Structural and pharmacological characterisation of positive allosteric modulator, BAY2413555 and ASP8302 at M2 and M3 muscarinic acetylcholine receptors

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The muscarinic acetylcholine receptors are family of five class A G protein-coupled receptors (GPCRs) and plays critical role in the central and peripheral nervous systems. Current mAChR-targeting drugs exerts cholinergic side effects due to poor subtype selectivity and targeting allosteric sites has emerged as a promising strategy. BAY 2413555 and ASP8302 are recently identified positive allosteric modulators of M_2 and M_3 mAChRs, however, their pharmacological mechanism and structural basis of subtype selectivity remains poorly understood. We used cryo-electron microscopy combined with molecular pharmacology to characterise these compounds across all five mAChR subtypes and determined a high-resolution cryo-EM structure of the M_3 mAChR in complex with Acetylcholine and ASP8302.

Visualization of dysregulated proteins involved in motor neuron disease using electron cryo-microscopy.

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A GGGGCC hexanucleotide repeat expansion in C9ORF72 is a major genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Repeat-associated non-AUG (RAN) translation of the expanded RNA generates five dipeptide repeat (DPR) proteins, among which the arginine-rich poly(GR) and poly(PR) species are particularly toxic. These DPRs perturb multiple cellular processes, including protein synthesis, yet the structural mechanisms underlying their effects on human ribosomes remain poorly understood.

Here, we investigate the structural basis of GR101-induced translational disruption in human cells using single-particle cryo-electron microscopy (cryo-EM). Ribosomes isolated from cells expressing GR101 were resolved to 2.34 Å resolution. Extensive three-dimensional classification revealed a heterogeneous translational landscape, including a prominent population of rotated ribosomes containing hybrid tRNAs. These ribosomes displayed continuous nascent-chain density extending from the peptidyl-tRNA into the polypeptide exit tunnel, providing structural evidence of GR101 translation at the point of ribosomal arrest. Strikingly, this GR101-associated hybrid state persisted despite cycloheximide treatment, indicating that these ribosomes remain trapped in a pre-translocation configuration rather than being completely redistributed into classical cycloheximide-stabilized states.

Our findings support a mechanism in which nascent GR101 engages the ribosomal exit tunnel and stabilizes a hybrid pre-translocation state, thereby interfering with productive elongation. These structures provide a structural framework for understanding how arginine-rich DPRs disrupt human translation and contribute to C9ORF72-associated neurodegeneration.

Structural Insights into M_2 mAChR Antagonism

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G protein-coupled receptors (GPCRs) are preeminent class of drug targets accounting for approximately one-third of FDA approved drugs. Despite this success, achieving receptor subtype selectivity has proven difficult, as many GPCRs are activated by similar types of ligands. This is especially true for the muscarinic acetylcholine receptors (mAChRs), a class A GPCR family comprising five subtypes (M_1-M_5) that mediate acetylcholine (ACh) signaling throughout the central and peripheral nervous systems. Ach binds at the orthosteric pocket which is highly conserved among the mAChR subtypes. It is a major obstacle to achieve subtype selectivity across the family. Yet roughly 95% of reported mAChR ligands, and every clinically approved mAChR drug, binds at this pocket. Determining how selectivity can be achieved at the most druggable pocket of these receptors is therefore an important question and insights gained from this study can be used to design ligands across other subtypes and conformational states. We aimed to address this using the M_2 mAChR as a case study.

We pursued this structurally, engineering a BRIL-fused M_2 construct and forming a complex with an anti-BRIL Fab and an anti-Fab nanobody to make the receptor rigid and improve particle alignment for cryo-EM. From this we determined high-resolution cryo-EM structures of the M_2 mAChR, each in complex with a distinct selective antagonist: SCH211803 (2.96 Å) and PG1000 (2.76 Å). In both structures the antagonist occupies the orthosteric pocket while simultaneously contacting the less-conserved extracellular vestibule (ECV), which offers a plausible structural explanation for their subtype selectivity. Together, these inactive-state structures supply a framework for designing selective modulators of the M_2 receptor.

Allosteric modulators at Dopamine Receptor 2

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The Dopamine Receptor 2 (D2R) has emerged as a promising target for the treatment of disorders such as Schizophrenia, Parkinson's Disease and Attention Deficit/Hyperactivity Disorder (ADHD). While medications for each disorder are in clinical use, their various limitations mean fundamental treatment needs remain unmet. Drugs in current use for D2R disorders target the receptor's orthosteric binding site. This site is highly conserved across all five dopamine receptors. Ligands designed to bind the orthosteric site are hence not specific to the D2R, and off-target binding can cause dangerous side-effects.

Allosteric sites are binding pockets outside of the orthosteric site, which are more unique to receptor subtypes. Targeting allosteric sites specific to the D2R would circumvent non-specific activation of other dopamine receptors, thus reducing the risk of side effects. Molecules that bind allosteric sites can have an activating or inactivating effect. These are known as Positive or Negative Allosteric Modulators (PAMs or NAMs). Activation of the D2R causes inhibition of dopamine binding. PAM binding has potential to decrease dopamine signalling, which is helpful in diseases of dopamine excess like Schizophrenia. Conversely, NAM binding could increase dopamine signalling, with potential to counter the dopamine deficiencies of Parkinson's Disease and ADHD. Our research group has identified a PAM (UCB-PAM 2) and a NAM (SB269652) with therapeutic potential at the D2R.

The D2R couples to inhibitory Gi/o proteins, and its activation suppresses adenylyl cyclase activity and cAMP production. A PAM enhances the affinity and/or efficacy of dopamine at the receptor, amplifying this response to endogenous dopamine. PAMs therefore offer a way to boost D2R signalling in states of dopamine deficiency such as Parkinson's disease and ADHD, while preserving the spatial and temporal pattern of endogenous dopamine release. Conversely, a NAM dampens dopamine's effect at the receptor, providing an alternative to the complete blockade produced by current antipsychotics in disorders of dopamine excess such as schizophrenia. Two such modulators, are the PAM UCB-PAM 2 and the NAM SB269652.

To our knowledge, no experimental structure of the D2R bound to an allosteric modulator has been published. My PhD project aims to purify the D2R in complex with both UCB-PAM 2 and SB269652 and visualise the respective active and inactive structures with cryo-EM.

Work so far has focused on screening D2R constructs incorporating fusion proteins and stabilising nanobodies. This has yielded a 2.7 Å cryo-EM structure of the active-state D2R bound to the orthosteric agonist rotigotine. Work towards a UCB-PAM 2–bound structure is ongoing, alongside an inactive-state structure with SB269652.

Distinct ICL2 and Helix 8 Configurations Define Non-canonical G-protein Engagement by GCGR

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Glucagon receptor (GCGR), a class B1 G protein-coupled receptor central to glucose homeostasis, primarily signals through Gs but can also engage non-canonical Gi/o and Gq/11 pathways. How GCGR accommodates these distinct G-protein subtypes at the structural level remains incompletely understood. Here, we combine cryo-electron microscopy with signalling pharmacology to compare GCGR complexes formed with the endogenous ligands glucagon (GCG) or oxyntomodulin (OXM) and representative Gs, Gi1 and Gq-family transducers. We report the first GCGR–Gq-family structures and sub-3 Å structures of OXM-bound GCGR coupled to Gi1, providing a structural framework for comparing canonical and non-canonical coupling.

