Acute activation of rat dorsal root ganglion neurons and their satellite glial cells by the serum of patients suffering from fibromyalgia

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Abstract

Objective

The dorsal root ganglia (DRG) may play an important role in fibromyalgia pain. The DRG house the nucleus of somatosensory neurons carrying painful stimuli from different body parts. Each neuronal soma interacts with its enveloping immune-competent satellite glial cells (SGCs). Different mediators may activate SGCs, inducing stronger SGCs-neuron coupling and leading to chronic pain. Mice injected with immunoglobulin G of patients suffering from fibromyalgia develop hyperalgesia and neuropathy; in these instances, immunoglobulin G is deposited in DRG SGCs. Our aim was to determine whether the serum of women suffering from fibromyalgia induces more immediate stimulation of DRG neurons and/or their SGCs than does the serum of healthy women.

Methods

Sera from 6 women suffering from fibromyalgia and from 6 healthy controls were tested on Wistar rat DRG neuron and SGCs primary cultures. Fluo-4 was used as intracellular calcium concentration reporter.

Results

Among the 1477 DRG neurons studied, 625 were activated by human serum. Neuronal activation in patient serum was not different from that in control serum. A total of 558 SGCs were activated by human serum. Compared with that in control serum, a greater proportion of ATP-insensitive SGCs were stimulated by patients' serum (45% vs. 34%. Fisher's exact test, p=0.0092). Furthermore, patients' serum induced significantly greater SGCs calcium influx.

Conclusions

Serum of patients suffering from fibromyalgia induces more intense and widespread acute stimulation on ATP-insensitive SGCs. DRG SGCs may play a role in the pathogenesis of fibromyalgia.

Key words

fibromyalgia, dorsal root ganglia, satellite glial cells, small fibre neuropathy

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Introduction

The mechanisms leading to fibromyalgia pain have not been elucidated (1). The predominant hypothesis views fibromyalgia as a centralized nociplastic pain syndrome (2). Nevertheless, mounting evidence backs an alternative theory of fibromyalgia as a stress-evoked, sympathetically maintained neuropathic pain syndrome. This scheme situates dorsal root ganglia (DRG) at the epicentre of fibromyalgia pain (3), DRG are neuro-immune hubs lying all along the spine and housing the bodies of somatosensory neurons, whose long nerves transmit painful stimuli from the skin, muscles, and internal organs. Each individual neuronal cell body is tightly enveloped by several metabolically active, immune-competent satellite glial cells (SGCs). Different mediators stimulate SGCs, resulting in increased cytokine release and stronger SGCs-nociceptive neuron coupling, leading to chronic pain (4). ATP is a major SGCs activator working through inotropic and metabotropic purinergic receptors.

The DRG lie outside the brain blood barrier; nevertheless, they remain covered by meningeal layers. Viruses and antigen-specific antibodies are frequent DRG residents (3). The following evidence highlights the prominent role of the DRG in fibromyalgia pain: i) the link between severe fibromyalgia and a particular DRG sodium channel genotype, ii) the clear relationship between small-fibre neuropathy and fibromyalgia (5), and iii) experiments showing that mice injected with immunoglobulin G of patients suffering from fibromyalgia develop hyperalgesia and neuropathy; in these instances, immunoglobulin G is deposited in dorsal root ganglia SGCs (6).

Accumulating evidence suggests that human serum can acutely activate murine DRG neurons (7). It remains to be established whether human serum can also stimulate DRG SGCs.

The aims of our work were i) to determine whether human serum can acutely activate not only murine DRG neurons but also their SGCs, ii) to determine whether the serum of women suffering from fibromyalgia has more pronounced acute stimulation of DRG neurons and/or their SGCs than does the serum of healthy women.

Methods

Animals

Female Wistar rats were bred, raised and housed in our institutional vivarium. Animals weighed between 210 and 270 g at the time of the study, a total of 46 animals were used in this research. The research protocol was approved by the ethics and research committees of the participating institutions (INCAR-DG-DI-CI.-DICT-023-2021) and by the National Institute of Psychiatry *ad hoc* committee for laboratory animal use and care (CICUAL 01/2021).

