Biochemical Society Summer Vacation Studentship Report 2023

Using single-chain fragment variables to investigate the cardiac voltage channel, Nav1.5

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Background and project aims

The voltage-gated sodium channel, Nav1.5, is a large trans-membrane protein which initiates the cardiac action potential. Mutations in Nav1.5 are linked to cardiac arrhythmias, including long QT syndrome, Brugada syndrome, and atrial fibrillation (Song *et al*., 2012). Understanding the channel's mechanics, and how they are altered in pathological states, is a focus of molecular cardiology. Agents that can modulate the activity of Nav1.5 are of therapeutic interest.

There are nine Nav isoforms, but a unique sequence located in an extracellular loop of Nav1.5, named E3, has been previously identified (Figure 1). A rabbit polyclonal antiserum against this sequence inhibited peak channel conductance by 60% (Xu *et al*., 2005). However, the antiserum is no longer available. To investigate the E3 region, the Jackson Group used phage-display to isolate monoclonal single-chain fragment variable antibodies (scFvs) that targeted the E3 peptide in vitro.

The project aimed to identify anti-E3 scFvs that would specifically bind to Nav1.5 expressed on a cell membrane, and alter channel properties, to replicate and build on the findings from the polyclonal antiserum paper. The key focuses were:

1. Validate scFv binding to the E3 peptide *in vitro.*
2. Test scFv binding on the complete Nav1.5 protein.
3. Assess the impact of scFvs on cell electrophysiology.

Longer term aims were to develop a Nav1.5-specific scFv that could be modified for proximity labelling or as a general research tool, and to use computational approaches to investigate how protein-E3 interactions may alter channel mechanics.

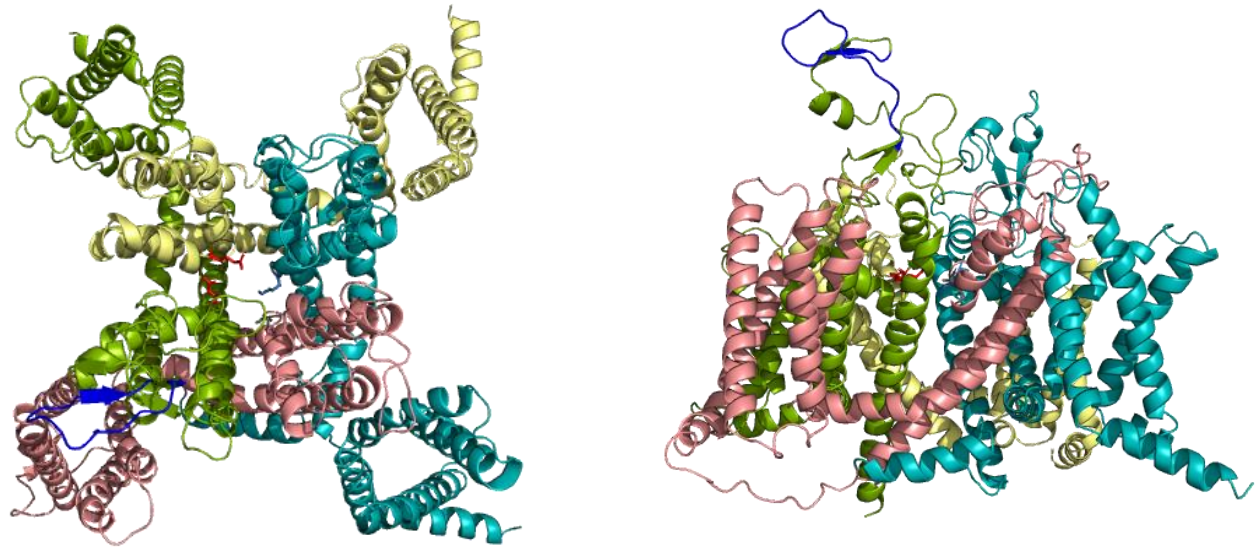

Figure 1. Cryo-EM depictions of Nav1.5 show the accessible E3 peptide.
Nav1.5 consists of four homologous domains, each with six trans-membrane helices. A central pore exists in the channel, where sodium ions are recognised by a DEKA selectivity filter that binds a hydrated sodium ion. The left-hand side is a top view, and the right is a side view of the protein. These cryo-EM depictions lack the C terminal domain, which is intracellular and connected by a disordered linker to domain IV. The navy-blue peptide that projects from the top of the protein is the E3 region of domain I.
Figures were generated with PyMOL and have been taken from the Jackson Group, Department of Biochemistry, University of Cambridge.