Across the complexes, the receptor transmembrane core retains a broadly conserved active-state architecture, whereas the intracellular coupling interface differs between G-protein subtypes. A prominent distinction is the upward-oriented intracellular loop 2 (ICL2) in the Gi1- and Gq-coupled complexes, contrasting with the downward ICL2 configuration and direct ICL2 engagement observed in the Gs complex. The Gq-family transducer nevertheless maintains a contact near ICL2, including a van der Waals interaction with GCGR residue E260, whereas the OXM–GCGR–Gi1 interface contains markedly fewer ICL2 contacts. These observations suggest that ICL2 adopts subtype-dependent configurations rather than serving as a uniform interaction surface across transducers.

A second distinguishing feature is the engagement of the receptor's C-terminal helix 8 (H8) region. The Gq-family complex forms contacts involving the TM7–H8 region, including residues Y400 and E406, while the Gi1 interface is more restricted and shows little engagement with TM7–H8. Comparisons of the relative orientations of the G-protein α5 helix and H8 further reveal distinct arrangements across the complexes. These are structural differences between resolved complexes and do not, by themselves, establish a dynamic movement or a causal role in coupling selectivity.

Together, these structures identify ICL2 configuration and TM7–H8 engagement as notable features of non-canonical GCGR–G-protein interfaces. Combined with functional profiling across multiple G proteins and ligands, the work provides a basis for testing how intracellular receptor elements contribute to GCGR transducer recognition and signalling.

Kinetic analysis of cell–cell recognition mediated by A-subclass Eph receptor tyrosine kinases and ephrin ligands

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Eph receptor tyrosine kinases and their membrane-anchored ephrin ligands constitute the largest family of receptor tyrosine kinases and are critical regulators of cell-cell communication across a wide range of cell types [1]. Signalling is initiated by the formation of receptor-ligand complexes across cellular junctions, which can further aggregate into higher order clusters or biomolecular condensates that amplify downstream phosphorylation cascades [2]. These interactions mediate bidirectional signalling in both the receptor- and ligand-expressing cells, resulting in context-dependent cell adhesion or repulsion involved in numerous physiological and pathological processes [3]. Despite their diverse functions, the molecular determinants of ligand recognition in the Eph family remain poorly defined, and the mechanisms by which specific receptor-ligand pairs elicit distinct cellular responses are not well understood.

To establish a comprehensive resource for ligand recognition within the Eph family, we recombinantly produced the extracellular domains of A-subclass Eph receptors (EphA1-EphA5, EphA7, EphA8, EphA10) and ephrin ligands (ephrinA1-ephrinA5). Binding kinetics of each receptor-ligand pair was determined via surface plasmon resonance and revealed promiscuous recognition ranging 10 nM-1 μM. Fast association and dissociation kinetics suggest that receptor activation is regulated through repeated binding events or kinetic proofreading, consistent with the formation of biomolecular condensates in the plasma membrane. Binding kinetics of ephrin ligands to full-length EphA10 overexpressed on the surface of HEK293 cells were validated via fluorescence-activated cell sorting. Integration of this kinetic data with available structural information and sequence conservation analyses identified molecular features that govern ligand specificity and binding affinity. Together, these findings provide a framework for understanding the redundant versus non-redundant functions of Eph receptors and ephrin ligands, their complex mechanisms of regulation, and inform the rational development of therapeutics targeting this receptor-ligand signalling axis.

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The Structural and Pharmacological Characterisation of the Neuropeptide S Receptor 1

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Introduction. Endometriosis affects 1 in 9 people, and a key symptom is chronic severe pelvic pain (Crump et al, 2024). The current treatments are limited and include surgery and hormonal contraceptives, which are suggested to be inadequate for pain management and suppress fertility, respectively (Crump et al, 2024). Recently, a neuropeptide S receptor 1 (NPSR1) antagonist was demonstrated to reduce abdominal pain in an endometriosis mouse model (Tapmeier et al, 2021), highlighting its therapeutic potential as a drug target. Currently, there are no available atomic structures of the NPSR1 and this potentially contributes to the lack of NPSR1 targeted drugs progressing to clinical studies.

Aims. To structurally determine the active state structure of the NPSR1 in complex with its endogenous agonist neuropeptide S (NPS). To pharmacologically characterise NPSR1 antagonists in functional assays.

Methods. Active state NPSR1 constructs were designed and screened in insect cells, followed by protein expression and purification in complex with G$\beta\gamma$ protein. Additionally, NPSR1 antagonists were pharmacologically characterised using second messenger assays distal to $G\alpha_q$ protein activation in mammalian cells.

Results. Here, we report that the NPSR1 fused mini-Gα protein (NPSR1-mGisq) construct exhibited adequate expression at 48 h and was subsequently purified with G$\beta\gamma$ protein. Size exclusion chromatography and Western blot expression revealed that the purified NPSR1 protein complex was heterogenous. Additionally, pharmacological characterisation showed that NPSR1 antagonists SHA 68, NCGC84 and MIPS-54170 inhibited NPS-induced calcium mobilisation, extracellular signal-regulated kinase (ERK) phosphorylation and inositol monophosphate accumulation.

Discussion. The purified NPSR1 protein was heterogenous due to potential receptor dimerisation and/or cleavage. For structure determination, further receptor modifications e.g., N- and/or C-terminal truncations may be required to reduce protein flexibility and promote sample homogeneity. We aim to gain a deeper understanding of the molecular interactions involved in ligand binding at the NPSR1 for receptor activation and inactivation. Further in vitro profiling of NPSR1 antagonists will enable us to directly compare their pharmacological activities. Collectively, this knowledge may help guide the development of new NPSR1 compounds for the treatment of pain associated with endometriosis.

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Structural insights into GD1a presentation by CD1c and human T cell recognition

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CD1c is a cell-surface antigen-presenting protein that displays a wide range of lipid-based antigens to human T cells. T cells use diverse T cell receptors (TCRs) to recognise CD1c itself or a combination of CD1c and the presented lipid antigen. Previous structures revealed sideways presentation of the relatively simple gangliosides GM3 and GD3, but whether CD1c can accommodate larger, branched gangliosides and how their presentation affects T cell recognition remain unclear.

Here, we determined the crystal structure of CD1c bound to the branched ganglioside GD1a at 1.9 Å resolution. GD1a adopts a sideways orientation, with its ceramide anchored within the F′ pocket and its glycan directed laterally through the G′ portal, while spacer lipids occupy the A′ pocket. The resolved glucose moiety of GD1a contacts Gln87 in a position conserved across GM3- and GD3-bound structures. However, the remaining branched glycan is unresolved, consistent with conformational flexibility. Comparison with CD1c presenting an upwards-oriented mycobacterial lipid reveals a more closely apposed α1–α2 helical arrangement. Relative to the mock-loaded structure, GD1a presentation is associated with local displacements of approximately 2–3 Å in the loop adjoining the α1 helix. Relative B-factor comparisons indicate greater loop disorder than in GM3- and GD3-bound structures, suggesting that GD1a provides less stabilisation of this region.

To examine the immunological relevance of these differences, we compared ganglioside-loaded CD1c tetramer staining of human T cells. Selected ganglioside-reactive TCRs showed weaker staining with GD1a than with GM3 or GD3, often comparable to endogenous-lipid controls. A tonsil-derived T cell line showed stronger tetramer staining with GM3- and GD3-loaded CD1c, whereas staining with GD1a-loaded CD1c was comparable to the endogenous-lipid control.