Cell culture

The animals were deeply anesthetised with an overdose of isoflurane and then decapitated. DRG cell isolation protocol followed the previously described method (8). Under a stereoscopic microscope DRG from the thoracic and lumbar levels were dissected, the collected DRG were immediately placed in cold (4°C) Dulbecco's modified Eagle (DMEM) culture media. DRG cells were isolated by enzymatic treatment (porcine trypsin and collagenase IA at 1 mg/mL dissolved in DMEM, for 60 min at 37°C) with subsequent mechanical dissociation via fire-polished Pasteur pipettes, this last procedure was repeated three times with a washout between each mechanical dissociation. The last resuspension of the isolated cells was done in DMEM supplemented with N2. Cells were seeded on coverslips treated with poly-L-lysine and maintained in a 10% CO_2 incubator at 37°C. The experiments were performed 18-24 hours after cell plating.

Calcium imaging

The coverslips were incubated with Fluo-4AM (5 μM) for 50 min at 37°C. Recording of intracellular Ca^{2+} microfluorometry was carried out with a Nikon inverted microscope (TE-300, Japan) equipped with a monochromator illumination source (Cairn, UK) and a 20x fluorescence objective. Fluo-4 was illuminated at a wavelength of 488 nm, and the emission was filtered at 510

nm. The images were captured at a frequency of 0.33 Hz with Metafluor 6.0 software (Molecular Devices, USA).

An extracellular solution with the following composition (in mM) was used during the DRG cell Ca^{2+} imaging experiments: NaCl 140, KCl 5, $CaCl_2$ 1.8 $MgCl_2$ 1.2, HEPES 5 and glucose 10. The pH of the solution was adjusted to 7.4, with an osmolarity of 310 mosmol/L. Cellular recognition was initially achieved by visual inspection, where DRG neurons were observed as round or ovoid somas that were refractive to phase contrast light. SGCs were identified as flat cells surrounding neurons (3). The neuronal nature of the cells was confirmed via physiological assays demonstrating elevated calcium intake in the presence of a high-K^+ solution (composition in mM: NaCl 80, KCl 60, $CaCl_2$ 1.8, $MgCl_2$, HEPES 5 and glucose 10), Capsaicin (1 μM) and ATP (10 μM), which help us to distinguish peptidergic from nonpeptidergic DRG neurons lineage, respectively. A high proportion of SGCs responded to ATP perfusion with an increase in the intracellular Ca concentration. As expected SGCs did not respond to high K^+ stimulus. We ran a dose-response curve of ATP on DRG cells in primary culture (n=9 for each point in SGC and DRG neurons). The results showed that 10 μM elicited most of the possible responses from SGC (EC_{50}=6 μM; not shown). The response to ATP is one of the best methods for detecting SGCs during Ca imaging recording, however not all SGCs express purinergic receptors (9, 10).

Complete human serum was diluted in the extracellular solution at a concentration of 1:30. The experimental flowchart was as follows: the basal fluorescence was recorded for one minute and a half, and then, human serum was perfused by gravity for 30 s and washed for 3 min. Subsequently, ATP was perfused for 15 s, and washed for 5 min, capsaicin was perfused for 15 s, and it was washed for 5 min. Finally, high-K solution was perfused for 5 s. A single flow of serum was used on each coverslip to avoid artificial desensitisation of DRG cells to repeated serological stimuli.

Table I. Demographic and clinical features of 6 patients suffering from fibromyalgia and 6 matched controls.

