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Reduced intraepidermal nerve fiber density after a sustained increase in insular glutamate: a proof-of-concept study examining the pathogenesis of small fiber pathology in fibromyalgia

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Abstract

Introduction: Neuroimaging reveals increased glutamate within the insula of patients with fibromyalgia (FM), suggesting a link between FM symptoms and increased central excitatory neurotransmission. Many patients with FM also present with decreased intraepidermal nerve fiber density (IENFD), consistent with small fiber pathology. It remains unknown, however, whether either of these mechanistic findings represent a cause or a consequence of the other. This study tests the hypothesis that an excitatory imbalance within the insula leads to small fiber pathology.

Objectives: This is a proof-of-concept study to examine whether a chronic, bilateral increase in insular glutamate can be a causal factor in the development of small fiber neuropathy in FM.

Methods: The glutamate transport inhibitor L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC), which increases endogenous levels of glutamate, was dissolved in Ringer solution and bilaterally delivered into the insula of rats for 6 weeks. Naive rats that did not undergo any surgery or treatment and rats administered Ringer vehicle solution into the insula served as controls. Multimodal nociceptive sensitivity was assessed weekly. Hind paw tissue biopsies were collected for IENFD assessment, at the end of the experiment.

Results: Compared with controls, increasing endogenous glutamate in the insula with PDC caused sustained decreases in mechanical paw withdrawal threshold and thermal paw withdrawal latency, increased aversion to noxious mechanical stimulation, and a decrease in IENFD. Cold reactivity was not altered by PDC administration.

Conclusion: Bilateral insular PDC administration produced a persistent increase in multimodal pain behaviors and a decrease in peripheral nerve fibers in rat. These preclinical findings offer preliminary support that insular hyperactivity may be a casual factor in the development of small fiber pathology in FM.

Keywords: L-trans-Pyrrolidine-2,4-dicarboxylic acid, Osmotic pump, Chronic delivery, Chronic pain, Rat, Central pain

1. Introduction

Fibromyalgia (FM) is a chronic pain condition with well-described central nervous system (CNS) mechanisms.[49] Over a decade, human neuroimaging has revealed augmented activation of, and functional connectivity between, pronociceptive brain regions, including the insula and anterior cingulate cortex (ACC), contribute to FM pain.[12,24,31,41] Elevated levels of excitatory neurotransmitters (ie, glutamate) and decreased levels of inhibitory neurotransmitters (ie, gamma-aminobutyric acid) have also been identified in the insula of patients with FM,[17,26–28] suggesting that an excitatory inhibitory neurochemical imbalance may also play a role in FM. In support of this hypothesis, pregabalin, a treatment efficacious in FM, reduced glutamate levels and polysensory activation in the FM insula.[25,29] Preclinical studies substantiate insular involvement in nociception[1,40] and that the balance of excitatory and inhibitory neurotransmission in the insula contributes to the modulation of nociceptive reactivity.[33,55] Together, these findings suggest that insular hyperactivity may be a prominent underlying feature of FM and similar chronic pain conditions.[8,53]

In addition to these findings in the CNS, we and others have also identified reductions in intraepidermal nerve fiber density (IENFD) in FM.[5,35,39] Debate continues with respect to the meaning of these findings.[9] Some suggest that these changes