Summary of work

Phage display against the Nav1.5 E3 peptide had been previously done by Dr Samir Hamaia to identify candidate scFvs. Eight scFvs with distinct sequences were chosen for further investigation. The *E. coli* monocultures (strain BL21) that expressed the scFvs were grown in 1 litre volumes of LBE autoinduction media, before bacterial pellets were lysed and scFvs were obtained through periplasmic protein purification. Proteins were buffer swapped into TBS, and concentrations were estimated using the using the A280 method (Kielkopf *et al*., 2020).

scFvs were then used in ELISAs against the human E3 peptide to assess binding activity. Cross-reactivity was also assessed with the mouse E3 peptide.

xCELLigence RTCA assays were employed to detect scFv binding to the full Nav1.5 protein, expressed in a HEK-293 cell line. After multiple attempts, the positive control failed to yield a result, and an alternative assay was chosen to assess scFv-Nav1.5 interactions. A protein pulldown with HEK-293-Nav1.5 cell lysates, followed by western blotting, was attempted instead.

Automated patch clamp electrophysiology was performed with HEK-293-Nav1.5 cells with/without a pre-incubation in scFvs (1 µM). Voltage-current characteristics were obtained.

Deviations from original proposal

While we were trying to troubleshoot the xCELLigence RTCA assay, I carried out some work on the C-terminal domain of Nav1.5, screening scFvs against a unique helix, reinforcing skills in protein purification and ELISA. Due to issues that arose during the project, we ran out of time to screen all scFvs and perform computational investigations.

Results and outcomes

Result 1: scFv 2 has cross-reactivity with the mouse E3 peptide construct.

An ELISA carried out with both human and mouse E3 constructs found scFv 2 to have affinity to the mouse peptide (Figure 2). Other scFvs showed potential as well. Differences in cross-reactivity suggest that scFvs bind different epitopes within the small E3 region.

Result 2: scFv 2 binds to the full Nav1.5 construct expressed on mammalian cells.

Affinity pulldowns and western blotting portrayed interaction between scFv 2 and Nav1.5 (Figure 3). FLAG-conjugated scFvs were incubated with EZ-view red anti-FLAG beads, prior to incubation with HEK-293-Nav1.5 cell lysates. Western blotting with an anti-Nav1.5 antibody was used to assess whether scFvs could pulldown Nav1.5. Pulldowns suggested that clone 55, the positive control for xCELLigence, has probably denatured.

Result 3: scFv 2 does not appear to alter peak conductance in Nav1.5

Due to time restraints, only scFv 2 and 8 were tested via automated whole-cell patch-clamp electrophysiology. Initial results suggested no impact on IV-curves for HEK-293-Nav1.5 cells, when scFvs were tested at 1 µM with a 1-hour pre-incubation.