		Fibromyalgia n=6	Control n=6	*p*
Female (n)		6	6	0.999
Age (years)		37 ± 7	38 ± 8	0.818
Body mass index		24 ± 1	25 ± 2	0.131
Disease duration, (median, (IQR)		8 (3-10%)	-	NA
Pain visual analog scale (mm)		85 ± 18	22 ± 34	0.004
Tender points		17 ± 3	3 ± 3	0.002
Widespread pain index		16 ± 3	4 ± 4	0.002
Symptom Severity Score		9 ± 2	4 ± 2	0.009
Polysymptomatic Distress Scale		25 ± 3	8 ± 6	0.002
Revised Fibromyalgia Impact Questionnaire		57 (44-72)	4 (0.7-9)	0.002
Autonomic symptoms (COMPASS31)		38 17	13 9	0.015
Small-Fiber Symptom Survey Questionnaire		52 (41-61)	8 (3-11)	0.004
Neuropathic pain symptoms (S-LANSS ≥ 12)		6 (100%)	0	0.001
Depression (PHQ9)		10 (5-13)	5 (2-6)	0.093
Anxiety (GAD7)		4 (3-9)	4 (0-7)	0.394
Physical Activity (IPAQ)	Mild	1 (16.7%)	0	
	Moderate	2 (33.3%)	1 (16.7%)	
	High	3 (50%)	5 (83.3%)	0.400
EuroQol Health Thermometer		45 (35-50)	90 (50-97)	0.132
Decreased corneal nerve thickness: (< 5.6 microns)		4 (66.7%)	-	NA
Decreased anterior corneal plexus density: (< 87.1nerves/$mm^{2)}$		5 (83.3%)	-	NA

The intensity of the DRG cell response was defined as the fluorescence change normalised against the baseline fluorescence ($\Delta F/F_0$), and from this coefficient the area under the curve (AUC) was calculated in arbitrary units (a.u). Image analysis was carried out with MetaFluor 6 software. ImageJ was used to measure neuronal soma diameter. The researchers who conducted the *in vitro* experiments and data analyses were blinded to the serum identity (patient or healthy woman); the serum tubes were identified with an unrelated alphanumerical code.

Patients and controls

We studied the serum of 6 women diagnosed with fibromyalgia and 6 healthy controls matched by age, sex, and body mass index. In each set of experiments, a patient serum and a matched control serum were tested. All patients fulfilled the 1990 American College of Rheumatology fibromyalgia criteria and the 2016 Wolfe *et al*. criteria. Patients with concurrent rheumatic, metabolic or neurological diseases were excluded, as were those taking medications potentially causing neuropathy. The same exclusion criteria were applied to the control group. At the time of blood sampling, it was verified that patients were at least 5 half-lives free from any pharmacological treatment for fibromyalgia. A rheumatologist evaluated both the fibromyalgia group and the control group to rule out any concurrent systemic autoimmune disease. All participants filled out the following clinimetric questionnaires: 2016 Revisions to the 2010/2011 fibromyalgia diagnostic criteria, Revised Fibromyalgia Impact (FIQ-R), Autonomic symptoms (COMPASS-31), Small-Fiber Symptom Survey, Neuropathic pain symptoms (S-LANSS), Depression (PHQ9), Anxiety (GAD7), Physical Activity (IPAQ) and EuroQol Health Thermometer (Table I). All participants signed a written consent form.

Statistical analysis

Data are expressed as the mean ± standard error of the mean or the median with interquartile range. The chi-square test was used to compare groups, Fisher's exact test was used to compare categorical variables, Student's *t* test was used to compare continuous variables, and the Mann-Whitney U test was used to verify distribution equality. A p value <0.05 was considered statistically significant. Analysis was performed with GraphPad Prism v 9 and 10. OriginPro 8.0 was used for AUC analysis.