Together, these findings reveal how CD1c accommodates a complex branched ganglioside and suggest that differences in glycan architecture and associated CD1c flexibility may modulate the strength of human T cell recognition.

Structural and Functional Studies of Zinc Resistance Associated Proteins (ZraP) from *Klebsiella pneumoniae*

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Two-component systems (TCSs) are a major class of bacterial signal-transduction pathways that enable adaptation to fluctuating environmental conditions. The Zra system is a specialised TCS involved in zinc homeostasis in *Escherichia coli* and *Klebsiella pneumoniae*. Classical TCSs consist of a sensor kinase (SK) and a response regulator (RR). However, the Zra system uniquely incorporates an additional periplasmic protein, ZraP. In *Salmonella enterica*, ZraP has been reported to function both as a molecular chaperone and as a repressor of Zra signalling[1]. In contrast, the structural features and functional roles of *K. pneumoniae* remain unknown, thereby limiting mechanistic understanding of the entire Zra system.

This project uses single-particle cryo-electron microscopy (cryo-EM) alongside X-ray crystallography to determine the structure of *K. pneumoniae* ZraP (*Kp*ZraP). By combining ongoing cryo-EM structural analyses with biophysical characterisation of its metal-binding properties, this work aims to clarify the role of ZraP in bacterial zinc homeostasis and stress adaptation.

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High-resolution structure of soluble HIV-1 envelope (SOSIP) with bovine bNAbs using cryo-EM

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Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), has resulted in more than 40 million deaths worldwide and currently affects approximately 39 million people. Although antiretroviral therapy (ART) has significantly improved life expectancy, HIV infection remains incurable, and long-term treatment is complicated by drug resistance and the continual emergence of viral variants. To date, RV144 remains the only HIV vaccine trial to demonstrate partial protective efficacy, highlighting the need for improved vaccine strategies.

The HIV envelope glycoprotein (Env) is the primary target of broadly neutralising antibodies (bNAbs). However, HIV evades immune recognition through extensive sequence variability, dense glycan shielding, and conformational masking. Notably, naturally arising bNAbs generated during chronic HIV infection predominantly target the state-1 (closed) conformation of Env. To better understand these vulnerable epitopes and inform vaccine design, we generated bovine bNAbs against HIV Env through sequential immunisation strategies.

MEL-1872 is a bovine broadly neutralising antibody that targets the CD4-binding site (CD4bs) of HIV-1 Env. Cryo-electron microscopy (cryo-EM) data were collected for AD8 SOSIP in complex with MEL-1872 Fab fragments. The reconstructed cryo-EM map achieved a global resolution of 2.8 Å at a Fourier shell correlation (FSC) threshold of 0.143, enabling detailed visualisation of the epitope-paratope interface. Structural analysis revealed that MEL-1872 engages the AD8 SOSIP trimer primarily through CDRH3-mediated interactions with residues located within the CD4-binding loop, D loop, and V5 loop of gp120. These interactions are stabilised by an extensive network of hydrogen bonds and salt bridges. Collectively, these findings define the molecular basis of MEL-1872 recognition of HIV Env and provide insights that may contribute to the development of next-generation HIV vaccines and antibody-based prophylactic strategies.

Cryo-EM structures of the *Pseudomonas aeruginosa* MexB efflux pump in substrate-bound states

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Antimicrobial resistance in Gram-negative bacteria is an escalating global health threat, and *Pseudomonas aeruginosa* remains a problematic pathogen because its intrinsic and acquired resistance are strongly reinforced by the combination of low outer-membrane permeability and multidrug efflux. Central to this phenotype is the major constitutively expressed RND efflux system MexAB-OprM, in which MexB functions as the inner-membrane transporter and accommodates a broad range of clinically relevant substrates. Despite the importance of MexB in resistance, high-resolution structural insight into antibiotic recognition has remained limited, in part because substrate-bound states have been difficult to capture and detergent molecules used for membrane-protein solubilisation can occupy key binding regions. Here, we overcome these limitations using cryogenic electron microscopy and amphipol reconstitution to interrogate substrate-bound states of MexB. We report a ~2.2 Å cryo-EM structure of ampicillin-bound MexB, the highest-resolution substrate bound MexB structure reported to date, alongside high-resolution ciprofloxacin-bound and DDM-bound states, revealing how ligands are accommodated within the substrate-binding pockets. These findings provide a detailed framework for understanding substrate recognition by a clinically important RND transporter and establish a structural basis for the rational design of efflux pump inhibitors and combination therapies aimed at restoring antimicrobial susceptibility in multidrug-resistant *P. aeruginosa*.

A complete RXFP1–relaxin interaction model unlocks the design of potent mini-protein modulators

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Relaxin family peptide receptor 1 (RXFP1) is a multi-domain GPCR with compelling therapeutic potential [1], yet uncertainty surrounding the mechanism of its activation by the hormone H2 relaxin has hindered the development of selective modulators. Here, we combine deep learning based structural modelling [2] with de novo protein design [3,4,5] to overcome this barrier. We generate a high-confidence structural model of the RXFP1–relaxin complex that is strongly supported by existing biochemical and functional evidence [6]. This model reveals that relaxin engagement stabilises the RXFP1 extracellular linker, thereby triggering receptor activation. Guided by this model, we design mini-protein modulators that either block linker stabilisation or enforce it and induce an active receptor geometry. These molecules act as potent, selective RXFP1 antagonists or agonists, achieving low-nanomolar activity in both engineered and endogenously expressing cell lines despite adopting folds unrelated to relaxin. Together, these findings define the mechanistic basis of RXFP1 signalling, establish the first de novo agonists and antagonists of this receptor, and demonstrate how AI-enabled modelling and design can target structurally complex GPCRs previously inaccessible to structure-guided drug discovery.

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Title: Structural basis of bitopic ligands at the M1 muscarinic acetylcholine receptors.

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The muscarinic acetylcholine receptors (mAChRs) are class A G protein coupled receptors (GPCRs) consisting of five subtypes (M_1 to M_5), all subtypes are activated by the endogenous neurotransmitter acetylcholine (ACh). The mAChRs are expressed in the central nervous system (CNS) and periphery, but mAChR subtypes differ in their tissue distribution and physiological function. In particular, the M_1 mAChR subtype is highly expressed in the all major areas of the forebrain and play a critical role in synaptic plasticity, learning and memory processes, making it an attractive therapeutic target for Alzheimer's disease (Lebois et al., 2018).

However, therapeutic targeting of the M1 mAChR using traditional orthosteric agonists has proven challenging due to the highly conserved residues in the orthosteric binding site across all five mAChR subtypes (Vuckovic et al., 2019). The poor selectivity of orthosteric ligands can result in unwanted off-target side effects that hinder clinical translation (Brannan et al., 2020). Targeting allosteric sites provides an alternative strategy because these sites are less conserved and spatially distinct from the orthosteric pocket. Nevertheless, M1-selective allosteric modulators have shown limited clinical success due to poor drug-like properties, insufficient procognitive efficacy, and on-target cholinergic adverse effects (Mandai et al., 2025).

A promising alternative is the use of bitopic ligands, which combine orthosteric and allosteric pharmacophores into a single molecule (Valant et al., 2009). By simultaneously engaging both orthosteric and allosteric sites, bitopic ligands have the potential to achieve both high affinity and high subtype selectivity. Despite this promise, structural information about the mechanisms by which these bitopic ligands act on the M_1 mAChR remains limited.