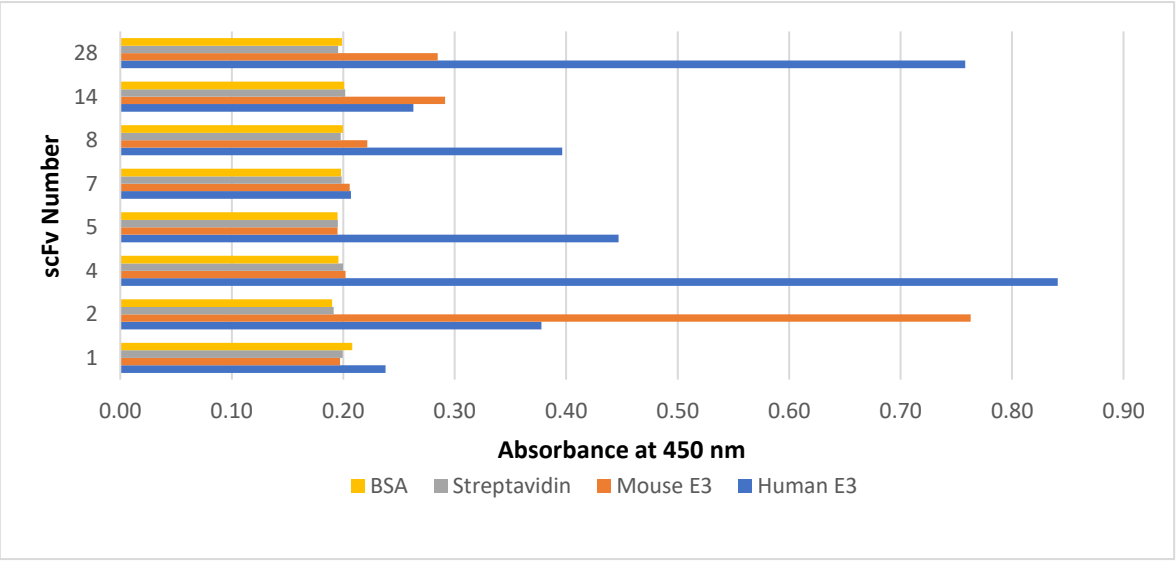

***Figure 2*. An ELISA with scFvs raised against human E3 peptide shows some cross-reactivity with mouse E3 peptide.**

In each well, scFv (2 µg) was screened for binding against streptavidin (1 µg), or human E3 peptide (1 µg), or mouse E3 peptide (1 µg), or a BSA protein negative control. HRP-conjugated secondary antibody (1 µg) was added, prior to addition of HRP substrate. This was followed by quenching with sulfuric acid, and plate reading at A450. Streptavidin was tested, because the E3 peptide was biotinylated and immobilised by streptavidin in the ELISA plate. The mean value of triplicate repeats is shown above.