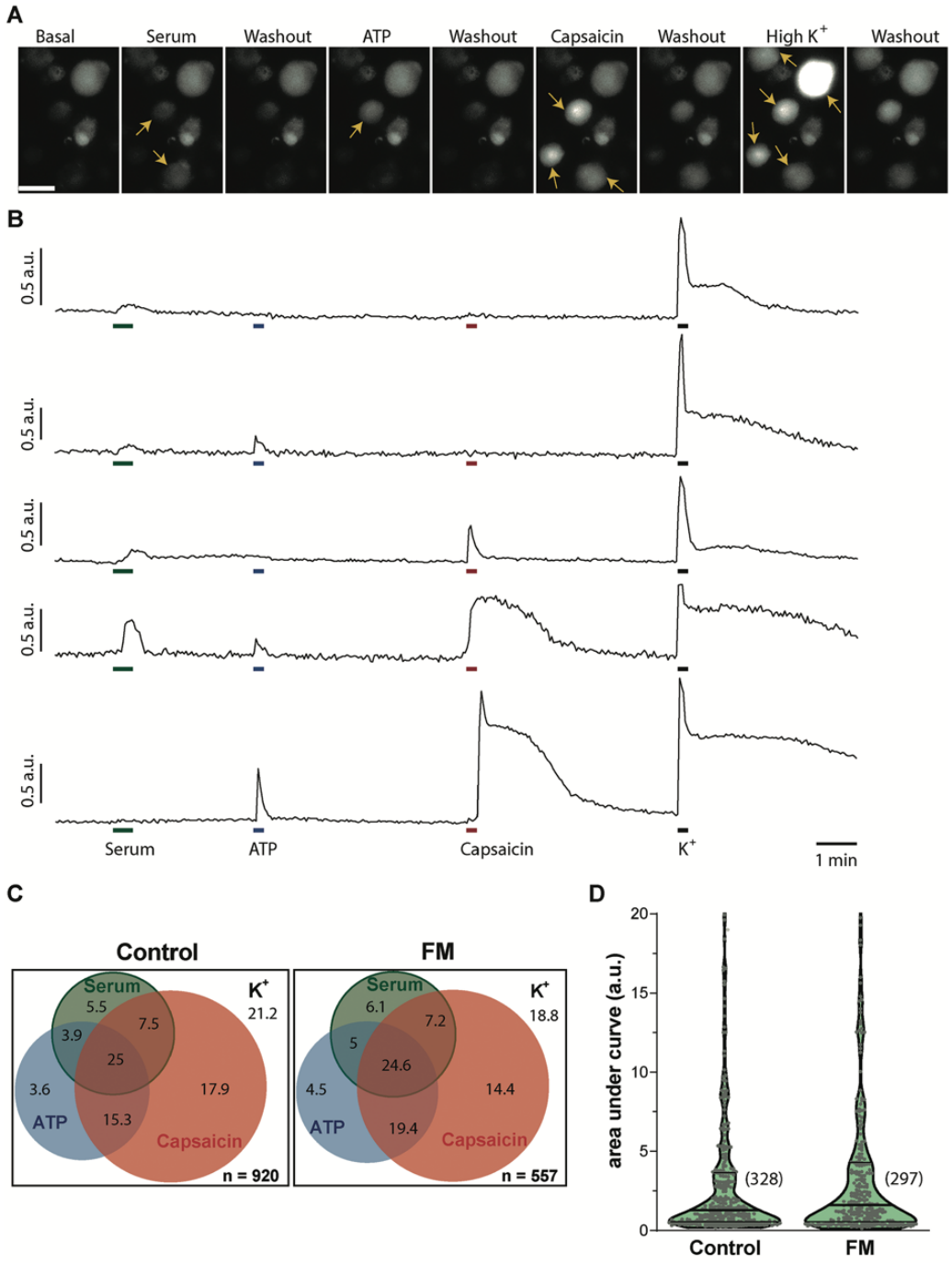

Fig. 1. Intracellular Ca^{2+} influx in rat dorsal root ganglion neurons after acute exposure to human serum.
A) Sequence of images showing two DRG neurons with increased intracellular Ca^{2+} in response to human serum (1:30 dilution), one of which was also activated by ATP (10 μM) and capsaicin 1 μM; (4th tracing in B), whereas the other neuron responded only to capsaicin (3rd tracing in B); the scale represents 30 μm. **B**) Representative time tracing showing the variability of DRG neuron intracellular Ca^{2+} influx response to human serum, ATP, capsaicin, and high-K^+ solution used as neuronal depolarisation stimuli. **C**) Venn diagrams comparing the percentage of neurons increasing intracellular Ca^{2+} influx in response to different stimulating substances including the serum collected from patients or controls. The total number of studied neurons is indicated at the bottom of the diagram. **D**) AUC of DRG neuron fluorescence intensity response to patients or healthy women serum. There was no statistical difference.