By utilizing advanced structural biology, data processing, residue mutagenesis, and molecular pharmacology techniques, our lab aims to provide in-depth insights into the molecular mechanisms of bitopic ligand interactions at the M_1 mAChR, such as the bitopic ligand TBPB. Up to date, the purification of the human M1 mAChR bound to TBPB in complex with a G-proteins (miniGsqi-$\beta1\gamma2$) and Nb35 and the processing of the cryo-EM dataset have been completed, positioning the project at model building and real-space refinement stage.

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Poster #14

Comparative cryo-EM analysis of RhsA and Rhs0240 reveals conserved toxin-encapsulating cage architecture

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Rhs (rearrangement hotspot) proteins are bacterial effectors associated with type VI secretion system (T6SS)-mediated interbacterial competition. Their conserved YD-repeat regions form protective cages that encapsulate diverse C-terminal toxins. However, the precise molecular mechanisms underlying toxin release and delivery into target cells remain poorly understood.

Here, we determined cryo-electron microscopy (cryo-EM) structures of RhsA from Escherichia coli K-12 at 2.5 Å and Rhs0240 from enterohaemorrhagic E. coli (EHEC) at approximately 3.7 Å. We compared their overall architecture, YD-repeat organisation and terminal sealing regions to identify conserved and divergent structural features.

Both proteins form highly conserved, multilayered YD-repeat cages surrounding a central cavity. The encapsulated toxin domains lack resolvable cryo-EM density, consistent with an unfolded or highly flexible state. Although their N-terminal autocleavage sites differ, the cleavage events are proposed to serve a common function: breaking the covalent linkage between the N-terminal seal peptide and the cage, potentially allowing seal displacement and subsequent release of the unfolded toxin.

Together, these structures provide a framework for understanding how conserved Rhs cages package diverse toxin cargoes and how N-terminal autocleavage may facilitate their release.

Investigating Biased Signalling at the μ-Opioid Receptor

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The opioid crisis has highlighted the urgent need for analgesics that provide effective pain relief with fewer harmful side effects. One proposed strategy is biased agonism: designing μ-opioid receptor (MOR) ligands that preferentially activate G protein signalling over β-arrestin2 recruitment. However, the apparent bias of the clinically approved agonist oliceridine, and of other reported G protein-biased ligands, may largely reflect low intrinsic efficacy rather than true pathway selectivity (Gillis et al., 2020). Whether MOR ligands can achieve genuine G protein bias without partial agonism, and what structural features would underlie it, remains unclear.

To address this, we pharmacologically characterised 16 novel MOR ligands using BRET-based assays measuring Gi2 protein activation, β-arrestin2 recruitment, and recruitment of Nb33, a nanobody sensor of the active receptor conformation. Ligand efficacy and signalling bias were quantified using area-under-the-curve analysis and the operational model of agonism.

Consistent with previous reports, most ligands showed reduced intrinsic efficacy across all readouts, indicative of partial agonism. In contrast, compound 13 showed a preference for G protein signalling while retaining high intrinsic efficacy, suggesting that its bias cannot be explained by partial agonism alone.

To investigate the structural basis of this profile, we have obtained a cryo-EM map of MOR in complex with compound 13. Ongoing structural analysis aims to identify receptor–ligand interactions that distinguish compound 13 from oliceridine and other partial agonists, and that may explain its signalling preference.

This work identifies compound 13 as a rare example of G protein-preferring MOR agonism with preserved efficacy. Defining its structural basis could reveal new mechanisms of biased signalling and inform the rational design of safer opioid analgesics.

Integrated fluorescence-guided fib-milling and cryo-electron tomography to study intercellular interactions

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Cryo-electron tomography (cryoET) is a powerful method capable of determining protein structures at subnanometer resolutions in situ. Although some biological specimens are thin enough (<300 nm thick) to be directly visualised by cryoTEM, many are too thick and require additional sample preparation. Specimens larger than 300 nm include eukaryotic cells, bacteria biofilms, and most other non-microbial samples. Consequently, investigating intercellular interactions by cryoET necessitates methods for producing thin sections that preserve regions of interest.

Cryo-FIB-SEM is a method for generating a thin (<200 nm thick) cryo-lamella by milling away unwanted sample with a focused ion beam (FIB). Despite considerable technological developments, several elements of this workflow remain challenging, such as the vitrification of thick samples, localisation of targets within thick samples, and automation. We use high pressure freezing, for the vitrification of thick samples; cryo-light microscopy, to localise region of interest and target cryo-lamella preparation; and milling procedures, to create an optimized workflow for faster cryo-lamella preparation.

This allows us to visualise ultrastructural details of intercellular interactions and molecular machines in action at nanometer resolution. The development of cryo-lamella preparation techniques underpins the utility of cryoET and *in situ* structural biology and broadens its target range and diversity.

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Unravelling the Activation and Pore Formation Mechanisms of Insecticidal Proteins from Ferns

Insecticidal proteins from *Bacillus thuringiensis* (*Bt*) have been the foundation of biological pest control for several decades. Their use in transgenic crops has substantially reduced reliance on chemical insecticides; however, long-term deployment has led to the emergence of resistant insect populations across multiple pest species. This growing resistance highlights the need for novel insecticidal proteins with distinct mechanisms of action. Recently, a new class of insecticidal proteins has been identified in ferns, including Fip_Cow from *Colysis wrightii*, which shares structural similarity with *Bt* Cry toxins. These proteins protect crops against *Bt*-resistant insects and therefore represent promising candidates for next-generation bioinsecticides.

Given its structural resemblance to *Bt* toxins, Fip_Cow is hypothesised to function as a pore-forming protein, although the molecular mechanisms underlying this process remain poorly understood. In its crystal structure and in solution, Fip_Cow forms a stable dimer, suggesting that activation involves structural rearrangement or dissociation. Ongoing work aims to characterise the activation and pore formation mechanisms of Fip_Cow through a combination of biochemical and structural approaches, including cryo-electron microscopy (cryo-EM). Together, these studies aim to establish the molecular basis of Fip_Cow toxicity and to provide broader insight into the mechanisms of this emerging family of fern insecticidal proteins.

Reference: Wei, J.Z. et al. (2023). Novel insecticidal proteins from ferns resemble insecticidal proteins from *Bacillus thuringiensis*. Proceedings of the National Academy of Sciences, 120(44).

Allosteric drug discovery for muscarinic acetylcholine receptors against lymphatic disorders

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For decades, the standard of care for lymphedema and many other lymphatic diseases has remained limited, focusing on symptom management through lifestyle modification, compression garments, or surgery. The muscarinic acetylcholine receptor subtype 3 (M3R) regulates peripheral lymphatic circulation and therefore represents a promising novel drug target for lymphatic disorders. Developing orthosteric, subtype-selective ligands for the muscarinic receptor family is notoriously difficult. The subtype-unique allosteric sites of this family, by contrast, offer an opportunity to fine-tune signaling at the target receptor in the presence of local endogenous ligand. Here we combined biochemical, structural, pharmacological, and biophysical approaches to discover allosteric ligands targeting M3R. Pharmacological profiling of the endogenous agonist acetylcholine together with the novel ligand MIPS-4542 revealed a positive allosteric effect on acetylcholine signaling. Ligand-induced thermostability assays, biochemical purification, and structure determination were then used to investigate the mechanism underlying this effect at M3R. Together, these findings advance allosteric drug discovery at muscarinic receptors for the treatment of lymphatic disorders.

