



The molecular basis of subtype selectivity of human kinin G-protein-coupled receptors

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G-protein-coupled receptors (GPCRs) are the most important signal transducers in higher eukaryotes. Despite considerable progress, the molecular basis of subtype-specific ligand selectivity, especially for peptide receptors, remains unknown. Here, by integrating DNP-enhanced solid-state NMR spectroscopy with advanced molecular modeling and docking, the mechanism of the subtype selectivity of human bradykinin receptors for their peptide agonists has been resolved. The conserved middle segments of the bound peptides show distinct conformations that result in different presentations of their N and C termini toward their receptors. Analysis of the peptide-receptor interfaces reveals that the charged N-terminal residues of the peptides are mainly selected through electrostatic interactions, whereas the C-terminal segments are recognized via both conformations and interactions. The detailed molecular picture obtained by this approach opens a new gateway for exploring the complex conformational and chemical space of peptides and peptide analogs for designing GPCR subtype-selective biochemical tools and drugs.

GPCRs respond to a wide variety of stimuli, for example photons, amines, ions, peptides, as well as small proteins, and trigger downstream signaling pathways by activating heterotrimeric G proteins¹. They form the most important class of signal transducers in higher eukaryotes. In recent years, the structural characterization of GPCRs by X-ray crystallography has contributed to an unparalleled understanding of their molecular architecture and the structural aspects of ligand binding, receptor activation and allosteric modulation^{2–4}. The wealth of newly obtained structural data has created a strong demand for advanced spectroscopy such as solution and solid-state nuclear magnetic resonance (ssNMR) to gain insights into the mechanism of signaling bias, structural plasticity^{5–7}, ligand binding and ligand–receptor interactions^{8–12}.

Despite these major advances in understanding the molecular basis of GPCR signaling, the foundations of subtype selectivity, especially for peptide ligand GPCRs, remains poorly understood, which hampers mechanistic understanding and rational drug design for peptide receptors. GPCR subtypes are closely related receptors with high sequence similarity, but they can differentiate between sets of ligands that are highly similar in structure or sequence by binding to them with substantially different affinities^{13,14}. Recently, subtype selectivity of rhodopsin-like GPCRs has been studied with non-native, small-molecule ligands, revealing rearrangements of the seven transmembrane bundles to confer binding specificity^{15,16}. In the case of peptide ligands, however, this situation becomes more challenging because of their size and inherent complexity.

Here, we address the molecular basis of subtype selectivity for kinin peptides by human bradykinin receptors (BRs). The peptides kallidin (KD) and bradykinin (BK) are derived from different kininogen isoforms. KD differs from BK only in the presence of one additional N-terminal lysine residue¹⁷ (Fig. 1). Both are high-affinity agonists for the human bradykinin 2 receptor (B₂R), which

regulates vasodilation, and thereby blood pressure, as well as other cardiovascular functions¹⁸. *In vivo*, carboxypeptidases convert KD and BK into desArg¹⁰-kallidin (DAKD) and desArg⁹-bradykinin (DABK) by removing their C-terminal arginine residues. The resulting peptides display only weak binding affinity to the B₂R. However, KD and DAKD bind to the human bradykinin 1 receptor (B₁R) as high affinity-agonists and trigger downstream signaling related to inflammation and pain¹⁹. In contrast, BK and DABK, which lack the additional N-terminal lysine residue, exhibit rather low affinity to the B₁R (Fig. 1). Both receptors share a high overall sequence identity (41%), and it is assumed that the residues forming the peptide-binding pocket of the BRs are highly conserved¹⁴. It is therefore puzzling how these receptors differentiate between peptides with high sequence similarity in such a selective manner.

In the absence of 3D structures for B₁R and B₂R, we address this question by comparing structures of bound peptide agonists determined by ssNMR and combining these data with advanced molecular modeling and docking. Because wild-type, non-engineered human B₁R can only be prepared in small quantities that are insufficient for conventional NMR studies, we made use of dynamic nuclear polarization (DNP) for enhancing the detection sensitivity of our ssNMR experiments by approximately 100-fold. DNP makes use of unpaired electrons in the form of stable radicals added to the sample as a polarization source to increase the NMR signal (Fig. 2a). DNP-enhanced ssNMR with magic-angle sample spinning (MAS) has just recently emerged as a tool in membrane protein research. The signal enhancements enabled challenging applications suffering from small spin numbers. Examples include the analysis of trapped photointermediate states^{20,21}, visualizing cross-protomer interactions²², ligand-binding studies on mammalian transporter complexes²³ or even studies on proteins directly within the cellular context^{24–26}.

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Here, DNP-enhanced ssNMR reveals a substantially different fold of DAKD bound to human B₁R in comparison to earlier reported BK bound to human B₂R¹⁰. The combination of NMR data with advanced docking and modeling enabled a comparative analysis of peptide-binding GPCR interactions. Overall, selectivity is controlled by peptide interactions with nonconserved residues in the binding pockets of B₁R and B₂R. Our findings show that subtype selectivity in peptide receptors is distinct from that in small ligand receptors and indicate that the subtype selectivity of BRs is the result of multiple chemical and conformational factors, which act together in a complex and synergistic manner.