Results

DRG neuron activation by human serum

DRG neurons response to different activating substances reflected, as expected, diverse neuronal populations (Fig. 1A and B). The pharmacological profile of 1477 neurons was recorded. DRG neurons elicited an increase in intracellular calcium in response to high K^+, capsaicin, ATP, human serum, or a combination of these substances (Fig. 2B). The response profile of the different neuronal subgroups was similar to what has been previously reported in cultured DRG nociceptors (Fig. 1C) (10). Furthermore, isolated cells showed negative resting membrane potentials, incoming and outgoing ionic currents and discharge of action potentials upon current injection, demonstrating that cells are viable for microfluorometric assays (Supplementary Fig. S1).

The number of neurons whose intracellular calcium concentration increased with the perfusion of fibromyalgia patient serum (43%, n=297) did not differ from the response rate to control serum (42%, n=328; p=0.492, Fischer's exact test). The size of the neurons reactive to human serum was not different between fibromyalgia patients (mean diameter of 26.8±0.3μ) and controls (26.4±0.3μ; p=0.3351, unpaired t test). There was no significant difference in the relative magnitude of the response calculated by the AUC analysis between patient serum (median=1.6 a.u.,

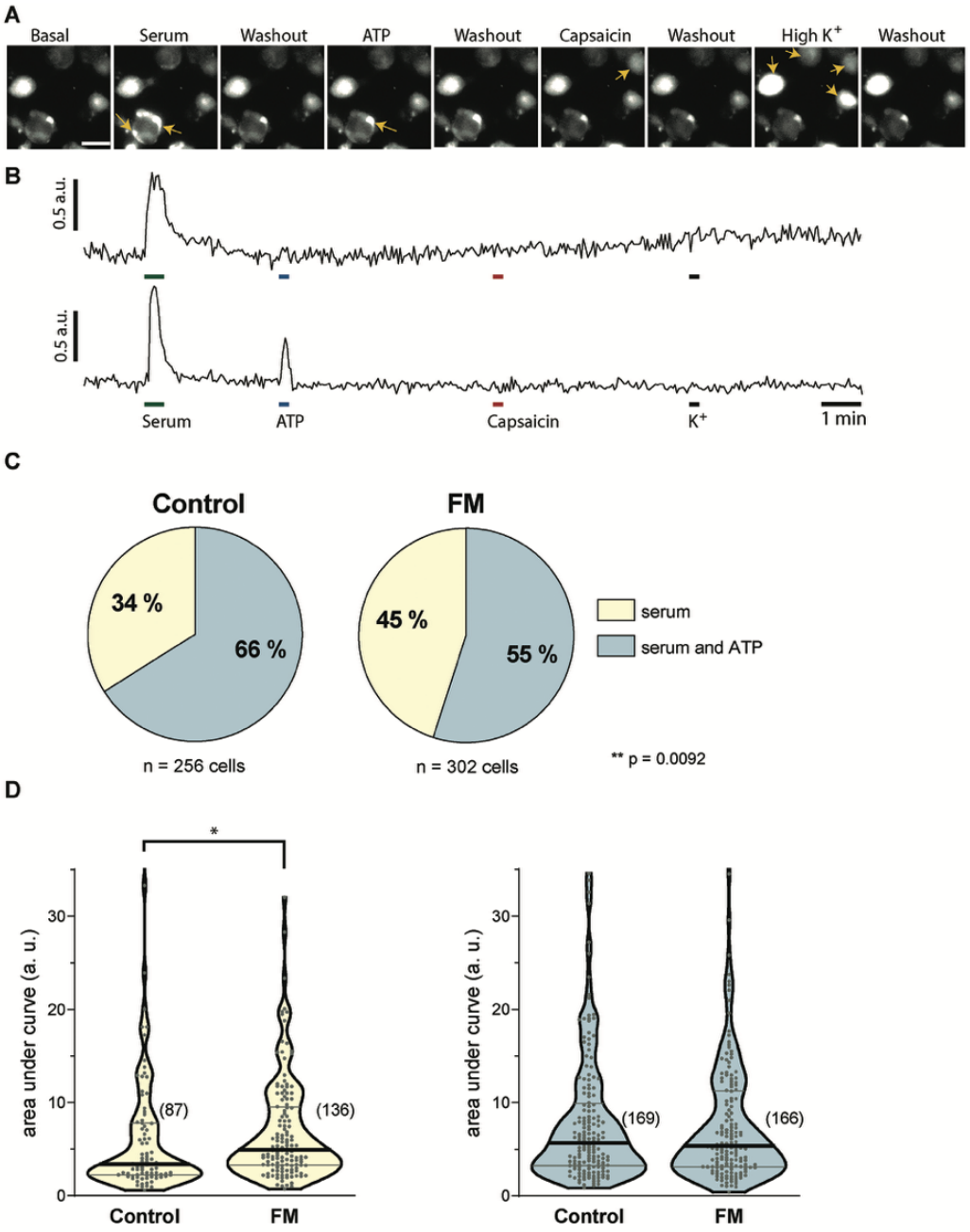

Fig. 2. Intracellular Ca^{2+} influx in dorsal root ganglion satellite glial cells after acute exposure to human serum.
A) Image sequence showing intracellular Ca^{2+} influx response of SGCs to human serum (arrows), but not to ATP, capsaicin or high K^+; (scale length = 30 μm). DRG neurons responding to capsaicin and high K^+ are seen in the upper part of the micrographs. **B**) Representative plots of SGCs intracellular Ca^{2+} concentration after acute perfusion of human serum showing a SGC responding only to serum (upper tracing) and to serum and 10 μM ATP (lower tracing). **C**) Proportion of "serum-only" activated SGCs (yellow) compared SGCs activated by both serum and ATP (blue) in the group of patients (right) and controls (left), more SGCs were activated in the "serum only" group (Fisher's exact test p=0.0092). The total number of SGCs is indicated at the bottom of the diagrams. **D**) AUC of increased fluorescence in "serum only" activated SGCs (yellow background) and serum + ATP-activated SGCs (blue background). The AUC of increased fluorescence was greater in "serum only" activated SGCs (Mann-Whitney test, p=0.0122). The number of analysed SGCs is indicated in parentheses.

percentile 25=0.5, percentile 75=4.3) and control serum (median 1.2 a.u., percentile 25=0.6, percentile 75=3.6; Mann-Whitney U test, p=0.1646) (Fig. 1D). The subpopulation of neurons that responded only to human serum but not to capsaicin or ATP was similar in both groups (Fig. 1C).

DRG satellite glial cell activation by human serum

Flat cells attached peripherally to neurons were considered SGCs, a great proportion of these SGCs responded to 10 µM ATP (Fig. 2A) (8). In our experiments, the response to human serum perfusion with an increase in intracellular Ca^{2+} concentration was recorded in 558 SGCs. 256 SGCs were activated by control serum, of which 169 (66%) responded to serum and ATP and 87 (34%) responded only to serum. On the other hand, a total of 302 SGCs responded to patients' serum with intracellular Ca^{2+} increment, of which 166 responded to serum and ATP (55%) and 136 reacted only to patients' serum (45%) (Fig. 2C). The proportion of SGCs activated by serum but not by ATP was greater after exposure to fibromyalgia patients' serum versus controls serum (Fisher's exact test, p=0.0092) (Fig. 2B, C). Serum from patients induced significantly greater calcium influx in ATP-insensitive SGCs, with an area under the curve =4.95 (3.27–9.5) median (quartile 25–75) (n=136) *versus* an area under the curve of 3.4 (2.23–7.8) (n=87) of controls. Mann-Whitney U test, p=0.0122 (Fig. 2D).