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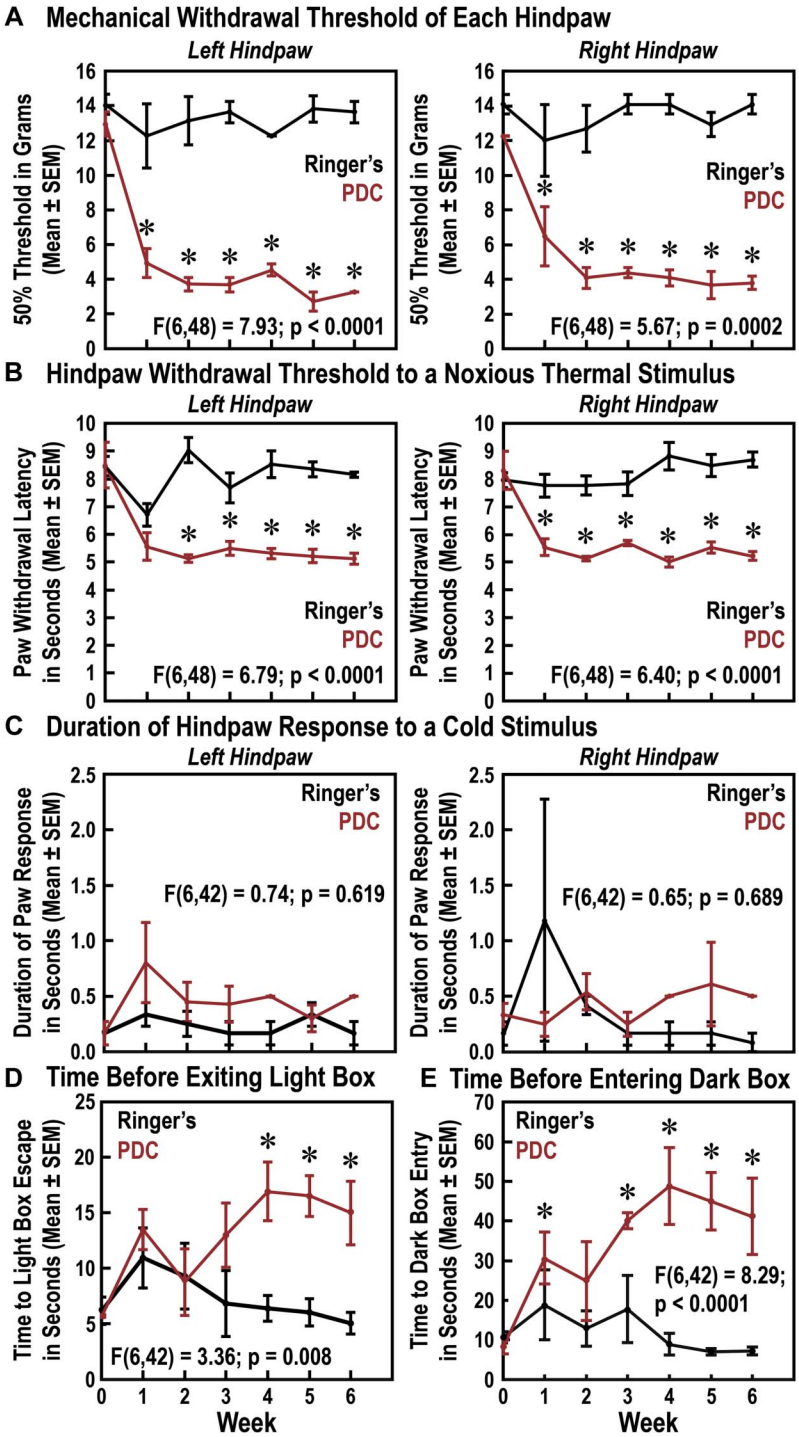

