May 2023 Progress Report

Pain Relief Foundation Research Grant

Title: “Using live sensory neurons to assess the pathogenicity of autoantibodies from pain patients”

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1. Background and aims

There is now strong clinical and preclinical evidence to support the idea that autoantibodies (-Abs) targeting antigens within the nervous system are a mechanism to cause pain. For example, CASPR2-Abs from neuropathic pain patients are causal to pain via disrupting ion channel function leading to increased neuron excitability and enhanced pain sensitivity; one key step in their pathogenicity is their binding to sensory neurons.
We are currently taking a wider, explorative approach using sera from a range of chronic pain patients in combination with live sensory neurons to determine IgG binding and therefore identify putative pathogenic autoantibodies.
We have pre-obtained samples from a range of chronic pain patient to identify binding of patient IgGs to sensory neurons, including fibromyalgia (FMS; N=50), diabetic neuropathy (DN; N=16), sciatica (N=35), and complex regional pain syndrome (CRPS; N=50), to be juxtaposed with a large healthy control (HC) cohort (N=148).

Cohort	***N***
Fibromyalgia (FMS)	50
Diabetic neuropathy (DN)	16
Sciatica	35
Complex regional pain syndrome (CRPS)	50
Healthy controls (HC)	148

To test for binding of sera-IgG on live sensory neurons, cell-based immunofluorescence binding assays are performed with a dual-species approach (**Figure 1**):

(A) primary mouse dorsal root ganglia (DRG) sensory neurons cultured from C57/BL6 mice (6-8 weeks of age), used at 2 days in vitro; and
(B) human induced pluripotent stem cell (iPSC)-derived sensory neurons (iPSNs) used 40-50 days following the onset of differentiation, either in a monoculture format or in a myelinating coculture with rat Schwann cells.

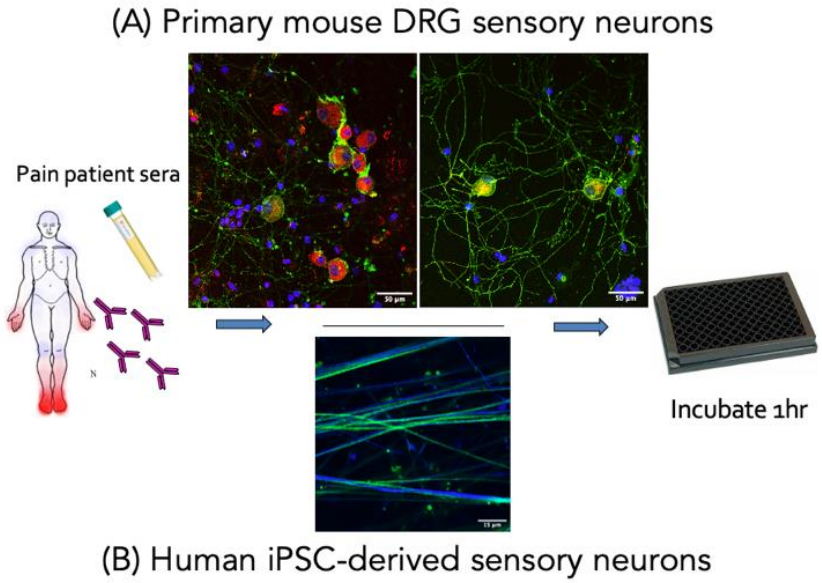


Figure 1. Schematic of the experimental approach

Sera samples were tested at different titres and were scored by experienced scorers according to an established, consistent scoring system with well-controlled inter-rater validity of an average agreement rate of 97.5% between trained scorers.

The ordinal scale comprised of 0: negative, 1: intermediate, 2: positive (moderate), and 3: positive (strong). Averaged scores for each sample were then used for further evaluation of binding, with the following bin boundaries: [0,1) [1.0,1.5) [1.5,2.0) [2.0,2.5) [2.5,3.0] .

2. Project progress

The project has achieved good progress in the first 6 months of the 1-year grant (Oct 2022 start) and is generally progressing in line with the original timetable and estimated costings. The following section describes the current data obtained.

2.1 Primary mouse DRG neuronal cultures

During the optimisation phase of the primary DRG neuronal culture-based binding assays, we tested sera samples at different titres including 1:100, 1:400, 1:1000, 1:1600, and 1:6400 – eventually settling on testing at 1:100 and 1:400 dilutions. **Figure 2** shows sample confocal images with the corresponding category for level of IgG binding to βIII-tubulin-positive cells (neuronal marker) in primary mouse DRG cultures.

