induced by an ultrasound protocol that consisted simply of iFU that is on for the same net time as the TAP protocol (that is, a total

of 375 ms not divided into 5×75 ms pulses). For such a monotonic protocol, the associated tissue temperature grows monotonically until cessation of iFU application at which point the temperature falls exponentially.

We calculated the predicted maximum temperature change for two tissue types found in the paw (muscle and fat)—Table IV and Fig. 5, associated with a BAP and a TAP. We found that the predicted BAP and TAP temperature maxima do not differ in a statistically significant way.

IV. DISCUSSION

We observed reliable withdrawal of the inflamed paws of rats after application of iFU, using each of a single pulse of iFU (a BAP) or rapidly applied iFU pulses (a TAP). This is supported by our ability to determine a minimum amount of iFU to induce withdrawal—an “iFU threshold value” for all our rats—and that was always associated with the inflamed paw, never with the contralateral paw. Our observation of reliable withdrawal induction by iFU stimulation is also consistent with our measured values of sensitivity and specificity associated with iFU application (each greater than

TABLE III. The average and standard deviation of predicted maximum displacement values of the data set collected for each acoustic protocol in the four different tissues types that were modeled.

Intensity (W/cm ²)		Predicted tissue displacement (μm)	
		Muscle	Fat
TAP			
Average	308.0	11.8	6.2
Standard deviation	96.6	3.7	2.0
BAP			
Average	1295.5	49.6	26.2
Standard deviation	354.4	13.5	7.2

FIG. 3. Threshold results for each acoustic protocol in CFA injected rats in units of acoustic dose.

TABLE IV. The average and standard deviation of predicted temperature change values of the data set collected for each acoustic protocol in the four different tissues types that were modeled.

Acoustic dose ($\text{W}/\text{cm}^2 \cdot \text{s}$)		Predicted temperature change ($^{\circ}\text{C}$)	
		Muscle	Fat
TAP			
Average	115.5	22.2	20.8
Standard deviation	36.2	7.0	6.3
BAP			
Average	97.2	25.6	20.7
Standard deviation	26.6	7.0	5.7

90% for a TAP; for a BAP—sensitivity around 75% and specificity greater than 90%). Moreover, iFU stimulation had no influence on the Hargreaves latency times, a measure of the sensitivity of tissue to stimulation by light-induced heat. This is a functional measure of the iFU safety; the rat's response to a standard heat source did not change after iFU application. This amount of iFU is also consistent with that applied to humans by a variety of authors (Dickey *et al.*, 2012; Dalecki *et al.*, 1995; Gavrilov *et al.*, 1977a,b; Gavrilov *et al.*, 1996). Also, our recent work (Garcia *et al.*, 2013) showed that the iFU stimulation threshold generated by single pulses of iFU for inflamed tissue tracks the known diurnal component of inflammatory pain. Taken together, these results support the translation of the view articulated by Gavrilov and colleagues (reviewed in Gavrilov *et al.*, 1996) in the context of neuropathic injury to that of inflammatory disease, namely, that iFU might one day find utility in diagnosing inflammatory pain conditions.

We further hypothesized that an iFU protocol consisting of a BAP would create an observable withdrawal by the animal at minimal values of iFU when applied to damaged tissue with greater stimulation than an iFU protocol incorporating rapidly repeated bursts of iFU (again, a TAP), each with the same duration as a BAP. Our results support this hypothesis when given in terms of *intensity*: The iFU threshold intensity for BAP is very much larger than that of

FIG. 4. The time course of predicted temperature change in tissue (here, for muscle) associated with TAP and the predicted temperature induced by an ultrasound protocol consisting of a single pulse with the same net time as the TAP protocol (375 ms). See Table II for reports of the average maximum value of the predicted temperature change induced by iFU.

FIG. 5. Predicted maximum temperature change ($^{\circ}\text{C}$) at threshold for each acoustic protocol in CFA injected rats using muscle as model tissue.