Figure 1. Chronic elevation of endogenous insular glutamate produced a sustained increase in pain behavior. For the assessment of pain behavior, rats were acclimated to experimental apparati weekly before and during the experiment for a minimum of 30 minutes on nontest days. Baseline behavioral responses were measured before surgery (week 0). Tests began between 9.30 and 10.30 hours. Mechanical paw withdrawal threshold (PWT) was assessed by sequentially testing von Frey monofilaments in ascending or descending intensity order, based on negative or positive paw withdrawal responses, respectively (up-down method).[6,55] Once 6 responses were recorded, an equation was used to determine the 50% PWT in grams (g); 15 g was recorded as the PWT after 4 negative responses to the 15-g filament. Thermal paw withdrawal latency was assessed using a Plantar Analgesia Meter with a heated glass floor (30°C). For this test, the thermal source is focused onto the plantar surface of a hind paw and then the light source and a timer are simultaneously activated. Immediately on paw withdrawal, the thermal source and timer are deactivated. Each test includes 10 measurements (5 on each paw, alternating paws for each measurement). To evaluate cold sensitivity, 100 μL of acetone was applied to the plantar surface of the hind paw. The duration of time the paw was elevated in response to acetone over the course of a minute was recorded for analysis. Pain affect was assessed using the Mechanical Conflict Avoidance System. The conflict was a choice between (1) escaping an aversive but nonnoxious stimulus (light compartment) by crossing a field of noxious mechanical probes to reach a dark compartment or (2) remaining in the light compartment to avoid noxious stimulation. Bilateral administration of L-trans-Pyrrolidine-2,4-dicarboxylic acid (n = 4) significantly decreased PWT (A) and paw withdrawal latency (B) in both hind paws compared with Ringer (n = 6) administration over the course of the 6-week experiment. No difference in cold reactivity was observed (C). Latency to escape the Mechanical Conflict Avoidance System light compartment (D) and duration to cross noxious mechanical probes (E) significantly increased in rats receiving bilateral infusions of L-trans-Pyrrolidine-2,4-dicarboxylic acid, suggesting that rats with increased insular glutamate perceived the noxious field as more nociceptive than the rats receiving Ringer vehicle solution. *$P \leq 0.024$ compared with Ringer's.