Structural and biochemical investigation of the SETDB1 methyltransferase

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Abstract

SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) catalyses histone H3 lysine 9 trimethylation (H3K9me3) to establish repressive chromatin, and its dysregulation is implicated in acute myeloid leukaemia, triple-negative breast cancer and neurodevelopmental disorders. Although individual SETDB1 domains have been characterised in isolation, the architecture of the full-length enzyme is unresolved and the regions required for nucleosome engagement have not been defined. We generated Strep-tag II constructs of full-length SETDB1 (1–1291) and seven truncations spanning its triple Tudor domain (TTD), methyl-CpG-binding domain (MBD), pre-SET, SET and post-SET domains: 1–1266 (lacking post-SET), 1–802 (TTD, MBD, pre-SET), 1–665 (TTD, MBD), 1–593 (TTD, N-terminal part of MBD), 594–1291 (C-terminal part of MBD, pre-SET, SET, post-SET), 727–1291 (pre-SET, SET, post-SET) and 803–1291 (SET, post-SET), together with the SETDB1-binding region of ATF7IP (553–1270). Constructs were expressed in Sf9 and ExpiSf9 insect cells using the Bac-to-Bac baculovirus system. Anti-Strep western blotting (P1) and Strep pull-down (P2) detected most constructs, with expression levels varying between them. Trial purification by Strep-Tactin affinity and heparin ion-exchange chromatography, assessed by SDS-PAGE and mass photometry, indicated that purity and homogeneity require further optimisation before large-scale preparation. Preliminary electrophoretic mobility shift assays with a 182-bp nucleosome suggested that full-length SETDB1, 1–1266 and 1–593 bind to the nucleosome. Ongoing work includes replicate binding assays for all constructs, H3K9me3 methyltransferase assays, co-expression with ATF7IP and USP17, and negative-stain electron microscopy of SETDB1–nucleosome complexes. This study will lay the foundation for structural basis of SETDB1-nucleosome complex and explain why mutations in this gene lead to human diseases.

Amylin receptor subunit interactions are modulated by agonists and determine signaling

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Three amylin receptors (AMYRs) mediate the metabolic actions of the peptide hormone amylin and are drug targets for diabetes and obesity. AMY_1R, AMY_2R, and AMY_3R are heterodimers consisting of the calcitonin receptor (CTR), a G protein–coupled receptor, paired with a RAMP1, RAMP2, or RAMP3 accessory subunit, respectively, which increases amylin potency. Here, we found that the AMYRs had distinct basal subunit equilibria that were modulated by peptide agonists and determined the extent of cAMP signaling downstream of receptor activation. By developing a biochemical assay that resolves the AMYR heterodimers and free subunits, we found that the AMY_1R and AMY_2R subunit distributions favored free CTR and RAMPs and that rat amylin promoted association of the constituent subunits of AMY_1R and AMY_2R. The agonist αCGRP also induced AMY_1R subunit association. A stronger interaction between the CTR and the RAMP3 transmembrane domains yielded a more stable AMY_3R, and human and salmon calcitonin agonists promoted AMY_3R dissociation. Similar changes in subunit association and dissociation were observed in live-cell membranes, and G protein coupling and cAMP signaling assays showed how these changes altered signaling. Our findings have implications for AMYR biology and drug development and reveal regulation of heteromeric GPCR signaling through subunit interaction dynamics.

Interaction of Glycerol with Proteins: Implications for the use of Glycerol as Cryoprotectant in Cryo-EM Studies

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Abstract

Cryo-EM has revolutionised our ability to determine the solution structures of proteins. Because cryo-EM involves rapid freezing that can cause aggregation and artefacts, cryoprotectants such as glycerol can be included to alleviate these effects. Despite its widespread use in biochemistry, the cryoprotective mechanism of glycerol remains debated in literature, and the extent to which its inclusion impacts ensemble properties of proteins remains to be determined. Taking the protein Sa1V90T as a model, the mechanism by which glycerol acts as cryoprotectant has been examined in atomic detail using Molecular Dynamics (MD) simulation. Protein properties in water and a 15% glycerol-water mixture have been examined at 303 K, and during rapid cooling to cryogenic temperatures, mimicking the process of vitrification used in cryo-EM. We find that glycerol interacted with the protein directly, displacing water which was excluded from the protein-solvent interface. Simulations of rapid cooling mimicking cryo-EM sample preparation suggest that glycerol prevents cold denaturation by limiting the interaction of water with hydrophobic residues. The effect of cooling and glycerol on the range of conformations sampled during vitrification was also examined shedding light on the potential effects of cooling artefacts and glycerol on the extent of structural heterogeneity observed in cryo-EM ensembles.

Understanding the Structural Assembly and Signalling Dynamics of IL-6 Family Cytokines Utilizing LIF-Receptor

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Leukemia Inhibitory Factor (LIF), Oncostatin-M (OSM), Cardiotrophin-1 (CT-1), Ciliary neurotrophic factor (CNTF) and Cardiotrophin-like cytokine (CLC) belong to the IL-6 family cytokines. All these cytokines are known to signal through a complex formed with gp130 and LIFR receptors. Past studies have revealed the structure of LIF and OSM cytokines and identified their signalling partners. LIF forms its signalling complex with LIFR and gp130, while OSM forms two different types of signalling complex employing gp130 with either LIFR or OSMR. The structure and assembly dynamics of CT-1 signalling complex is not well understood with ambiguity being presented on the presence and identity of a third non-signalling α receptor. LIF and OSM display numerous common and distinct biological activities and have also been associated with pro-tumorigenic characteristics in various cancers. The focus of the current study is to discern the dynamics of signalling complex assembly and structural characteristics of LIF, OSM and CT-1 cytokines by employing biophysical assays and cryo-electron microscopy.

Structural and Pharmacological insights into novel dual agonists of the GLP-1R and SecR

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Obesity and type 2 diabetes are major global health challenges, highlighting the need for more effective and sustainable therapies. In recent years, GLP-1R agonists have transformed metabolic disease treatment, but limitations including gastrointestinal side effects, weight regain on discontinuation of treatment, loss of lean mass and potential negative effects on bone density remain. The secretin receptor (SecR), a class B1 GPCR, has emerged as a promising complementary metabolic target through its roles in enhancing thermogenesis, energy expenditure and satiation. This highlights the potential in co-targeting the GLP-1R with the SecR. Despite the GLP-1R being a well validated drug target, structural understanding of SecR remains limited. Here, we designed and pharmacologically characterised novel dual agonists of the GLP-1R and SecR and used cryo-electron microscopy to determine the molecular basis of agonist specific mechanisms of receptor activation and dual receptor targeting.

Dual agonists were characterised pharmacologically at GLP-1R and SecR using a range of BRET assays to quantify agonist mediated signalling, and regulation. Following pharmacological profiling of a series of peptide designs, a dual agonist of the GLP-1R and SecR (SA5) was selected to progress into structural characterisation. For structural studies, GLP-1R or SecR were co-expressed with dominant negative Gαs (DNGs), Gβ1γ2 and Nb35 in insect cells. Receptor–G protein complexes were stabilised and purified using affinity and size-exclusion chromatography. Protein sample quality was assessed through SDS-PAGE, western blotting and negative-stain electron microscopy. High quality protein samples were subsequently vitrified and analysed by single-particle cryo-electron microscopy. Following image processing, 2D/3D classification, 3D reconstruction and refinement, atomic models were built and refined against the resulting cryo-EM maps to elucidate ligand–receptor interactions.