RESULTS

DNP-enhanced solid-state NMR on DAKD with B₁R

Human wild-type BRs, B₁R in particular, remain at low expression levels in eukaryotic hosts after intensive optimizations and have limited stability. Our initial attempts to reconstitute the B₁R into proteoliposomes or lipid cubic phase failed to yield samples suitable for DNP ssNMR studies because of either the difficulty of controlling protein orientation or phase destruction while cooling the samples to the DNP operating temperature (ca. 100 K). As B₁R in 1% *n*-dodecyl β-D-maltoside (DDM) and 0.1% cholesteryl hemisuccinate (CHS) mixed detergent micelles (Supplementary Fig. 1) shows a high DAKD binding affinity close to that in native membranes, we decided to directly characterize the DAKD–B₁R complex in homogeneous solution. This approach allows accessibility of all binding sites and also leads to high DNP signal enhancements (over 100 times; Fig. 2a), substantially better than those achieved on inhomogeneous liposome samples. The cryogenic conditions needed for DNP-enhanced ssNMR experiments also extend the sample lifetime remarkably, permitting time-consuming NMR experiments to be performed on a GPCR-peptide complex of low stability.

To alleviate signal overlap, the complete DAKD sequence was covered by six nonoverlapping isotope labeling schemes, which were designed for the optimized separation of peptide ¹³C signals based on the characteristic chemical shift dispersions of each site (Supplementary Table 1). To resolve the majority of the DAKD signals, we chose DQ-SQ (double-quantum single-quantum) ¹³C-¹³C and TEDOR (transferred-echo double resonance) ¹⁵N-¹³C 2D correlation spectroscopy as the main NMR methods. These experiments have been shown to be suitable for studying membrane proteins under DNP conditions, and both serve as efficient filters for selectively removing the natural abundance ¹³C signals from the receptor and detergent²³. As an example, ¹³C-¹³C DQ-SQ and ¹⁵N-¹³C TEDOR spectra of U-^[13C,15N]P₈F₉-DAKD (Fig. 2b) in complex with B₁R are shown in Figure 2c,d. Following the characteristic spectral patterns, most of the ¹³C and ¹⁵N signals on these 2D spectra could be assigned unambiguously. The same approach was applied to five other labeled peptide–B₁R complexes (Supplementary Figs. 2–5). Remaining overlapping signals were resolved by additional spectroscopic editing and filtering experiments. As shown in Figure 2e,f, the NMR signal of ¹³C in P₈, which is close to and therefore strongly dipolar-coupled to ¹⁵N of P₈, could be selectively detected in TEDOR-type experiments. On the contrary, the NMR signals of ¹³C nuclei that are further away from ¹⁵N, including the C-terminal carboxylate group, were resolved by applying a REDOR-type filter, which dephases the ¹³C magnetization that would be built up by TEDOR. Eventually, we unambiguously assigned almost all backbone and side chain ¹³C and ¹⁵N resonances of DAKD in complex with or in the absence of B₁R (Supplementary Tables 2 and 3) in DNP-enhanced ssNMR spectra.

By comparing the chemical shifts (Supplementary Table 4) of B₁R-bound and free DAKD peptides recorded under the same conditions, we could already identify some interaction areas. The N-terminal residues reveal the most pronounced ¹⁵N chemical shift perturbations (Supplementary Fig. 7a–c). The side chain and

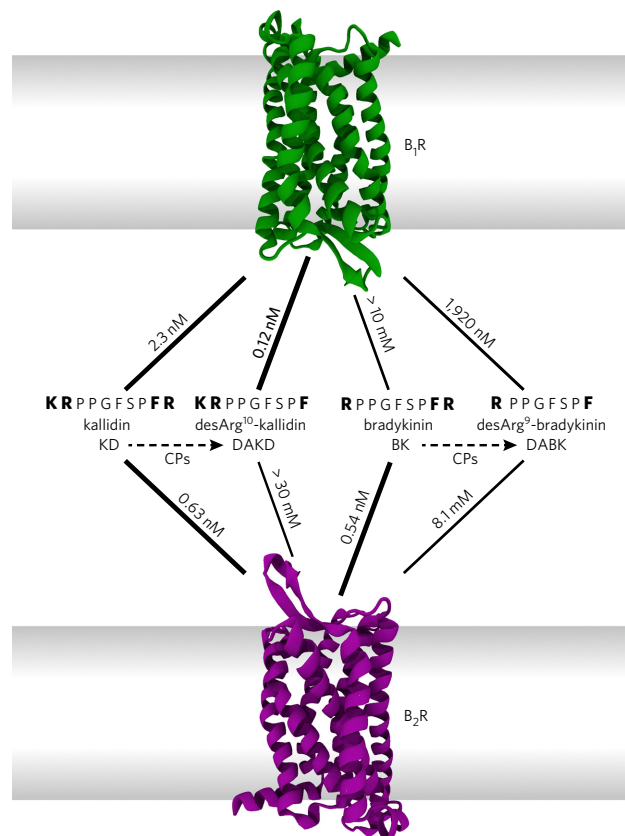


Figure 1 | Affinities of kinin peptides for their respective human

bradykinin receptors, B₁R and B₂R. Kallidin (KD) and bradykinin (BK) derive from kininogen by proteolytic cascades and differ only by an additional N-terminal lysine residue in KD. Both peptides are high-affinity ligands for B₂R. Removal of the C-terminal arginine (dashed lines) by carboxypeptidases (CPs) yields desArg¹⁰-kallidin (DAKD) and desArg²-bradykinin (DABK). Despite their similarity, only DAKD, but not DABK, binds with high affinity to B₁R¹³.

the N-terminal amine group of K1^{DAKD} show significant up-field shifts for their ¹⁵N resonances upon binding to B₁R, which would be in line with electrostatic interactions between this group and the receptor. Similarly, the guanidine group of R2^{DAKD} also exhibits a small observable shift in its ¹⁵N resonance (Supplementary Fig. 7b), which is also an indicator of similar interactions as for K1^{DAKD}. The binding-induced chemical shift perturbations exhibit a different pattern at the C-terminal residues. The presence of B₁R causes a considerable shift of the F9^{DAKD} Cα–N cross-peak in the TEDOR spectrum, which points to a defined conformational change of the C-terminal backbone structure (Supplementary Fig. 7d). We have also monitored this signal in a DAKD analog (DALK, KRPPGKSPL), which differs only at its C-terminal residue and acts as a high-affinity antagonist for B₁R. Furthermore, a significant shift of the L9^{DALK} Cα–N peak was detected in the presence of receptor (Supplementary Fig. 8). This finding resembles the observations for DAKD, and therefore suggests that the C-terminal part of these peptides forms a common motif for interaction with the B₁R.