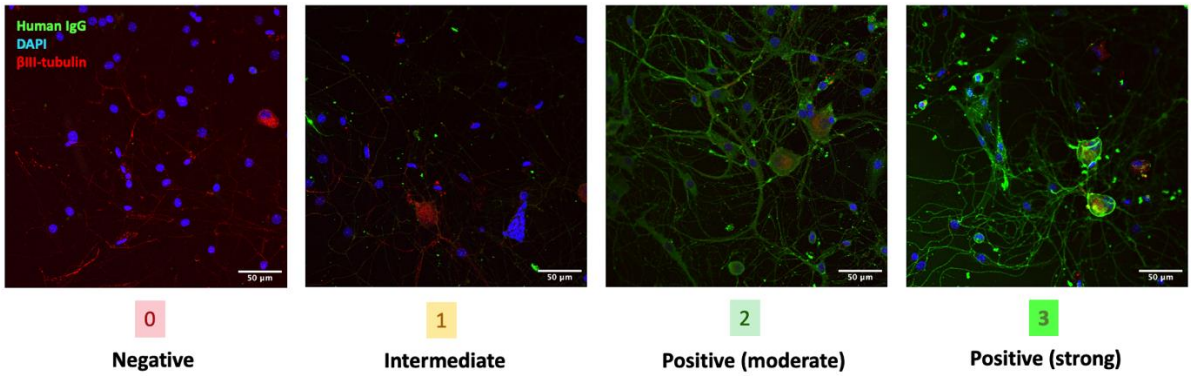
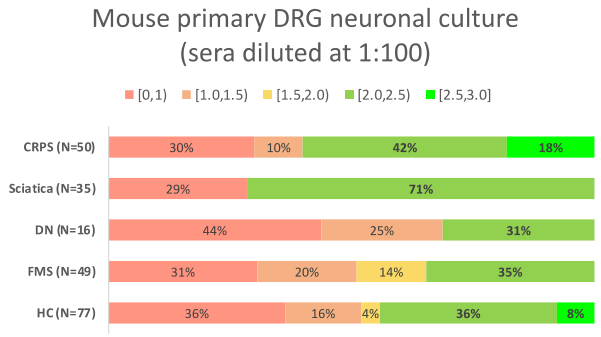


Figure 2. Sera-IgG binding on mouse primary DRG neuronal cultures.
We have now completed IgG-binding assays for all patient cohorts in primary mouse DRG neuronal cultures at 1:100 and 1:400 (**Figure 3A-B**). When tested at 1:100 dilution (**Fig. 3A**), CRPS and sciatica sera-IgG had stronger binding than that of the HC cohort; whereas when tested at 1:400 dilution (**Fig. 3B**), sera-IgG of some chronic pain cohorts (sciatica and FMS, but not CRPS and DN) had a higher level of binding to neurons compared to HCs.

(A)



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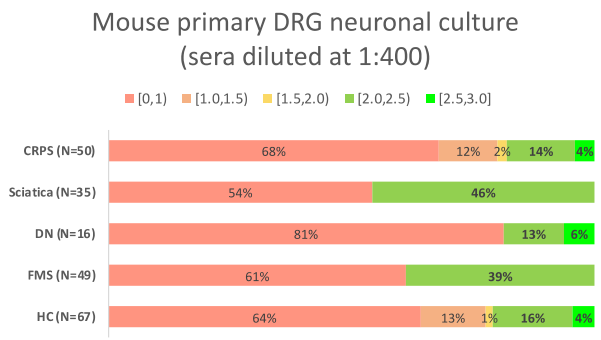

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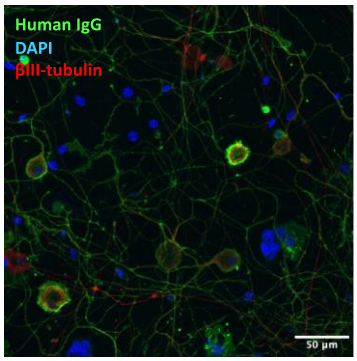

Figure 3. Distribution of averaged scores in mouse primary DRG neurons at (A) 1:100 and (B) 1:400 dilutions across the healthy control and pain cohorts including CRPS, sciatica, DN, and FMS. Non-parametric statistical testing pending, awaiting full completion of experiments planned. (C) Sera-IgG (green) binding to sensory neurons (red) in primary mouse DRG cultures.

2.2 Human iPSN monocultures

As described, we also generated human iPSN monocultures for IgG binding assays, where sera samples were used at a 1:100 dilution. Likewise, our preliminary results show that CRPS and FMS sera-IgG appeared to have a higher level of binding to AD3-cell line human iPSN monocultures in contrast to HC sera-IgG (**Figure 4**). We had however experienced unforeseen delays in this segment of the project due to technical issues, and experiments on the sciatica and DN cohorts will be performed shortly.