TAP. This is consistent with the fact that the maximum palpation induced by iFU is proportional to intensity (Myers, 2006) as well as with the observations of Gavrilov *et al.* (Gavrilov *et al.*, 1977a,b; Gavrilov, 1984) and of Dalecki *et al.* (1995) that for short bursts, iFU stimulation of the tissue of healthy volunteers occurs at least via the “push” generated by ultrasound.

We also observed, however, that the iFU threshold *dose* for BAP and TAP did not differ in a statistically significant fashion nor did the estimated temperature increase. The TAP protocol, therefore, produced lower maximum displacement values than the BAP protocol and comparable values of maximum temperature rise, yet each acoustic protocol caused the animal to withdraw from iFU stimulation.

Our work viewed in isolation therefore supports the hypothesis that iFU's temperature increase alone may have induced the animal's withdrawal of their inflamed tissue from iFU stimulation instead of palpation. Recall, however, the review of prior work discussed in the preceding text that shows that single and repeated bursts of iFU of the sort we used generate at least focal palpation. Our present results are therefore also consistent with the hypothesis that rapid and repeated stimulation by iFU may have caused our animals to withdraw from the iFU stimulus through a combination of temporal summation via focal palpation and heat induction at least for inflamed tissue.

How might we tease out these potential contributors to the withdrawal response to iFU stimulation of inflamed tissue that we observed? This will require an understanding of which sensory and/or nociceptive pathways carry iFU-induced sensations from the periphery into the central nervous system tissue (muscle, bone, tendon) for iFU stimulation can each have specialized neural pathways as well as receptors (e.g., for bone, see Mach *et al.*, 2002; Ivanusic, 2009; Jimenez-Andrade *et al.*, 2010); also consider the Golgi tendon organ or GTO, located where skeletal muscle and tendon meet, that plays a role in proprioception at least (Mancall and Brock, 2011). Future work should consider use of ultrasound protocols that emphasize heat induction (generally long pulses, for example) versus palpation induction

(generally short pulses, for example) applied to animals that have been genetically modified so shut off the ability of knock-out mice to sense heat (Caterina *et al.*, 2000) or mechanical input (reviewed in del Valle *et al.*, 2012). This is clearly beyond the scope of the present work.

V. CONCLUSION

Wright *et al.* (2002) and Dalecki *et al.* (1995) showed that application of a series of short and rapidly applied pulses of iFU to the tissue of healthy volunteers can generate sensations. We have extended this work to *inflamed* tissue, tested *in vivo*, in anticipation of eventual use of iFU stimulation to localize deep, inflamed peripheral pain generators, following up on our recent work using single pulses of iFU for diurnally varying inflamed tissue (Garcia *et al.*, 2013), and detection of focal neuropathic injury (McClintic *et al.*, 2013). Gavrilov and colleagues anticipated this diagnostic application of ultrasound for neuropathic injury.

We also assayed the possibility that iFU could generate temporal summation after its application to inflamed tissue. We found that further work is required to show unambiguously that iFU can induce temporal summation through its palpation of tissue, likely through use of electrophysiological studies performed simultaneously with iFU stimulation by TAP-like protocols of mice genetically ablated for heat sensation or for mechanical sensation. Nonetheless, our work and that of Wright *et al.* point to the possibility that deep peripheral stimulation of a sensitized nociceptive field by iFU-induced temporal summation may one day offer a useful approach to study a variety of chronic inflammatory pain syndromes, all of which are associated with deep but not cutaneous tissue, and induction of large temporal summation responses relative to healthy tissue due to the action of central sensitization.

As such, iFU stimulation, likely under image guidance, may eventually find a place not only as a means of localizing peripheral tissue contributing to pain at all stages (acute and chronic) but also represent a means of probing the central nervous system for the presence and level of effect of central sensitization, a major contributor to the pathophysiology of *chronic* pain.

ACKNOWLEDGMENTS

Funding was provided by the Life Sciences Discovery Fund of Washington State, National Institutes of Health (R41 NS 049719-01), and the Veterans Administration (Office of Research and Development Rehabilitation R&D Program). We'd like to thank Rowen Tych for his invaluable assistance with the animal model.

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