April 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.

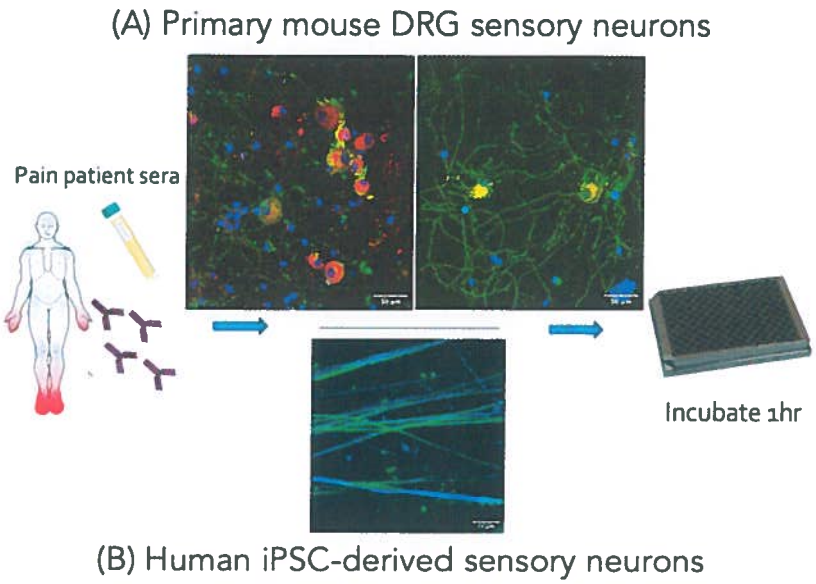

***Figure 1**. Schematic of the experimental approach*

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).

2. Project progress

There have been some delays to the project progression due to issues relating to culturing of monocultures of IPSNs and the development of the automated analysis pipeline. These issues have now largely been resolved and all experiments relating to binding have been completed and are currently being run through the analysis pipeline. 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.

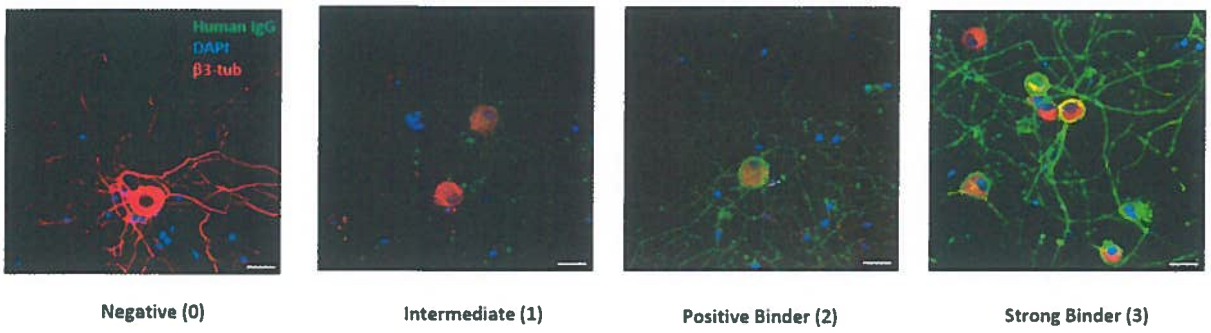

***Figure 2**. Examples images for scoring of Sera-IgG binding on mouse primary DRG neuronal cultures.*

We have now completed IgG-binding assays for all patient cohorts in primary mouse DRG neuron cultures at 1:100 and 1:400 with by-eye scoring (**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.