The receptor-bound structure of DAKD

Despite the significant signal enhancement provided by DNP, all attempts to record long-range distance restraints were unsuccessful because of the limited coherence lifetime under our experimental conditions. Therefore, the DAKD backbone structure in the

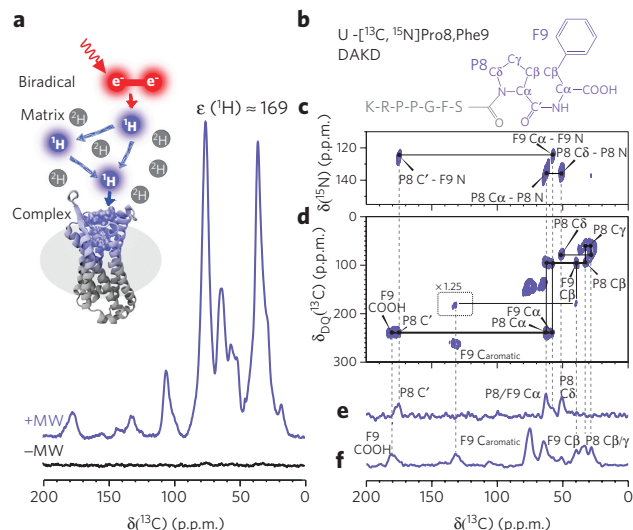


Figure 2 | Experimental setup and exemplary spectra of B₁R in complex with DAKD. (a) A sample containing the DAKD–B₁R complex doped with the biradical AMUPol is subjected to magic-angle sample spinning under continuous wave microwave irradiation, resulting in polarization transfer from electrons via protons to the sites of interest. As a result, a large signal enhancement of the B₁R–DAKD complex (purple) is observed in comparison to conventional NMR (black) (see Online Methods). (b) One of the DAKD labeling schemes used here: U-[¹³C,¹⁵N]P₈F₉ DAKD. The chemical shift assignment was accomplished by following the connection of the signals on TEDOR ¹⁵N–¹³C spectra (c), DQ–SQ ¹³C–¹³C (d), TEDOR-filtered ¹³C–¹³C spectra (e), and DQ/REDOR doubly filtered ¹³C–¹³C spectra (f). The pulse program used for f is presented in **Supplementary Figure 6**. C_{aromatic} carbons on a phenyl ring. Dashed lines guide the chemical shift connectivity among different spectra.

B₁R–DAKD complex had to be calculated based on the backbone ¹³C and ¹⁵N chemical shifts.

To obtain a converging and verified solution using these sparse data, we calculated the DAKD backbone structure from torsion angle restraints directly predicted from chemical shifts (‘backward’ approach). The obtained solution was examined by an extensive ‘forward’ protocol based on random structural libraries from which chemical shifts were predicted and statistically compared to experimental values. The best matching set was finally clustered. Combining both approaches allowed us to assess the robustness of this methodology.

The determined DAKD backbone conformation is depicted in **Figure 3a**, displaying a V-shaped fold bearing a characteristic β-turn-like structure in the middle part of the peptide (P3^{DAKD}–F6^{DAKD}). This structural motif was verified biochemically using an engineered DAKD analog in which the amide of F6^{DAKD} was methylated. This modification disrupts the β-turn-like structure and should therefore stretch the peptide conformation. Indeed, our binding assay shows that this DAKD analog has a 1,000-fold lower binding affinity for B₁R than the native peptide (**Fig. 4a**).

To elucidate the molecular origins of subtype selectivity between B₁R and B₂R, we calculated the backbone structure of BK bound to B₂R from our previously reported NMR data¹⁰ using the method described above. B₂R-bound BK shows a conformation strikingly distinct from that of B₁R-bound DAKD. It features an S-shaped structure with a ₃₁₀-helix-like segment in the middle (**Fig. 3b**). Moreover, the C-terminal part in BK is folded in a turn-like structure, whereas an extended open conformation is observed for DAKD. Interestingly, binding of BK to the B₂R causes a major conformational rearrangement of the peptide, whereas

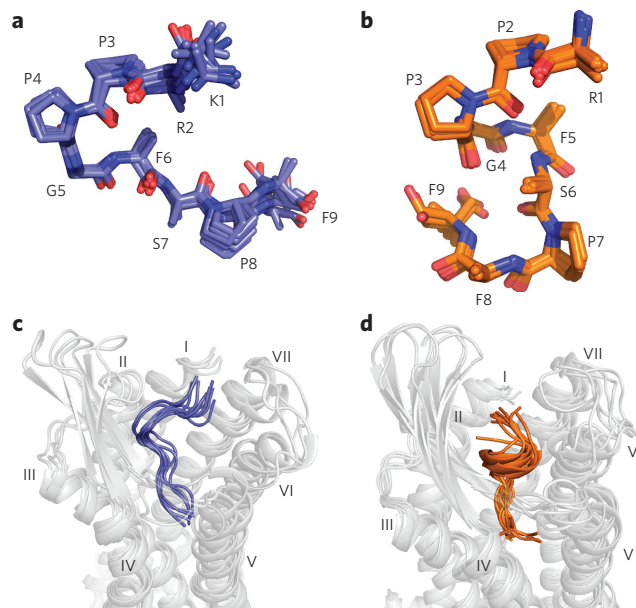


Figure 3 | Backbone structures of DAKD in complex with human B₁R in comparison to BK bound to human B₂R. Only backbone and Cβ atoms are shown. (a) The backbone structure of DAKD calculated from NMR data features a V-shaped fold with a β-turn-like structure around P3–F6. (b) The NMR-based backbone structure of BK is characterized by an overall S-shape with a ₃₁₀-helix-like segment (P2–F5) in the middle. (c,d) Rosetta modeling of DAKD in B₁R (c) and BK in B₂R (d) reproduces the characteristic V-shape of DAKD and the S-shape fold of BK (see text and **Supplementary Fig. 10** for further details).