A cryo-EM structure of the Secretin Receptor Gs complex bound by dual agonist SA5 was determined at a global resolution of 3.31Å. This structure will be compared to existing secretin bound SecR structure to understand how agonists with distinct pharmacology bind the SecR.

A CRYO-EM STRUCTURE TO UNRAVEL THE UNEXPECTED PROMISCUITY OF CYTOKINES

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Interleukin-6 (IL-6) and interleukin-11 (IL-11) are two cytokines in the IL-6 family of cytokines. As the only two cytokines in this family utilizing a homodimer of gp130, IL-6 and IL-11 form higher-order, hexameric signalling complexes. The hexameric complexes consist of two cytokine molecules, two molecules of gp130, and two molecules of the respective alpha receptor, IL-11Rα or IL-6Rα. Studies suggest that IL-6 and IL-11 have overlapping but distinct biological activities. The functional differences can be partially explained by expression of IL-6Rα and IL-11Rα on different cell types; by targeting their specific alpha receptor, cytokines can selectively activate specific cell types. Our preliminary data challenge this assumption, suggesting that IL-6 may promiscuously recognize IL-11Rα-expressing cells by forming a hexameric complex with IL-11Rα and gp130. Our analytical ultracentrifugation (AUC) and biolayer interferometry (BLI) data demonstrated that IL-6 may interact with gp130 first as an unstable tetrameric complex. This tetramer is further stabilized by two molecules of IL-11Rα, resulting in a chimeric IL-6/IL-11Rα/gp130 complex. We present a cryo-EM structure of the hexameric IL-6/IL-11Rα/gp130 complex at 3.2 Å resolution and propose a hypothesis of how this complex overcomes the mismatch between IL-6 and IL-11Rα.

Poster #25

Development and characterisation of nanobody that negatively modulates ectodomain dependent RXFP1 activation

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Introduction. The relaxin receptor RXFP1 belongs to the class A GPCR subgroup of leucine-rich repeat (LRR) containing GPCRs which are defined by their large ectodomain comprised of LRRs. The peptide hormone relaxin-2, binding at the ectodomain, activates this receptor to mediate cardioprotective and tissue remodelling effects, particularly during pregnancy. Our current model of RXFP1 activation suggests that the binding mechanism of relaxin-2 facilitates engagement of an N-terminal ectodomain region essential to releasing transmembrane domain autoinhibition.

Aims. Variable region fragments of a heavy-chain only antibody, also known as nanobodies, have previously been developed to target the extracellular side of GPCRs and are often used for structural studies or as ligands. Nanobodies targeting RXFP1 could help inform the mechanism of RXFP1 activation and thereby development of novel relaxin-2 analogues.

Methods. Nanobodies targeting RXFP1 were generated through immunisation of alpacas with purified protein and cells expressing a thermostabilized high-expressing RXFP1 mutant. We used phage display and magnetic-based selection to identify nanobodies directed against the N-terminus of RXFP1. The lead nanobody, TL1, was characterised using biophysical and cell-based assays.

Results. Our nanobody development approach yielded a nanobody with affinity (K_D) in the nanomolar range. TL1 was found to inhibit relaxin-2 binding (pK_i=9.14±0.04) and -induced cAMP signalling (pIC_{50}=7.54±0.06), which was specific to the RXFP1 ectodomain. Further testing including on RXFP1-endogenously expressing lines and NanoBRET binding studies revealed the mode of antagonism on relaxin-2 binding and signalling was saturable, indicative of negative allosteric modulation. TL1 displayed no specificity to the homolog receptor RXFP2 and was able to inhibit mouse and rat RXFP1.

Discussion. Development of RXFP1 inhibitors has been limited by a poor understanding of the receptor activation mechanism. These results demonstrate a novel interaction site and support, for the first time, previous biophysical and mutagenic studies in a natively expressed system.

Title: Elucidating the Structural Architecture of Seed Storage Protein Bodies

Anik Basak[1,2], *Deepa Agarwal*[2], *Roman Buckow*[2] and *Ashwani Kumar Thakur*[1]

Most flowering plants store proteins (globulins and albumins) in cotyledon cells to fulfil the amino acid requirements of the embryo during germination events. These proteins are present as fibrous aggregates within specialized compartments called seed storage protein bodies (SSPBs). The aggregates appear as electron dense inclusions in transmission electron micrographs, a feature commonly observed in amyloid aggregates, which are associated with human amyloid diseases and functional amyloids in certain organisms.

Recent studies on cereals and legume seeds have shown that SSPBs have a composite signature of amyloid, amyloid-like and native secondary structures. However, structural information of the SSPBs at atomic level and organization of globulins and albumins in amyloid composites, in the native cellular environment is unknown.

To address the knowledge gap, my proposal is to employ cryo-electron microscopy (cryo-EM) to investigate the high-resolution structure of seed storage proteins within SSPB in near native cellular environment. Since seed storage proteins in SSPBs exist in composite structural forms in the cotyledons of seeds, Cryo-electron tomography (cryo-ET) in combination with focused ion beam (FIB) milling will also be utilized. These methods have been used to determine 3D structure of large complexes like viruses, ribosome, amyloid fibrils, huntingtin and α-synuclein inclusions.

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Verified by project guide

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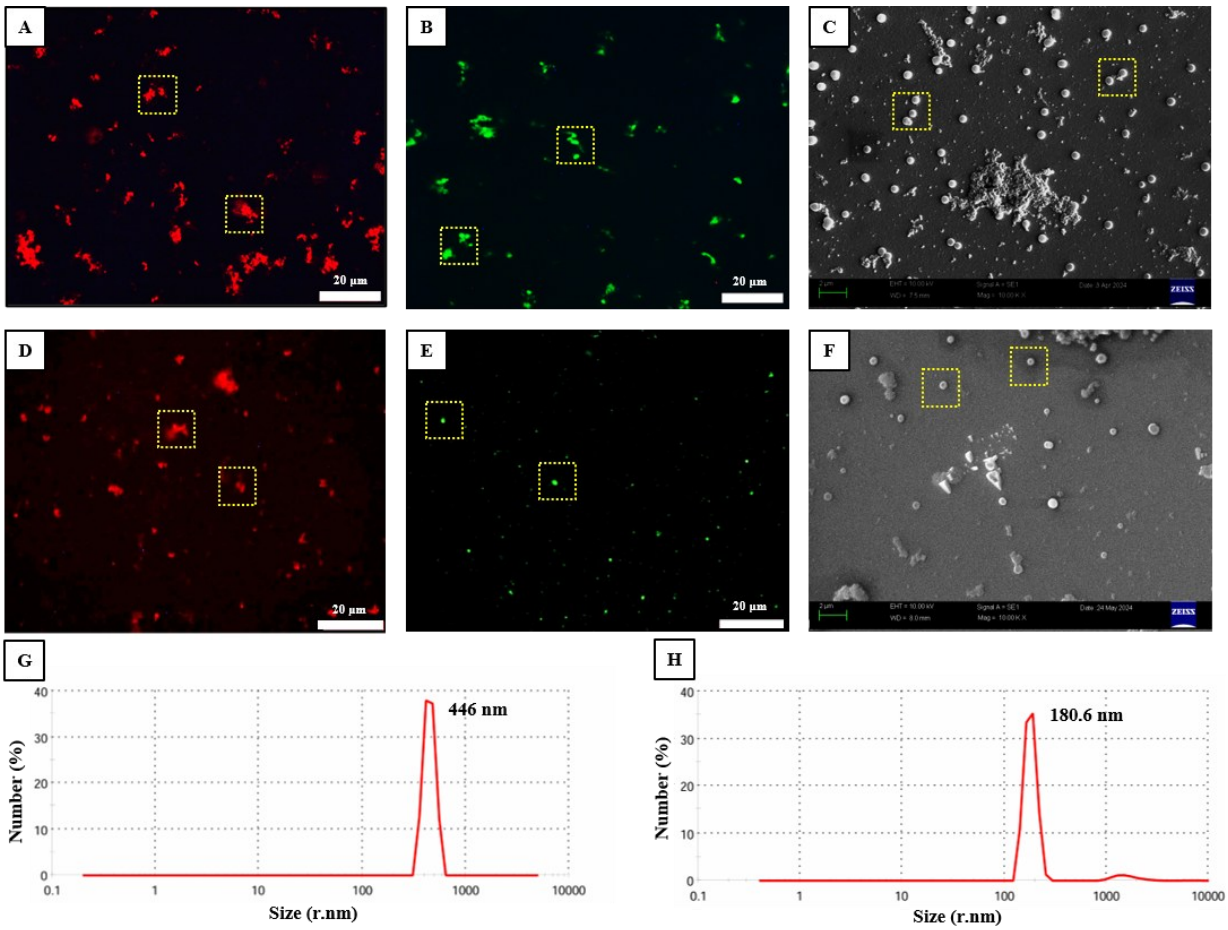