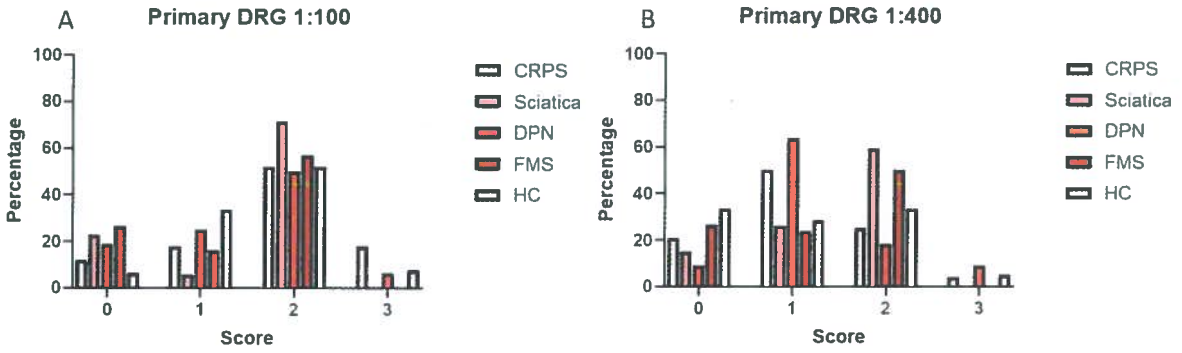

***Figure 3.** Percentage of samples scoring 0-3 for IgG binding to mouse primary DRG neurons at (A) 1:100 and (B) 1:400 dilutions across the healthy control and pain cohorts including CRPS, sciatica, DN, and FMS.*

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.

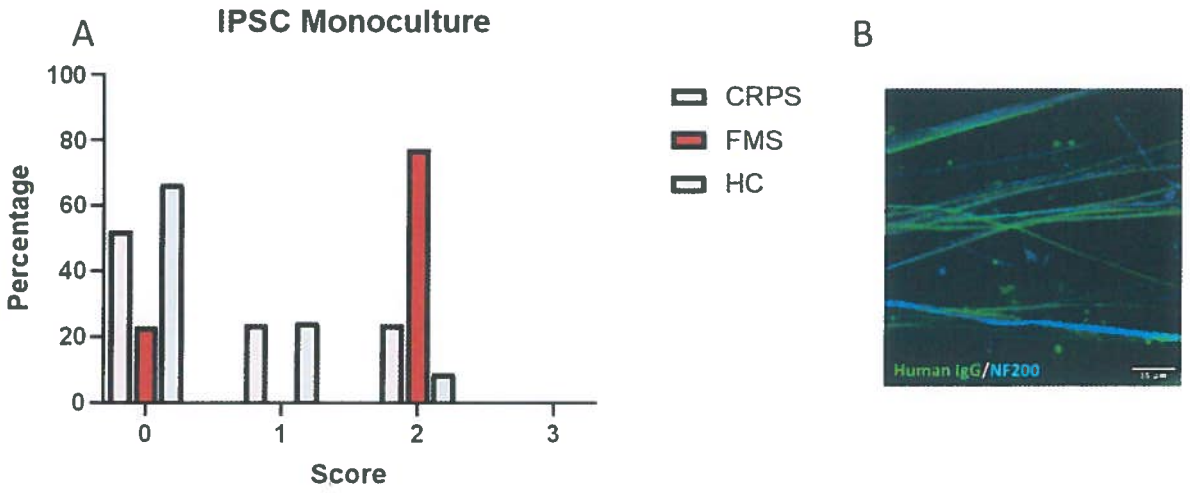

Figure 4. *(A) Percentage of scores across patient cohorts and HCs in human iPSN monocultures. (B) Sera-IgG (green) binding to iPSNs in the monoculture format (blue; neuronal marker).*

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**).