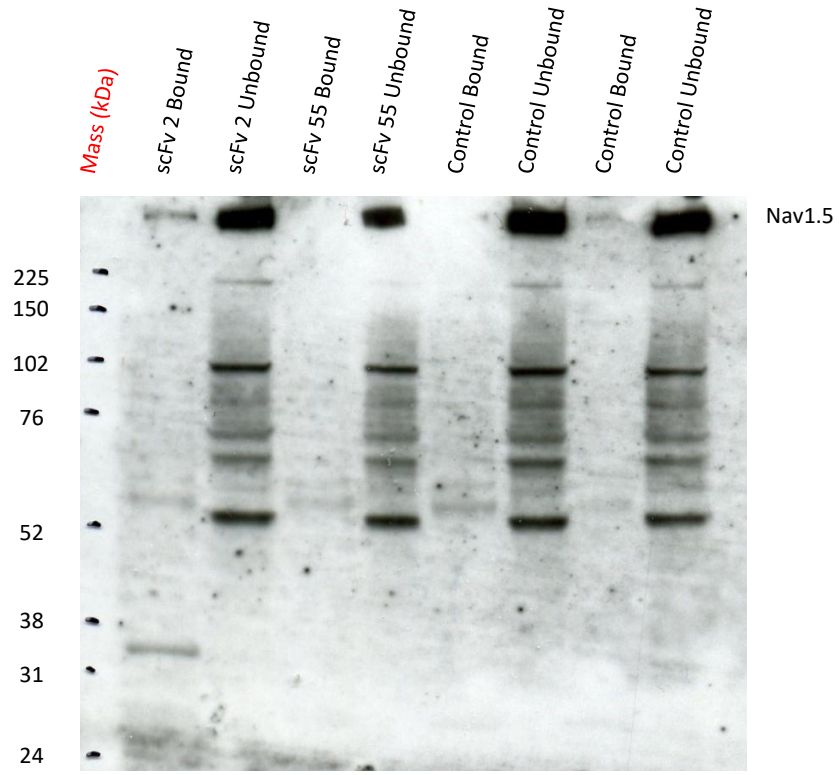

Figure 3. A Western blot shows that scFv 2 can pulldown Nav1.5.
scFvs were incubated with anti-FLAG beads, and then Nav1.5 cell lysates. Bound and unbound fractions were separated prior to washing of the beads in the bound fraction. Following electrophoresis and transfer to nitrocellulose, proteins were probed with anti-Nav1.5 primary antibody (rabbit) and then an anti-rabbit HRP-conjugated secondary antibody. Amersham ECL western blotting detection reagent was added, and this image was obtained after a 77-minute exposure. scFv 55 was a positive control for Nav1.5 which was used during xCELLigence assays. Nav1.5 is shown in the thick band above 225 kDa. Control fractions are cell lysates that were incubated with anti-FLAG beads, without scFv, to test for non-specific binding.

Future Directions

As well as completing the screening the other scFvs, scFv 2 has potential to be developed into a lab-tool due to its affinity to Nav1.5, and cross-reactivity with mouse E3-peptide. scFv 2 could be adapted for use in SPLATT for proximity proteomics studies or conjugated to GFP for use as a Nav1.5 specific marker. scFvs that successfully bind Nav1.5 could also be cloned into a full IgG construct, given that scFvs are the variable domains of an IgG antibody.

Importance and impact

Mutations in Nav1.5 are implicated in channelopathies, causing approximately 15-20% of Brugada syndrome and 5-10% of long QT syndrome (Ackerman *et al.*, 2004). A greater understanding of how these arrhythmias arise is desired, and developing biologics that alter channel function could be a way to tackle these conditions. Whilst I didn't report any scFvs that could modulate channel function, scFv

2 is still of potential use as a research tool. Furthermore, if scFvs are unable to have an electrophysiological effect on Nav1.5, whilst a polyclonal antiserum can, then this could give insight into the mechanism by which an anti-E3 IgG exerts an effect. Molecular dynamics simulations could investigate this perhaps.

Personal value of studentship

Within the Jackson Group, I have gained composure in the lab, and have leant new skills including pulldowns, different protein purification protocols, good cell culture technique, as well as the much more specialised process of patch-clamp electrophysiology. I have had to be resilient when navigating pitfalls and unexpected issues within the project, improving my adaptability, and learning from things going wrong. Weekly presentations and scientific discussion within the group has helped with my confidence to convey information.

Overall, the studentship has supported my personal career development, part of the Biochemical Society strategy, by allowing me to see how much I enjoy the laboratory atmosphere. I will be pursuing a new research project this year in my final year of undergraduate studies.

Acknowledgements

Many thanks to Professor Tony Jackson, who hosted me in his lab group and guided the project. Thanks to Dr Samir Hamaia, my day-to-day supervisor, for overseeing my laboratory work. Thanks also to Samantha Salvage for her expertise in patch-clamp electrophysiology and help with western blotting.

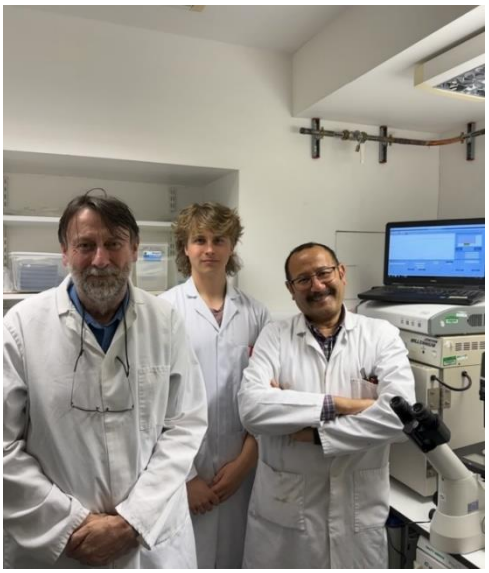

Figure 4. Myself, Tony, and Samir next to the xCELLigence machine.
Professor Tony Jackson is on the left, Dr Samir Hamaia on the right.

References

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