Figure 1: Isolation and validation of isolated SSPBs collected after sucrose density gradient centrifugation. We considered the presence of protein aggregates in a vesicular structure as proof of validation. **A–C.** Chickpea SSPBs visualised by acid fuchsin staining **(A)**, thioflavin T (ThT) fluorescence **(B)**, and scanning electron microscopy (SEM) **(C)**. **D–F.** Corresponding analyses of mung bean SSPBs using acid fuchsin staining **(D)**, ThT fluorescence **(E)**, and SEM **(F)**. Acid fuchsin staining reveals the protein-rich storage materials, and ThT fluorescence detects the presence of protein aggregates in SSPBs. SEM observations revealed that SSPBs were membrane-enclosed spherical particles. The detection of protein aggregates together with the spherical morphology observed by SEM confirms the presence of SSPBs. Yellow boxes in the microscopy images indicate representative SSPBs selected for visualisation. **G–H.** Dynamic light scattering (DLS) profiles showing the hydrodynamic diameter of chickpea **(G)** and mung bean **(H)** SSPBs. The predominant hydrodynamic diameter was approximately 446 nm for chickpea SSPBs and 180.6 nm for mung bean SSPBs, indicating a substantially smaller SSPB population in mung bean. DLS readings were consistent with the data referenced in previous researches conducted on SSPBs.

Scale bars: 20 µm (A, B, D and E), 2 µm (C and F).

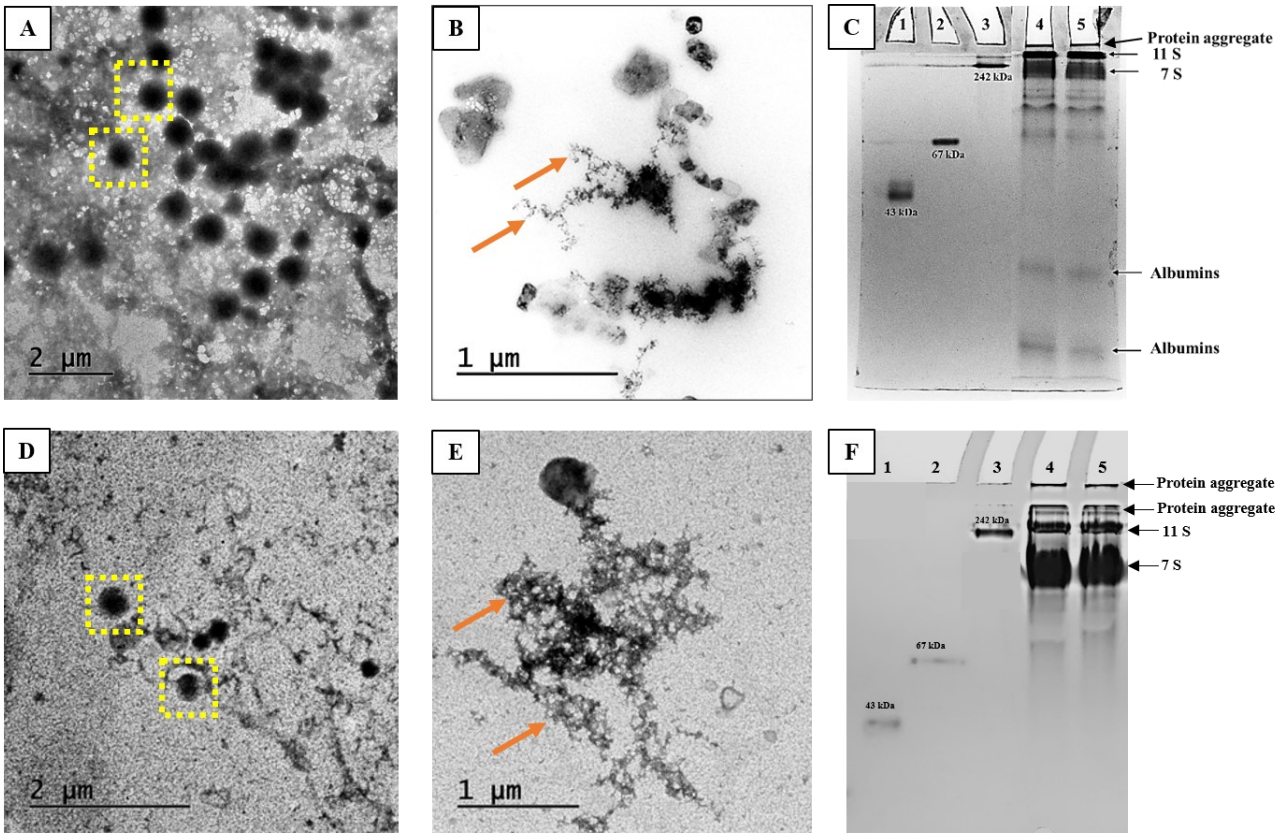

Figure 2. Microstructural and protein compositional characterisation of isolated seed storage protein bodies (SSPBs) from chickpea and mung bean. A. TEM image of isolated chickpea SSPBs showing predominantly spherical, electron-dense particles, a typical characteristics of inclusion bodies. **B.** TEM image of chickpea SSPBs after sonication, showing disruption of the SSPB structures and release of associated protein aggregates, supporting the composite organisation of the protein bodies. **C.** Native PAGE analysis of chickpea SSPBs. Lanes 1,2 and 3 was catalase, human serum albumin and ovalbumin, respectively, as reference proteins; lanes 4 and 5 represented SSPBs isolated by enzymatic lysis and mechanical homogenisation, respectively. The presence of high-molecular-mass in stacking gel indicates the retention of aggregated protein assemblies within the isolated SSPBs. The protein profile was dominated by 11S globulin, with a comparatively lower contribution from 7S globulin. **D–F.** Corresponding TEM, sonication and native PAGE analyses of mung bean SSPBs. Mung bean SSPBs showed a similar spherical morphology and release of protein aggregates following sonication. In contrast to chickpea, the native protein profile of mung bean SSPBs showed a greater contribution from 7S globulin and a lower contribution from 11S globulin, demonstrating distinct species-specific globulin composition within the SSPBs. Together, these observations confirm the microstructure of intact, protein-rich SSPBs containing protein aggregates and revealed differences in their globulin composition between chickpea and mung bean.