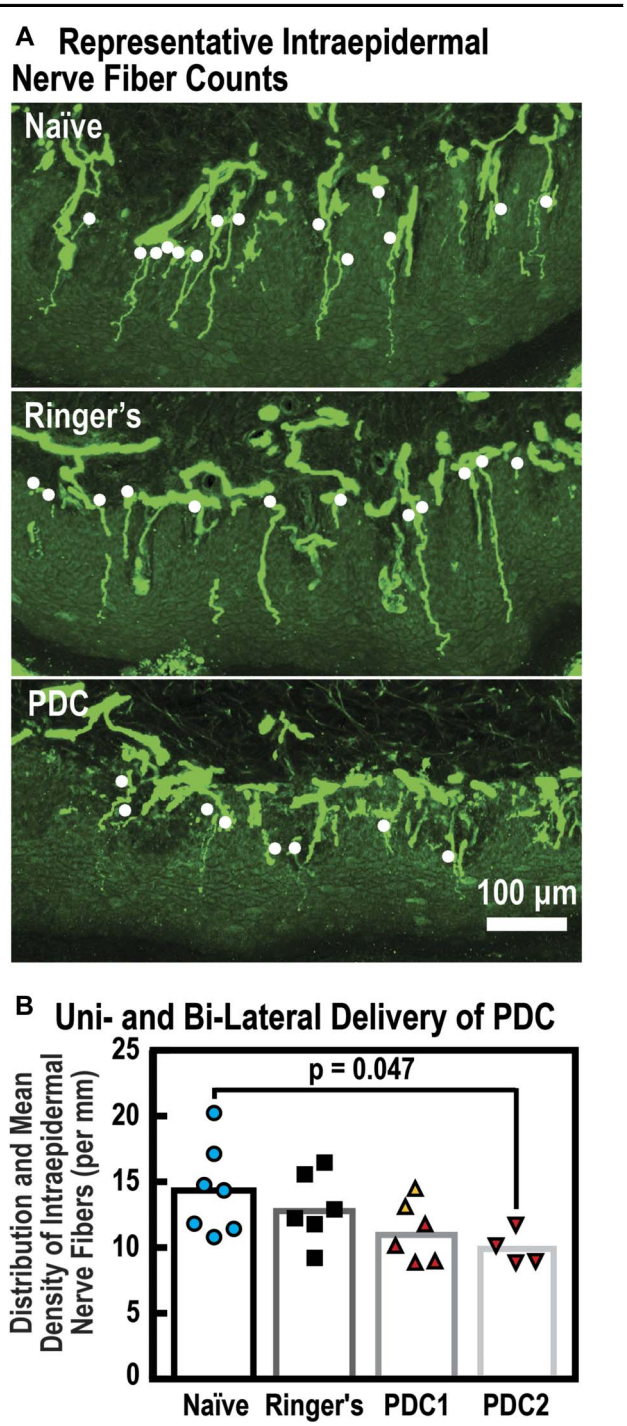

Figure 2. **Chronic elevation of endogenous insular glutamate decreased intraepidermal nerve fiber density (IENFD).** Immediately following animal dispatch, the most distal papillae from glabrous skin of both hindpaws was removed with a razor blade and placed in 2% Zamboni's fixative for 4–6 hours. Tissue was rinsed in 30% sucrose in 1X PBS overnight and embedded in OCT in 4565 Tissue-Tek molds the next day. Thirty micron cross-sections from the largest area of the papillae were sectioned and stained with protein gene product 9.5 (PGP9.5) at 1/2500, visualized with Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488; Thermofisher; Pittsburgh; PA; Cat #: A-11034) preadsorbed (ab150065), and mounted on slides with prolong gold with 4′,6-diamidino-2-phenylindole (DAPI). A technician blinded to experimental group, using a Nikon microphot FXA upright microscope with the X-Cite 120Q light source and 40x Plan Apo lens, counted images manually. Fibers that cross over the basement membrane and into the epidermis were counted as positive fibers (**A**; denoted by white dots on each image). Distance was measured in mm and final data is presented as fibers per mm. Hind paw IENFD was compared between experimental groups (**B**): Naïve (n=7; blue circles; no surgery/treatment; mean ± SEM: 14.36 ± 3.42), Ringer's (n=6; black squares; insula-administered Ringer's vehicle solution; 13.03 ± 2.65), and PDC1 (n=6; at least one microinjector in the insula delivering PDC; 11.22 ± 2.29) or PDC2 (n=4; both microinjectors in the insula delivering PDC; 9.23 ± 1.33). In the PDC1 column, the yellow triangles indicate rats that received unilateral injections and the red triangles represent rats that received bilateral injections from the PDC2 group. For each rat, IENFD was not different between left and right hind paws. A nonsignificant reduction in IENFD was observed in PDC1 rats compared to the Naïve and Ringer's groups ($H(3) = 3.25$, $p = 0.201$). In a secondary analysis restricted to PDC2 rats, IENFD was significantly reduced following PDC administration ($H(3) = 6.33$, $p = 0.034$), signifying that bilateral insular administration of PDC is necessary to significantly reduce hindpaw IENFD. Post hoc analysis revealed a decrease in IENFD in the PDC2 group compared to the Naïve group ($p = 0.047$). No other post hoc comparisons were significant.

are evidence of peripheral pathology driving the pain of FM. By contrast, we hypothesize that small fiber pathology is a consequence of FM that represents a functional reorganization of the peripheral nervous system in response to CNS hyperactivity.

We directly evaluated this hypothesis in the present pilot study by experimentally increasing glutamate in the rat insula for 6 weeks through infusion of L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC),[4,32] an excitatory amino acid transporter inhibitor that increases endogenous glutamate, and measuring hind paw IENFD and multimodal pain behavior.

2. Methods

Experiments were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC) and followed established guidelines.[11,58] Adult, male Crl:CD(SD) (Sprague Dawley) rats (n = 20; Charles River Laboratories; 275–300 g on delivery) were housed in an environmentally controlled facility (12 hour light/dark cycle, 0600 lights on) with free access to food and water. Anesthesia was isoflurane in 100% O_2 (3% for induction, 1.5%–2.2% for maintenance). Each rat was implanted with 2 subcutaneous, bilateral Alzet model 2006 osmotic pumps that were attached using PVC tubing to 2 bilateral microinjectors aimed for the insula (from bregma in mm: anterior–posterior [AP] = 2.52, medial-lateral [ML] = ±3.8, dorsal-ventral [DV] = −6.8).[44] Microinjectors were anchored with 6 screws and dental acrylic.[55,56] The pumps were filled with Ringer solution (in millimolar (mM): 150 NaCl, 2.68 KCl, 1.1 $MgSO_4$, 1.22 $CaCl_2$, 0.5 NaH_2PO_4, 1.55 Na_2HPO_4) or Ringer containing 2 mM of PDC (49 ng/h). Each pump delivered at a flow rate of 0.15 µL/h for 6 weeks.