the structure of DAKD is essentially the same both when in solution and when bound to the B₁R (see **Supplementary Fig. 9** for a comparison of all structures). All chemical shift values and the structure refinement statistics are summarized in **Supplementary Tables 2–8**.

Docking and modeling

To understand their subtype-specific binding behavior, models of DAKD in complex with B₁R and of BK in complex with B₂R were generated using Rosetta multiple-template comparative modeling and flexible peptide docking. The aim was to identify the binding interface of DAKD and BK at B₁R and B₂R, respectively. B₁R and B₂R models were created on the basis of 24 experimentally determined class A GPCR structures (**Supplementary Tables 9 and 10**). Both models show common structural features, as observed in the crystal structures of the other peptide-binding GPCRs, such as an α-bulge in helix V and proline-kinks in helices IV and VI. The extracellular loops (ECL) exhibit valid conformations with ECL₂, adopting a β-sheet, which is found in many peptide-binding GPCRs. The obtained models were found to be independent of the activation state of the used GPCR templates (see Online Methods).

DAKD and BK were simultaneously folded and docked into the B₁R and B₂R models, respectively, using the Rosetta FlexPepDock application²⁷. In both cases, ligand docking converged to a single solution. This approach was chosen over a direct docking of the NMR-derived structure into the receptor models, as it allows a better sampling of the conformational space by Rosetta. The Rosetta-derived models of docked DAKD and BK have the same distinct conformations as described above, i.e., a V-shaped backbone structure with a type-II β-turn for B₁R-bound DAKD and an S-shaped fold with a central ₃₁₀-helix for B₂R-bound BK (see **Fig. 3c,d** and **Supplementary Fig. 10**).

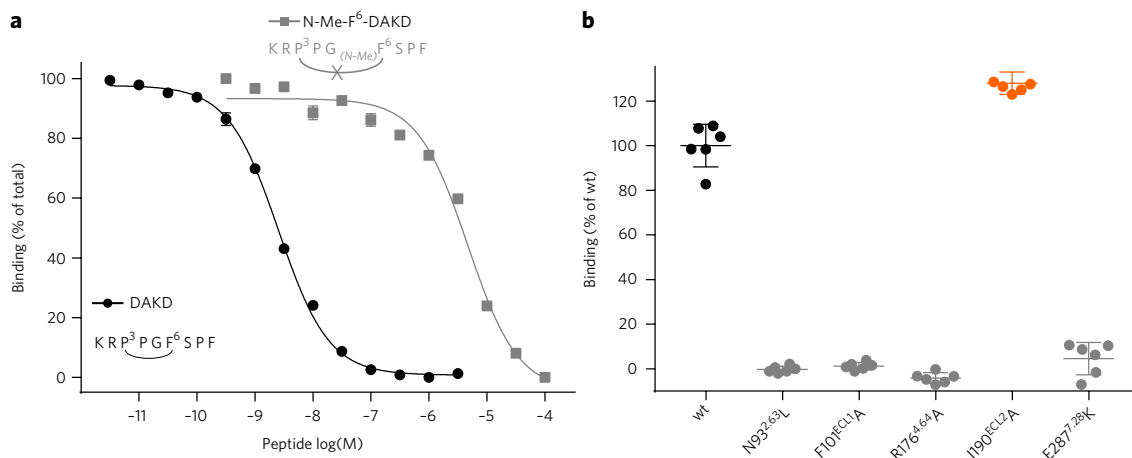


Figure 4 | Functional characterization of peptide variants and B₁R mutants. (a) Disruption of the central β -turn in DAKD results in a strong decrease in affinity for the B₁R. The methylation of the amide nitrogen of F6^{DAKD} disrupts the central β -turn and results in a 1,000-fold decrease of DAKD binding affinity (K_i DAKD: 1.11 ± 0.04 nM (circles); DAKD linearized: 2.03 ± 0.17 μ M (rectangles)). (b) Verification of key peptide interaction sites predicted from B₁R-DAKD models by site-directed mutagenesis (maximal binding of DAKD normalized to wild type; $n = 6$). I190^{ECL2}A shows an increased binding activity (129%, orange scatter), whereas all other mutations result in a complete loss of DAKD binding. Expression levels of B₁R mutants were assessed by western blot (**Supplementary Fig. 13**).

These distinct peptide structures suggest some major differences within their respective binding pockets, which have been believed to be similar overall. Approximately 3/4 of the B₁R binding pocket residues (defined here for a region within 5 Å distance to any ligand atom) are sequence counterparts of B₂R. Within the group of counterpart residues, the sequence identity and similarity between B₁R and B₂R are 45% and 67%, respectively, which is slightly higher than those for the complete receptor (41% and 59%). Furthermore, DAKD and BK were found to occupy a similar sized region within the binding pocket. The change in the solvent-accessible surface area of B₁R and B₂R upon binding was quantified to be $1,713 \pm 45$ Å² and $1,814 \pm 129$ Å², respectively.