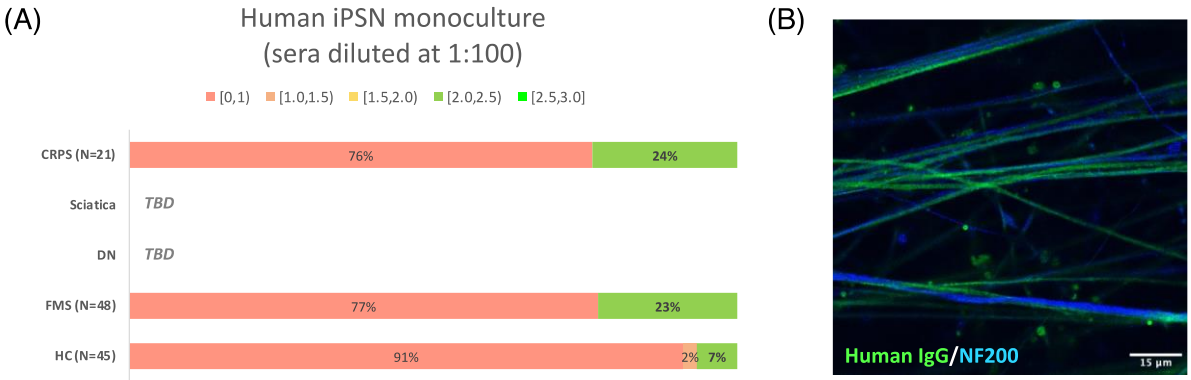

Figure 4. (A) Distribution of averaged scores across patient cohorts and HCs in human iPSN monocultures. (B) Sera-IgG (green) binding to iPSNs in the monoculture format (blue; neuronal marker).

2.3 Myelinating coculture of human iPSNs and rat Schwann cells

We have also performed binding assays with myelinating cocultures of human iPSNs and rat Schwann cells. Preliminary results show that CRPS and sciatica sera-IgG appeared to have a higher level of binding to live neurons than HC sera-IgG, but not DN and FMS sera-IgG (**Figure 5**).

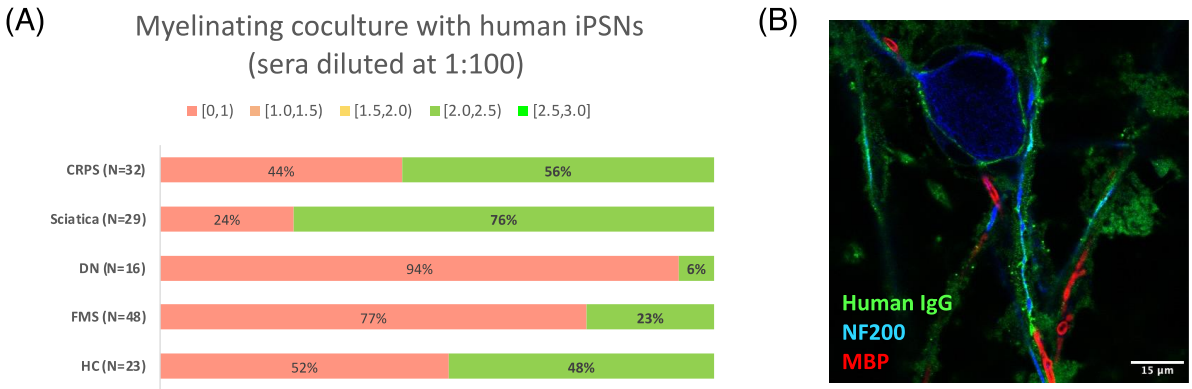

Figure 5. (A) Distribution of averaged scores across patient cohorts and HCs in myelinating cocultures. (B) Sera-IgG (green) binding to neurons in cocultures.

2.4 Conclusion and next steps

Our preliminary results reveal an overall trend of higher prevalence of IgG binding to sensory neurons in sera from pain patients than HCs. Autoantibodies have established roles in conditions such as FMS and CRPS, and our findings may help determine their cellular targets. These findings are also indicative of a novel autoantibody component in pain conditions such as sciatica.
We are currently refining the cut-off scores for bin boundaries for further evaluation, as well as establishing a semi-automated pipeline for the analysis of sera-IgG binding. Further analysis of positive binding samples will assess their impact on sensory neuron physiology and pain sensitivity to determine their

pathogenicity in pain. We will now proceed to performing subclass analysis on ‘positive binders’ to identify the subclass of autoantibodies (IgG1-4) using our live-sensory neuron-based assays.

3. Dissemination activity

1. Oxford Neuroscience Symposium 2023 – Poster Presentation (Marva Chan, March 2023, Oxford, UK)

2. British Pain Society Annual meeting 2023 – Workshop presentation ‘Using patient antibodies to better understand pain mechanisms’ (John Dawes, May 2023, Glasgow, UK).

3. NeuPSIG 2023 International Congress on Neuropathic Pain – Workshop and poster presentation (Prof John Dawes & Marva Chan, Sept 2023, Lisbon, Portugal)

4. Funding information

The project is predominantly supported by the PRF grant in conjunction with a Rosetrees Trust Seedcorn Award.