Mechanical paw withdrawal thresholds were assessed using the von Frey up–down method.[6,55] Paw withdrawal latency to a noxious thermal stimulus was determined using an IITC (Woodland Hills, CA) Plantar Analgesia Meter.[23,55] Cold sensitivity was tested using paw response duration to acetone application.[55] The affective-motivational aspect of nociceptive behavior was assessed using the Mechanical Conflict Avoidance System (Coy Laboratory Products, Grass Lake, MI).[30,37] See **Figure 1** legend for details on behavioral assays.

After the final experiment, brains were harvested, immediately frozen, coronally sectioned (40 µm), mounted on chrom-alum coated slides, fixed with 80°C paraformaldehyde vapor, and stained with cresyl violet.[55] Hind paw epidermal biopsies were also collected for IENFD assessment at this time (see **Fig. 2** and Ref. 50 for details). Microinjection sites were compared with a stereotaxic atlas of the rat brain[44] to determine coordinates of each microinjector.

Two-way repeated analyses of variance followed by Šídák multiple comparisons tests were used to compare the behavioral

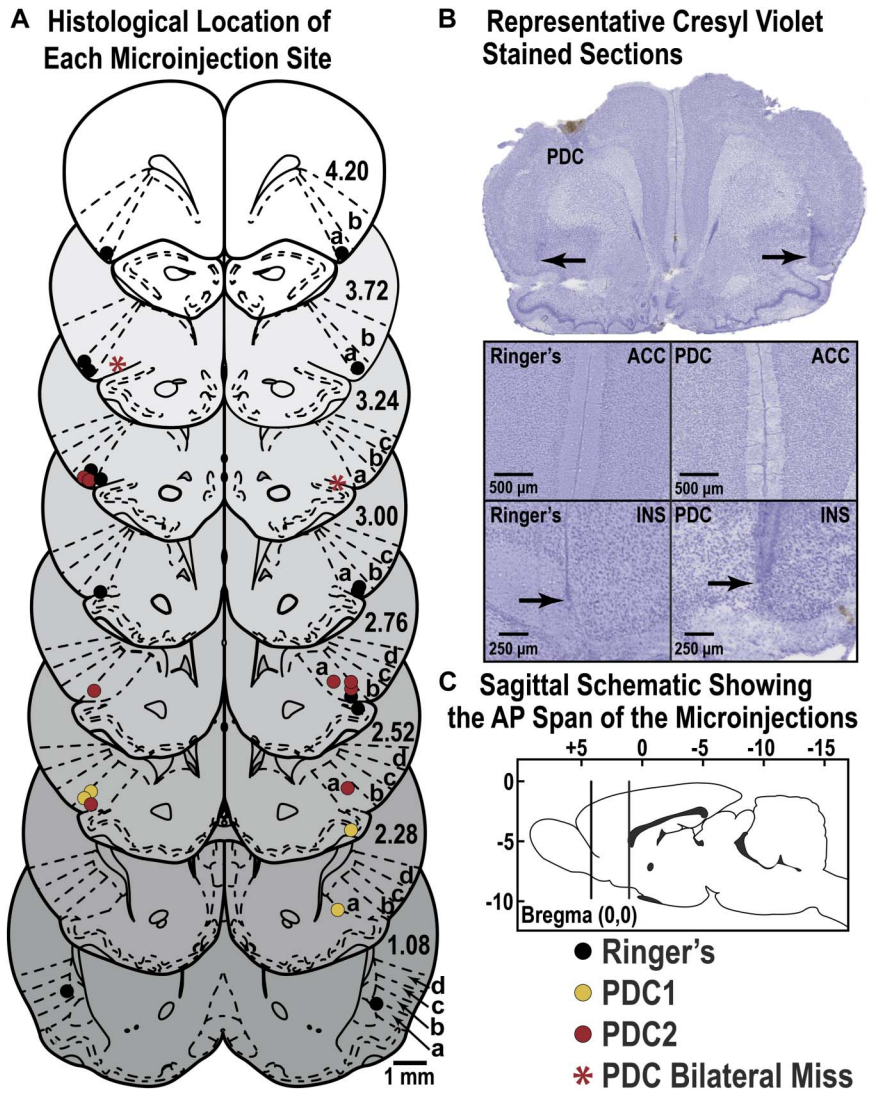

Figure 3. Chronic infusions of Ringer solution and L-trans-Pyrrolidine-2,4-dicarboxylic acid (PDC) did not produce significant morphological differences in the insula (INS) or anterior cingulate cortex (ACC). The histological localization of each microinjector site is diagramed in (A). Numbers indicate distance from bregma in millimeter. The letters indicate the following: a, ventral agranular insula; b, dorsal agranular insula; c, disgranular insula; and d, granular insula. (B) shows representative cresyl-violet stained photomicrographs of intracerebral microinjector placements. The top image shows a bilateral insula placement for PDC (AP = 2.76 mm from bregma). The bottom image in B shows a high-magnification view of the ACC and insula from a rat that received a chronic infusion of Ringer (left side) and one that received PDC (right side). Black arrows represent indicate where the bottom of the microinjector was located. Apparent morphological features are not substantially different between the ACC and insula of either rat. The AP span of all microinjection sites included in this study is depicted in (C). Schematic diagrams of the rat brain were modified from a rat brain atlas.[44] This figure was published in The rat brain in stereotaxic coordinates. 7th ed, Paxinos G, Watson C. © Elsevier (2014).

effects of bilateral PDC to Ringer administration. A Kruskal–Wallis test followed by Dunn multiple comparisons test was used to compare IENFD between the no treatment (Naive), vehicle-treated (Ringer), and PDC-treated groups. Analyses were performed in Prism 7.0a (Graphpad Software).

3. Results