B₁R-DAKD and the B₂R-BK receptor-peptide interfaces

To identify possible ligand-binding sites in B₁R and B₂R, we collected statistics of the contact frequency of peptide-receptor residue pairs. The number of pairwise residue interactions with a Rosetta score less than -1.0 Rosetta energy units (REU) across the 1,000 top-scoring models was counted (**Supplementary Figs. 11 and 12**), providing a likelihood of residue-residue interactions. For DAKD, we observed a binding mode that agrees well with previously reported mutagenesis^{28,29} and modeling studies³⁰ and fulfills most of the predicted contacts from which we had derived a set of upper distance restraints to guide ligand docking.

The N terminus of DAKD is facing transmembrane helices (TMH) VI and VII, with the N-terminal amine group and the side chains of K1^{DAKD} and R2^{DAKD} located next to a cluster of polar, acidic residues (**Fig. 5a,c**). K1^{DAKD} is coupled via electrostatic interactions with E287^{7,28}, D288^{7,29} and D291^{7,32}, whereas the side chain of R2^{DAKD} is involved in contacts primarily with E273^{6,58}, Q277^{6,62}, E287^{7,28} and D291^{7,32} (**Supplementary Fig. 11**). A role of E273^{6,58} and D291^{7,32} in DAKD binding was reported previously^{30,31}, and replacement of these residues by alanine decreased ligand-binding affinity. Similarly, the charge-inverting mutation E287^{7,28}K fully abolishes DAKD binding to the receptor (**Fig. 4b**).

The C terminus of the peptide is facing TMH III, V and VI, which are known to be involved in receptor activation². The C terminus of DAKD interacts electrostatically with the side chain of K118^{3,33}, and the phenyl ring of F9^{DAKD} is surrounded by a group of hydrophobic residues (I203^{5,39}, Y266^{6,51}, A270^{6,55} and L294^{7,35})

(**Fig. 5a,e**). These observations match previous experimental data²⁹ identifying K118^{3,33} as the B₁R key residue that discriminates between DAKD and BK binding.

In the middle part of the peptide, the interaction between the side chain of F6^{DAKD} and L294^{7,35} (**Fig. 5a**) occurs in 89% of our models (**Supplementary Fig. 11**). Meanwhile, the hydrogen bond interaction involving the backbone carbonyl of the neighboring residue, G5^{DAKD}, and the Ne atom of W93^{2,60} is the most frequently observed contact (94%) in our B₁R-DAKD models (**Supplementary Fig. 11**). The indole ring of W93^{2,60} is fixed in a preferred orientation for interaction with G5^{DAKD} by a π - π stacking with the F302^{7,43} phenyl ring, suggesting an indirect role for F302^{7,43} in ligand binding through concerted interactions involving residues in the binding pocket. This picture is compatible with previous observations that the F302A mutation in B₁R negatively modulates DAKD binding³⁰.

Another frequently detected interaction in our B₁R models is that between the hydroxyl groups of receptor residue Y266^{6,51} and S7^{DAKD} (**Fig. 5a**; **Supplementary Fig. 11**). When Y266^{6,51} is replaced with alanine or phenylalanine, no change in binding affinity is observed³⁰. We found that another polar residue, N298^{7,32}, adjacent to Y266^{6,51}, can possibly substitute a hydrogen bond to the OH group of S7^{DAKD}, which would require only a flipping of the S7^{DAKD} side chain, and this could compensate for the loss of a hydroxyl group of Y266^{6,51}.

In addition to reproducing previous experimentally determined interactions, our B₁R-DAKD models predict a series of new binding contacts, which we tested experimentally via site-directed mutagenesis. Proline residues P3^{DAKD} and P4^{DAKD} make hydrophobic contacts with F101 and I190 in ECL1 and ECL2, respectively (**Fig. 5a**; **Supplementary Fig. 11**), whereas the backbone at P4^{DAKD} is stabilized by a hydrogen bond with N96^{2,63}. Furthermore, R176 in ECL2 provides several hydrogen bond contacts to the backbone of F6^{DAKD} and P8^{DAKD}, although the exact geometry differs slightly between the different receptor models. It is conceivable that these residues have not been considered part of the receptor binding site in previous modeling studies³⁰⁻³³ that were based solely on the formerly available structures of bacteriorhodopsin or bovine rhodopsin. Our modeling approach using multiple genetically and functionally more closely related GPCR templates provides more confident receptor models, especially for their extracellular loop regions, supported