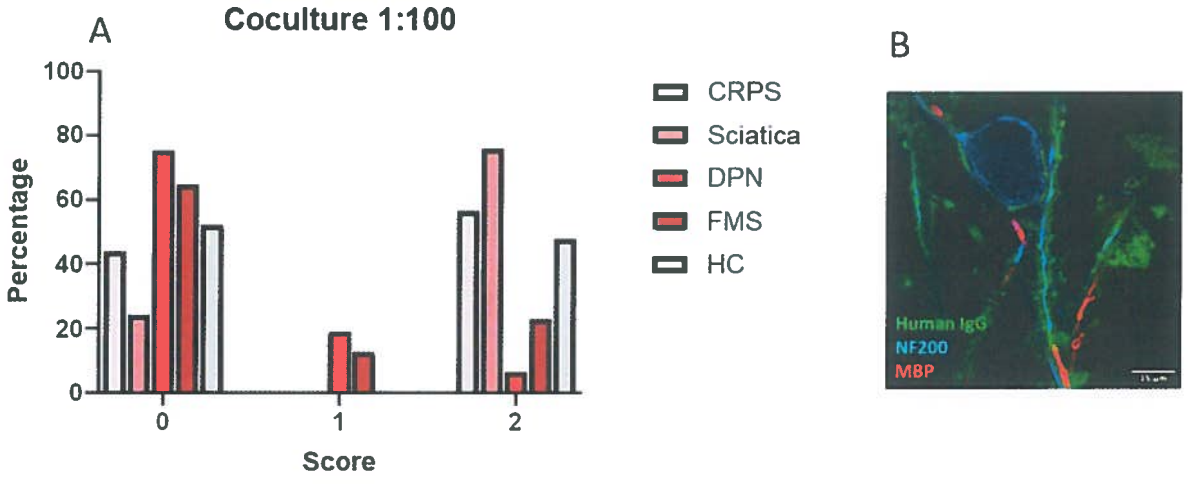

Figure 5. *(A) Percentage of scores across patient cohorts and HCs in myelinating cocultures. (B) Sera-IgG (green) binding to neurons in cocultures.*

2.4 Automated analysis pipeline:

While there is consistency between scorers for IgG-binding, this approach is time consuming and requires a high level of training. To streamline analysis, we developed an automated pipeline for

analysis of IgG-binding, reducing time constraints on the experimenter, standardising results and providing a more enriched data set (e.g. binding intensity, percentage of positive cells). Images were obtained following automated capture using a spinning disc confocal. We then employed Cellpose, a generalist algorithm for cell segmentation. Models were trained and then quality checked for high precision and accuracy, before performing mass segmentation. Segmented images were run through our analysis macro in imageJ (Fig 6). Positive cells were identified by having a ratio of > 1.0 for membrane vs intracellular IgG signal (8SDs above negative control) and the fluorescent intensity of membrane binding signal recorded.