Scale bar: 2 µm (A and D), 1 µm (B and E).

Structural Elucidation of Nanotubular Peptide Hormone Amyloid Fibrils via Cryo-EM

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Amyloid is a fibrillar protein conformation often associated with disease, yet it can also play essential roles in biology. Peptide hormones are key regulators of metabolism and utilise a functional amyloid state to concentrate and store peptides in a stable, inert conformation until external signals trigger release into receptor-active hormone monomers. Despite the fundamental biological importance of peptide hormones, their amyloid structure and high-resolution insights into their mechanism of assembly/disassembly remain poorly understood. To gain an understanding of the structure-formation relationship of peptide hormone amyloid, the human tachykinin peptides Substance P, Neurokinin A, and Neurokinin B were characterised using cryo-electron microscopy, as they display unique spectra compared to known amyloids. Five high-resolution tachykinin peptide amyloid structures were solved, with NKA and NKB forming two polymorphs each, and SP presenting with a single fibril type. These structures reveal the first nanotubular amyloids from proteins of eukaryotic origin and indicate the existence of a new structural class of amyloid fibril. The organisation of the peptides within the fibril suggest that the amyloid state prepares hormone monomers for rapid release in a conformation primed for tachykinin receptor activation. These findings mark a major step toward structurally defining the peptide hormone functional amyloidome.

De novo protein design of allosteric modulators targeting opioid receptors

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G protein–coupled receptors (GPCRs) are key therapeutic targets, yet most drugs act at orthosteric sites, often overriding endogenous signalling and contributing to adverse effects. Allosteric modulation offers a route to more precise control of receptor activity but remains challenging to achieve with small molecules for certain GPCRs, including opioid receptors.

Here, we establish a de novo protein design framework to generate synthetic binders targeting opioid receptors as candidate allosteric modulators. Computational protein design platforms (RFdiffusion/ProteinMPNN, BindCraft, and BoltzGen) were used to generate candidate binders targeting extracellular allosteric surfaces of the delta opioid receptor in its peptide-bound state. Candidate binders were expressed with fluorescent tags and evaluated using biophysical methods, including bio-layer interferometry (BLI) to detect binding to purified receptor, and cell-based approaches such as flow cytometry to assess binding to receptors expressed on living cells.

Initial results demonstrate the generation of structurally diverse binders with favourable in silico properties and detectable expression in experimental systems, supporting the feasibility of this approach. Ongoing work is focused on pharmacological and functional characterisation using BRET-based signalling assays and structural validation by cryo-electron microscopy. Together, this work establishes a scalable platform for engineering protein-based allosteric modulators of GPCRs and expands the toolkit for probing and tuning endogenous opioid receptor signalling.

Structural basis of synthetic immune synapse formation by a clinically approved TCR-based T cell engager

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Abstract

Tebentafusp is the first approved TCR-based T cell engager (TCR-TCE) and the only systemic therapy to improve overall survival in metastatic uveal melanoma. Yet it shows low response rates, modest survival benefit and a distinctive pharmacologic dose ceiling. Like other licensed and emerging TCEs, it is a fusion of an affinity-enhanced TCR and an anti-CD3 scFv, redirecting polyclonal T cells against melanoma cells presenting a gp100 peptide on HLA-A*02:01. The structural basis of TCR-TCE function is unknown, yet is critical to understanding synthetic synapse formation. We therefore sought to determine how tebentafusp bridges peptide-HLA on the target cell to CD3 on the T cell.

Here we present a 2.2 Å cryo-EM structure of tebentafusp in complex with gp100-HLA-A02:01 and CD3εδ. Tebentafusp precisely sets the geometry of the synapse, recapitulating a canonical TCR–pHLA synapse. This may explain how killing is triggered from only a handful of surface targets — the structural origin of both its potency and its on-target cytokine release. The engineered pHLA interface accounts for the drug's picomolar affinity. Moreover, extensive contacts with the α2 helix of HLA-A02:01 suggest a structural explanation for the reduced peptide specificity seen in coculture and killing assays. Crystal structures of the parental wild-type TCR from which tebentafusp was derived, and of intermediate CDR2-mutant design variants, support this interpretation. In parallel, we are using dSTORM super-resolution imaging to map the synapse tebentafusp builds at the living cell surface, linking near-atomic architecture to its organisation in situ.

This is, to our knowledge, the first structure of any TCR-TCE, and it defines how a clinically approved drug bridges tumour and effector cells. The results explain tebentafusp's potency and strict HLA-A*02:01 restriction, offer a framework for interpreting on-target toxicities such as cytokine release syndrome, and provide a rational template for engineering next-generation TCR-TCEs.

Keywords: cryo-EM, T cell receptor engineering, peptide-MHC recognition, immunological synapse, bispecific T cell engager

A Twist in the Tail: The Structural Basis of Kv7.2/Kv7.3 M-channel Assembly at Helix D

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Epilepsy is one of the most prevalent neurological disorders, affecting approximately 65 million people worldwide[1]. It is characterised by recurrent seizures, the severity of which can be influenced by genetic, environmental, or unknown factors. One in three individuals with epilepsy do not respond to current treatments[2], highlighting the need for better mechanistic understanding to develop more effective therapies.

The Kv7 family of voltage-gated potassium channels (Kv7.1–Kv7.5), encoded by *KCNQ* genes, has been implicated in epilepsy[3]. Kv7.2 and Kv7.3 form a majority of the neuronal M-channel, which regulates neuronal excitability by generating the stabilising M-current that prevents excessive firing. Mutations in the *KCNQ* genes, particularly within the transmembrane and regulatory domains of Kv7.2 and Kv7.3, are strongly associated with epilepsy.

A novel mutation has been identified in Kv7.3 within the proposed assembly domain, Helix D, resulting from a glutamine to leucine substitution (Q653L). Its effects on channel function remain unknown but may reveal a new mechanism. Additionally, Helix D has eluded structural studies of full-length Kv7 channels due to its location at the end of the highly flexible cytoplasmic C-terminal tail. On a domain level, crystal structures of Helix D from Kv7.1 and Kv7.4 reveal strong tetramer networks contributing to channel assembly[4,5]. However, size-exclusion chromatography of this isolated region shows Kv7.3 favours monomeric and homodimeric states[4]. Given that Helix D in Kv7.3 has a lower sequence conservation within the Kv7 family and plays a key role in heteromeric assembly, further investigation is needed to clarify its contribution to M-channel formation.

Here, we present the first crystal structures of Kv7.2 Helix D and a heteromeric Kv7.2/Kv7.3 Helix D complex, revealing key interfaces that likely mediate subunit association. Complementary biophysical analyses, including size-exclusion chromatography, multi-angle light scattering, mass photometry, and circular dichroism spectroscopy, show that Helix D forms a dynamic assembly capable of supporting distinct oligomeric states. These findings provide a framework for understanding Kv7 channel assembly and lay a strong foundation for ongoing cryo-EM studies of Kv7.2/Kv7.3 channels.

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