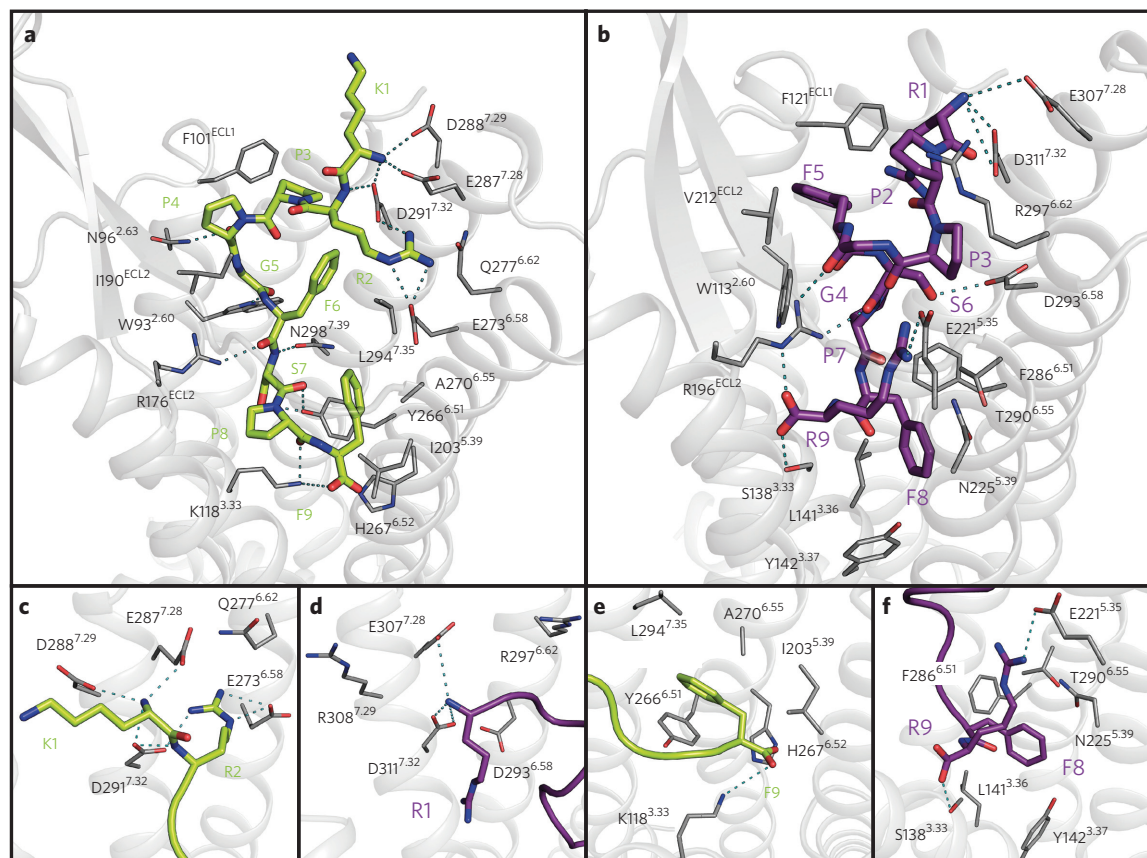


Figure 5 | Structural characterization of the B₁R-DAKD (green) and B₂R-BK (purple) binding pocket. (a) Top view of DAKD docked to a comparative model of B₁R. **(b)** Top view of BK docked to a comparative model of B₂R. **(c)** Side view of the DAKD and **(d)** BK N-terminal binding site at TMH VI and VII. **(e)** Side view of the DAKD and BK **(f)** C-terminal binding site between TMH3, 5 and 6. The ligand is shown as thick sticks. Receptor residues predicted to be involved in ligand binding are labeled and are shown as thin sticks. Predicted interactions are indicated by dotted cyan lines. Atoms are colored by type (oxygen, red; nitrogen, blue; sulfur, yellow).

by the biochemical validation of newly predicted receptor–peptide contacts. Indeed, replacing F101(ECL1) with an alanine residue fully abolishes DAKD binding to the B₁R. Polar contacts between N96^{2.63}–P4^{DAKD} and R176(ECL2)–F6^{DAKD} and R176(ECL2)–P8^{DAKD} are crucial for DAKD binding, as the introduction of hydrophobic residues in these sites (N96^{2.63}L and R176(ECL2)A) also results in a complete loss of binding affinity of DAKD (Fig. 4b). By contrast, introduction of an alanine at position I190(ECL2) maintains DAKD binding with an IC₅₀ comparable to that of wild-type B₁R (Fig. 4b), which is in agreement with the predicted frequency of the I190–P4^{DAKD} interaction (28%; Supplementary Fig. 11) and/or the ability of alanine to compensate the hydrophobic contacts provided by a flexible Ile side chain in a loop.

A similar analysis was also carried out for the B₂R–BK receptor–peptide interface (Fig. 5b,d,f and Supplementary Fig. 12). Compared to DAKD, BK adopts a similar overall pose within the B₂R binding pocket, with the N terminus facing TMH VI and VII and the C terminus bound to TMH III, V and VI. The observed B₂R–BK contacts, as obtained by a per-residue breakdown of the interface energy of our models (Supplementary Fig. 12), match well with residues found in a previous site-directed mutagenesis study of the B₂R binding pocket³¹. Furthermore, our B₂R binding model predicts several interactions that have not been described before, such as hydrophobic contacts between F5^{BK} and the aromatic side chains of W113^{2.60} and F121 in ECL1, as well as several hydrogen bonds between the central portion of the BK backbone and R196 in ECL2.

DISCUSSION

Our data indicate that the subtype selectivity of BRs is the result of multiple chemical and conformational factors, which act in a complex and synergistic manner.

BRs discriminate between the N-terminal parts of their respective peptides, BK and DAKD, mainly via their binding chemistry. Our work reveals that a cluster of acidic residues located at the extracellular side of TMH VI/VII and ECL3 are crucial for the binding of positively charged N-terminal residues. The location of such a cluster is supported by the previous work on B₁R/B₂R chimeric receptors^{30,31}. In B₁R, K1^{DAKD} and R2^{DAKD} interact with E287^{7.28}, D288^{7.29} and D291^{7.32} and E273^{6.58}, Q277^{6.62}, E287^{7.28} and D291^{7.32}, respectively (Figs. 5 and 6; Supplementary Figs. 11 and 12). These residues are all conserved between the B₁R and the B₂R except D288^{7.29} and Q277^{6.62}, which are replaced by R297^{6.62} and R308^{7.29} in the B₂R (Supplementary Fig. 14). The B₂R agonist BK carries only one charged N-terminal residue, R1^{BK}, which interacts mainly with residues D293^{6.58}, E307^{7.28} and D311^{7.32}. These residues coincide with those in B₁R that are responsible for binding to R2^{DAKD}. This means that R2^{DAKD} and R1^{BK} have a similar protein interaction interface, but the N-terminal residues K1^{DAKD} and R1^{BK} of both agonists have a different position within the binding pockets of both receptors.