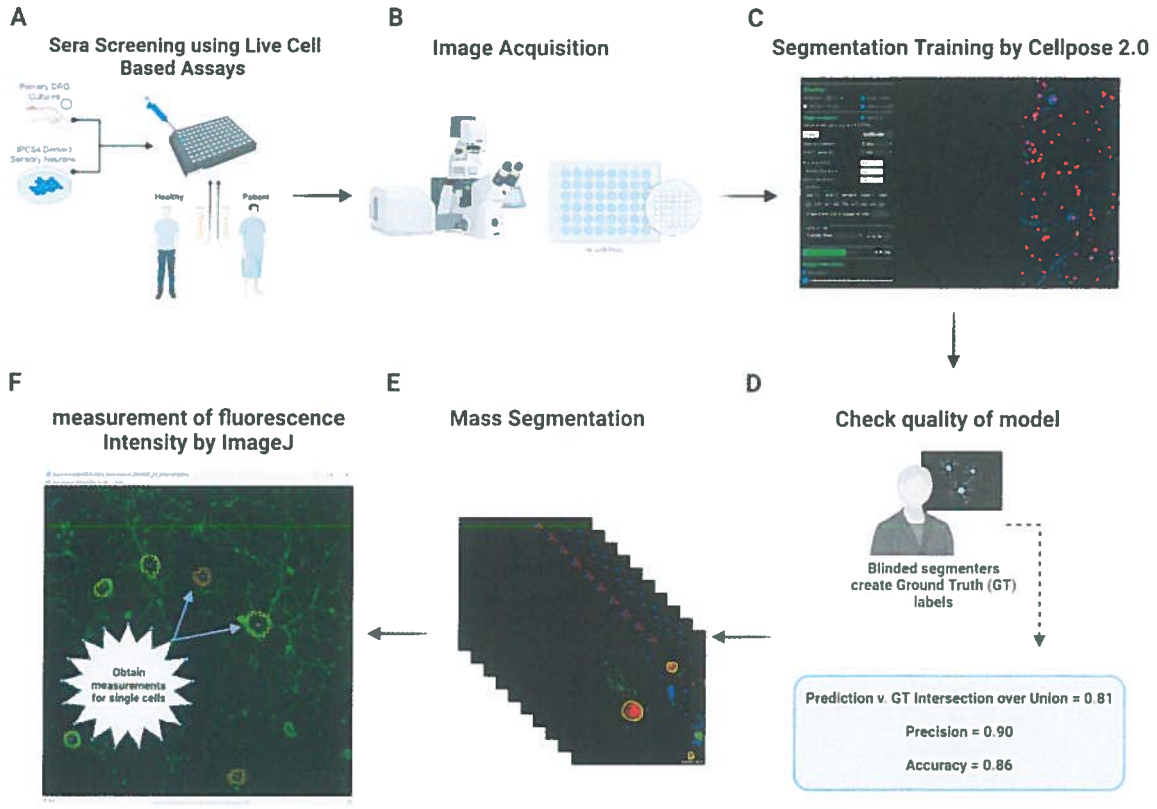

Figure 6. *Overview of the image analysis pipeline. (A) Live serum treatment assays are carried out. (B) Plates are imaged using spinning disc microscopy. (C) A model is trained though Cellpose2.0 human in the loop pipeline. (D) Quality of model is checked by comparing blinded manual segmentations to model predictions using online tool. (E) Segmentation of mass images through online graphics processing unit (GPU). (F) Outlines were matched to images and measurement carried out using ImageJ macro.*

Pipeline analysis has been completed for diabetic neuropathy samples (Fig 7) and compared to age and sex matched health controls. Using this automated approach we find that a higher proportion of DPN samples show positive binding to sensory neurons (Fig 7A) and these samples bind to a higher percentage of neurons (Fig 7B). When comparing positive samples between HC (n=3) and DPN (n=8) there is no difference in binding intensities (Fig 7C,D).

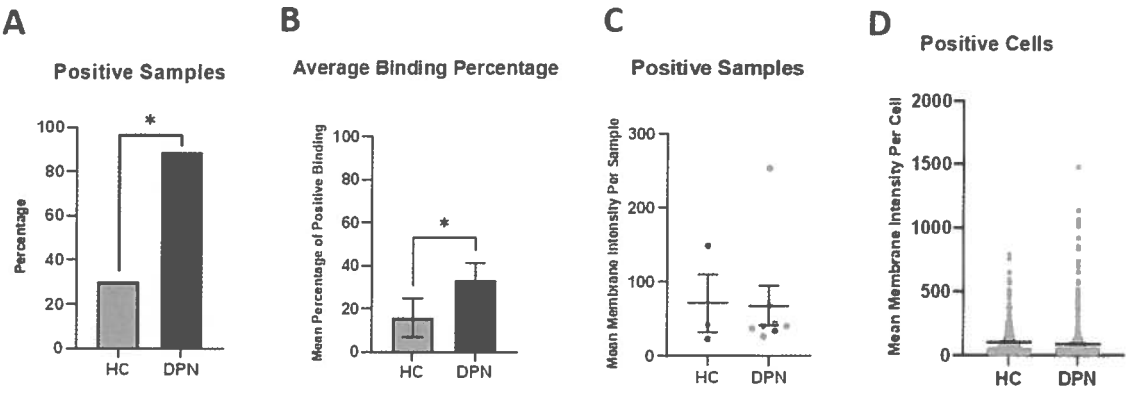

Figure 7. *Comparison of positive membrane binding between HC and DPN cohort using automated analysis pipeline. (A) Comparison of proportions showed significantly more positive samples in DPN compared to HC (DPN, n=9; HC, n=10), two-sided Fisher's exact test, * $p < 0.05$. n=number of samples. IgG from DPN samples bound to a higher percentage of DRG neurons in each sample binding percentage (33.9%, n=9) compared to HC samples (16.4%, n=10) Mann-Whitney test, * $p < 0.05$. n= number of samples. (C, D) Positive HC samples bind at a similar intensity to sensory neurons compared to DPN samples when comparing by sample (HC, n= 3; DPN, n= 8) (C) or by total cells (DPN mean= 89.9± 5.717, n= 687; HC mean= 103.4± 7.11, n= 316) (D).*

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 have now finished binding experiments for all samples on both mouse primary neurons and human IPSNs and are currently running these through our automated analysis pipeline. We will compare this to our by eye scores and positive samples will be analysed for IgG subclass and pathogenic activity.

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) Autoantibody Autoimmunity in Symptom-Based Disorders Workshop, platform presentation John Dawes, Liverpool, Sept 2023

5) Pain Research and Therapeutics conference, platform presentation John Dawes, University of Reading

4. Funding information

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