Discussion

Our results confirm a previous report showing that human serum is able to

stimulate rat DRG neurons (7). A novel finding is that human serum is also able to induce increased Ca^{2+} influx into murine SGCs, this finding suggests the presence of a regulatory humoral mechanism in SGCs physiology.

Our main result demonstrates that compared to serum from healthy women, the serum of patients suffering from fibromyalgia acutely activates more ATP-insensitive murine DRG SGCs; furthermore, patient-derived serum induced stronger SGCs kindling.

Previous studies have used a passive-transfer model injecting IgG (6) or neutrophils (11) from patients with fibromyalgia into mice inducing mechanical hypersensitivity 24 to 48 hours after injection. Both neutrophils and IgG are deposited in the DRG. Our protocol is different, we used non-fractionated serum in an acute *in vitro* model to measure real-time calcium influx into DRG cells. Our key novel finding is that serum from patients suffering from fibromyalgia activates a specific population of ATP-insensitive SGCs likely through a non-purinergic pathway. It is possible that the yet unidentified stimulating serum substance found in our study could be the end-product of antibody or neutrophil-dependent DRG inflammation.

The ATP solution used in our experiments was expected to induce a more generalized SGCs intracellular Ca^{2+} increase via P_2Y and P_2X receptors activation (12.15); however, in our assays only half of the SGCs responded to ATP. The possible reasons for this discrepancy include the different culture conditions, the SGCs isolation method and the low ATP (10 μM) concentration used in our experiments.

In contrast to human serum, which requires 30 seconds of perfusion to activate nociceptors, ATP and capsaicin induce an immediate, within second, increase in the calcium concentration, this kinetic difference suggests that the observed response of SGCs to serum could involve ligand-receptor interactions, possibly through G-protein coupled receptors. It remains to be established whether the purported activating factor in human serum is a single molecule that stimulates both SGCs and neurons or if there are multiple compounds with different targets. Our method for directly assessing acute DRG cell activation opens different avenues of investigation, including the definition of the molecular weight of the purported activating serum substance and its eventual identification through neutralising assays. More sophisticated experiments may determine the chemical characteristics of the substances responsible for SGCs activation.

Our results support the longstanding hypothesis proposing DRG cells as main player in fibromyalgia pain (3). Other recent evidence supports this contention; as already mentioned, IgG derived from fibromyalgia patients induces hyperalgesia in mice and is exclusively deposited in the DRG (6). In a mouse model of chronic widespread pain through hyperalgesic priming of muscle, neutrophils invade the DRG and transmit mechanical hypersensitivity to healthy recipient mice; neutrophils from patients suffering from fibromyalgia transferred to healthy mice invade the DRG, inducing hyperalgesia (11). Patients suffering from fibromyalgia with elevated levels of anti-satellite glia cell antibodies display more severe symptoms (16).

Limitations

Main limitations of this study are: i) We have not identified the molecule or molecules responsible for SGC and/or neuron activation. ii) The complexity of the assay precluded the study of greater number of patients and controls. This small sample size prevents meaningful statistical correlation between clinimetric parameters and the degree of intracellular calcium influx induced by patients' serum. Increasing the serum sample size may reveal differences not observed in the current experiments. iii) Prolonged activation assays would better simulate the chronicity of fibromyalgia clinical features.

Conclusions

Human serum can acutely activate murine DRG SGCs. Compared to serum collected from healthy women, serum of patients suffering from fibromyalgia induces more intense and widespread stimulation of ATP-insensitive DRG SGCs. This new finding reinforces the proposal that the DRG may be located at the epicentre of fibromyalgia pain.

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