Due to the replacement of Q277^{6.62} and D288^{7.29} in B₁R by R297^{6.62} and R308^{7.29} in B₂R, the negative charge density, and therefore the electrostatic binding capacity, of B₂R for peptide ligands with a positively charged N terminus is strongly reduced. This conclusion is consistent with the observation that replacement of these residues

by alanine or positively charged amino acids substantially impairs BK binding in B₂R³¹. Both the altered charge density and different N-terminal position in the binding pocket together could explain why B₁R selects peptides that contain two charged N-terminal residues K1 and R2 (DAKD or KD) over those starting with only positively charged N-terminal residue R1 (BK, DABK). The N-terminal peptide selectivity of B₁R in other species further supports this argument. In dog B₁R, for example, D^{7.29} is replaced by a neutral Asn residue (Supplementary Table 11), and the receptor shows no significant selectivity between DAKD and DABK³⁴. Furthermore, in some rodents, residue E^{7.28} is replaced by a Lys residue, which inverts the local charge and can impose a strong perturbation on the chemical architecture of the N-terminal binding cluster. Indeed B₁R of some of these rodents are known to have a diminished, or even reversed, N-terminal selectivity for human kinin peptides³⁴. Our mutagenesis study also validates the strong impact of this residue in species-specific subtype selectivity.

The presented data suggest that B₁R and B₂R discriminate between the C-terminal parts of their respective peptide ligands via specific peptide conformations and peptide–receptor interactions. The C-terminal segments of DAKD and BK fold into distinct conformations in B₁R and B₂R, respectively. The four C-terminal residues of BK form a turn-like structure in which the R9^{BK} residue flips back toward the extracellular surface. The formation of such a turn-like structure in DAKD is hampered by the lack of an additional C-terminal Arg residue, as present in BK. The drastic differences in the C-terminal folding are connected to the distinct receptor–peptide interactions. In B₁R, residues K118^{3.33}, I203^{5.39}, Y266^{6.51} and H267^{6.52} show contacts with F9^{DAKD}, and residues K118^{3.33} and R176^{ECL2} show contacts with P8^{DAKD} (Figs. 5 and 6; Supplementary Fig. 11). In B₂R, residues N134^{2.29}, S138^{3.33}, M192^{4.60}, R196^{ECL2}, E221^{5.35} and N225^{5.39} interact with R9^{BK}, and Y142^{3.37}, F288^{6.51} and Q287^{6.52} with F8^{BK} (Figs. 5 and 6; Supplementary Fig. 12). Residue H221^{5.35} in the B₁R is replaced by E221^{5.35} in the B₂R, which provides an electrostatic binding site in the B₂R for peptides with a C-terminal Arg. In addition, residue I225^{5.39} in the B₁R is replaced by N225^{5.39} in B₂R, which offers a better matching in binding pocket polarity for peptides with polar side chains at the C terminus. In agreement with our model, the previously reported N225^{5.39}A mutation decreases BK binding by a factor of 5 (ref. 31).

Moreover, the positively charged residue K118^{3.33} in B₁R is substituted by a neutral S138^{3.33} in B₂R, which is more compatible with the positively charged and flipped C-terminal Arg residue R9^{BK}. This finding is in line with earlier suggestions that residue 3.33 is key for triggering receptor subtype specificity²⁹. In addition, other sites with conserved differences between mammalian B₁R and B₂R also contribute to the binding of the peptide C-terminal region. Such a complex pattern indicates a highly integrated network for accommodating the distinct C-terminal folding of DAKD and BK. It can be envisaged that such a complex interaction network could also serve as a subtle regulator for functional switching between agonism and antagonism while not compromising the binding affinity, which has been indeed suggested for the DALK peptide³⁰.

The reconfiguration of the N- and C-terminal binding networks leads to different positioning of the N terminus of the peptide, as well as different orientation of the C-terminal residues in the receptor (Fig. 6). Together with these changes, residues in the middle part of the peptide switch to distinct binding sites on the receptor, allowing polarity matching as shown in Figure 6. The middle parts of the peptides, which share the common sequence, serve as linkers, allowing correct presentation of the peptide N and C termini with respect to the receptor. The importance of the conformation of this central segment is highlighted by the drastic loss of affinity of DAKD upon methylation of the backbone amide of F6, which is expected to impair the formation of the central β -turn (Fig. 4a).

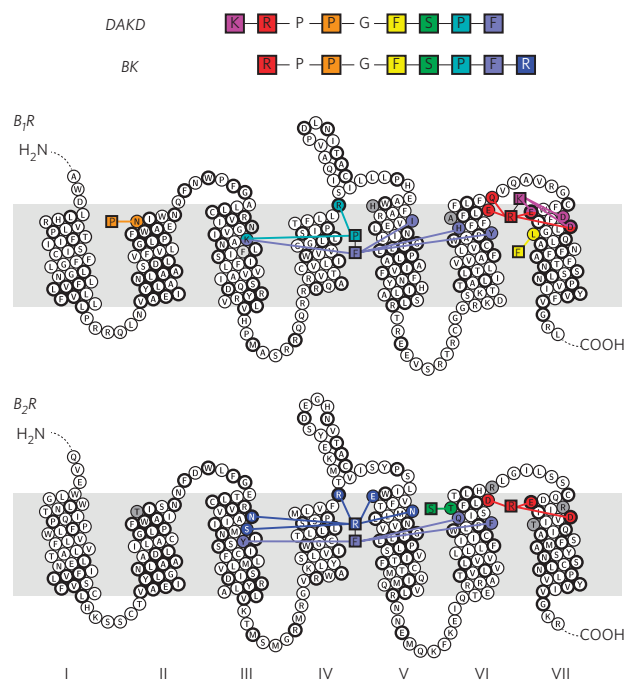


Figure 6 | Representation of key interactions responsible for high affinity binding of DAKD to B₁R and of BK to B₂R. B₁R discriminates between DAKD and BK mainly via electrostatic interactions at the N terminus, whereas B₂R selects via a complex interaction network as a result of different C-terminal structures of the BK and DAKD. The residues conserved among B₁R and B₂R are shown in bold circles.