L-trans-Pyrrolidine-2,4-dicarboxylic acid was used to increase endogenous levels of glutamate within the insula for 6 weeks. Compared with Ringer treatment, a bilateral increase in insular glutamate caused a significant decrease in mechanical paw withdrawal threshold (**Fig. 1A**) and thermal paw withdrawal latency (**Fig. 1B**), but had no effect on cold reactivity (**Fig. 1C**), in both hind paws starting at week 1. Aversion to noxious mechanical stimulation was also significantly increased starting at week 4 (**Fig. 1D**).

Based on histological findings, rats receiving PDC were divided into 2 groups: rats with at least 1 microinjector localized to the insula (PDC1) and rats with both microinjectors localized to the insula (PDC2). Rats in PDC1 included all rats from the PDC2 group. A partial but nonsignificant reduction in IENFD was observed in PDC1 rats compared with the Naive and Ringer groups (**Figs. 2 and 3A**); however, when examining only the subset of rats with bilateral insula placements (PDC2), a significant reduction in IENFD was observed after PDC infusion (**Fig. 2B**), signifying that bilateral insular administration of PDC is necessary to significantly reduce hind paw IENFD. Nerve fiber length was also reduced by PDC relative to the Ringer and Naive groups (**Fig. 2A**). A rat with both injectors outside the insula (**Fig. 3A**) had no apparent reduction in IENFD (15.15 fibers/mm), suggesting that the PDC effect may exhibit anatomical specificity to the insula. High-magnification photo-micrographs after 6 weeks of PDC treatment showed no

substantial morphological differences in the insula and ACC relative to Ringer administration (**Fig. 3B**), providing provisional evidence that the effect of PDC is not by producing excitotoxic lesions of pain processing regions.

4. Discussion

Bilateral insula administration of PDC for 6 weeks produced sustained increases in mechanical and heat sensitivity, increased aversion to noxious stimulation, and, most notably, a significant reduction in hind paw IENFD. To our knowledge, this is the first demonstration of an entirely "top-down" pathogenic mechanism by which increased CNS excitatory tone not only increased pain behavior but also altered the density and length of peripheral nerve fibers. Importantly, these effects do not appear to be the result of glutamate-induced excitotoxicity. Histological evaluation revealed intact insula and ACC cytoarchitecture after PDC administration. Moreover, previous studies suggest that lesioning the insula leads to decreased pain behavior,[2,3,10] not increased pain as was observed here. These findings offer preliminary support for our hypothesis that insular hyperactivity may be a casual factor in the development of small fiber pathology in FM.

Considerable debate exists regarding the relative contribution of central and peripheral nervous system factors in the development and maintenance of chronic pain. Here, a tonic elevation of endogenous glutamate in rats produced a behavioral and anatomical phenotype consistent with that observed in humans with FM. Patients with FM demonstrate diffuse mechanical and thermal hyperalgesia[21,24,45] and increased affective reactions to painful and innocuous sensory stimuli.[19,29,46] Multiple studies have also shown small fiber pathology in FM. Reduced peripheral nerve fiber density and other morphological and physiological abnormalities were identified in the skin[5,14,16,20,35,36,38,43,48,54] and corneal tissue[47] of patients with FM, as well as reports of abnormal evoked potentials.[22,54]

It remains unclear, however, whether peripheral nerve pathology causes pain in FM or whether it is an epiphenomenon of centralized dysregulation. Patients with FM respond poorly to peripherally directed interventions.[29] Small fiber pathology is found in a diverse spectrum of diseases not typically associated with pain,[13,34,42,57] and it is only observed in a subset of patients with FM.[36,43] Intraepidermal nerve fiber morphological changes, as well as the clinical phenotype in FM, are distinct compared with that observed in patients with classic, painful small fiber neuropathology.[16] Moreover, recent work in diabetic neuropathy suggests little to no association between small fiber pathology and pain.[7,18,51,52] Taken together, these data argue that small fiber pathology is a nonspecific finding unlikely to drive the diffuse pain and polysensory hypersensitivity seen in FM. Furthermore, it is even more unlikely that these peripheral findings could account for the fatigue and sleep, cognitive, and mood problems that are cardinal features of FM.

Functional neuroimaging reveals augmented nociceptive activity and excitatory neurotransmission in the FM brain, particularly within the insula, that is associated with clinical and evoked pain intensity.[12,24–27,31,41] We hypothesize that small fiber pathology in FM is a consequence of this CNS hyperactivity and represents a functional reorganization of the peripheral nervous system. Within this framework, the nervous system attempts to regain homeostasis after increased central excitability and pain by reducing peripheral nerve fiber density in an effort reduce afferent sensory input. An alternative hypothesis is that insular hyperactivity leads to major dysautonomia that in turn can cause small-diameter nerve loss.

This pilot study has limitations. Findings are based on a small number of male animals and require replication in a larger sample that includes females. The effect of PDC administration on sleep and cognitive function, as well as brain functional connectivity, was not evaluated. The duration of PDC-induced effects after the cessation of infusion and their response to antinociceptive treatment remain to be investigated. Last, morphological and molecular alterations in peripheral nerves after PDC were not assessed.

In summary, bilateral insular PDC administration produced a persistent increase in pain behaviors and a decrease in peripheral nerve fibers in rat. This study demonstrates that reverse translating one important feature of centralized pain in human chronic pain populations—increased excitatory tone in a pronociceptive brain region—appears sufficient to produce the small fiber pathology observed in FM and may represent a new animal model of FM.[15]

Disclosures

S. E. Harte has received research funding from Cerephex, Eli Lily, Forest Laboratories, and Merck and serves or previously served as a consultant for Pfizer, Analgesic Solutions, Aptinyx, and deCODE Genetics. He is coinventor of the Mechanical Conflict System. D. J. Clauw has received consulting fees from Pfizer, Eli Lilly, Nuvo, Cerephex, Tonix, Abbott, Forest Labs, Johnson & Johnson, Merck, Purdue Pharma, Samumed, Zynerba, Astellas Pharma, Williams & Connolly LLP, and Theravance. He has also received research support from Pfizer, Cypress Biosciences, Forest, Merck, Nuvo, and Cerephex. The remaining authors have no conflicts of interest to declare.

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