It may be peculiar that free and bound structures of DAKD show high similarity, but one could speculate about potential biological reasons: B₁R shows high basal activity comparable to that of agonized B₂R^{13,35}. Therefore, B₁R could already adopt or sample a ‘partially activated’ conformation, which is ready to bind a prestructured DAKD without the need of major structural reorganizations. In contrast, B₂R shows larger activity differences between its ground and activated states.

In summary, although the human B₁R and B₂R show high sequence identity, many nonconserved residues in the peptide-binding pocket reshape the binding landscape by casting distinct interactions to selectively accommodate peptides that have similar sequences but distinct structures. Whereas the mechanism of subtype selectivity of some GPCRs is evident from major structural divergence of the orthosteric binding pockets, for example the opioid receptors³⁶, other receptor subtypes such as orexin and muscarinic receptors only display minor rearrangements of specific residues and subtle changes of the size and shape of the binding pocket^{15,16}. Recent crystallographic studies on the orexin¹⁵ and endothelin B receptors³⁷ furthermore attempted to attribute the subtype selectivity of peptide ligand GPCRs to both the N-terminal region of these receptors¹⁵ and their TMH cores³⁷. However, the mechanism of human BR subtype selectivity of peptide ligands is strikingly more complex than previously assumed, which is caused by the intrinsic complexity of the conformational and chemical space of peptides. The diversity in the receptor sequence, the polymorphism of peptide conformation and the distinct binding chemistry are all required to create the unique subtype selectivity in these peptide receptors. The requirements of both N- and C-terminal binding at distal sites on the receptor also justify the tremendous difficulty of efficiently developing small-molecule regulators of BRs. Small sized ligands, which lack the capacity to establish distal interactions within the

receptors emulating the peptide N- and C-terminal binding modes, are less likely to be promising antagonists.

As demonstrated in this work, the integration of DNP-enhanced ssNMR with advanced molecular modeling and docking techniques offers a powerful and novel way to obtain structural and mechanistic insights into challenging GPCR targets.

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METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the [online version of the paper](#).

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Author contributions

C.G. and H.M. conceived the project. L.J. expressed and purified B₁R and performed radioactive ligand binding assays. J. Mao designed and performed DNP-ssNMR measurements, analyzed data, determined structural models of the kinin peptides and performed the analysis of B₁R homolog sequences. G.K. performed molecular modeling and computational docking studies. C.R. and T.K. carried out expression of mutants and additional binding assays. H.R.A.J., C.R. and H.S. provided and analyzed supplementary liquid-state NMR data on nonbound DAKD. J.P. assisted with receptor purification and sample preparation. J. Meiler, H.M., C.G. supervised the overall project. L.J., J. Mao, G.K. and C.G. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the [online version of the paper](#). Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to J. Mao, H.M. or C.G.

ONLINE METHODS

Peptide synthesis and labeling schemes. Uniformly ^{15}N - ^{13}C -labeled variants of DAKD (KRPPGFSPF) and DALK (KRPPGFSPFL) were ordered from Eurogentec, Cologne, Germany and Thermo Fisher Scientific, Ulm, Germany. Linearized DAKD (KRPPGFSPF, methylation of the amide nitrogen at F6) was from Thermo Scientific. Radiolabeled DAKD (3,4-PROLYL-3,4- $^3\text{H}(\text{N})$) was from PerkinElmer, Rodgau, Germany. Unlabeled DAKD for binding assays was obtained from Eurogentec. Peptides used in this study are summarized in **Supplementary Table 1**.

GPCR production in Sf9 cells. The cDNA encoding the full-length human B_1R was codon optimized for expression in insect cells and cloned into the pOET1 transfer vector (Oxford Expression Technologies, Oxford, UK) via 5' BamHI, 3' HindIII restriction sites. The receptor was flanked with an N-terminal decahistidine and FLAG tag as well as a C-terminal StrepII tag. Recombinant baculoviruses were generated using the flashBAC kit according to manufacturer's instructions (Oxford Expression Technologies). High titer baculoviruses were used to infect Sf9 cells at a cell density of 1.75 to 2×10^6 cells/ml cultured in TMN-FH medium (c.c.pro GmbH, Oberdorla, Germany) supplemented with 2 mM glutamine (PAA Laboratories, GE Healthcare, Munich, Germany), 5% (v/v) FCS (BioWest, Nuaille, France), 7.5 nM vitamin B12 (Sigma-Aldrich), 50 $\mu\text{g}/\text{ml}$ gentamicin (Thermo Fisher Scientific), 0.1% pluronic F-68 (Applchem, Darmstadt, Germany). Cells were harvested 96 h post infection and stored at -80°C until further use. Cell pellets were resuspended in 50 mM HEPES-NaOH (pH 7.6), 100 mM NaCl, 10 mM EDTA (supplemented with protease inhibitors: 5 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM EDTA, 1 μM E64, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ apronin, 1 mM PMSF) and lysed by nitrogen decompression. Nonlysed cells and debris were collected at 1,000g for 10 min at 4°C and membranes were pelleted by ultracentrifugation (210,000g for 90 min at 4°C). Membranes were resuspended in 50 mM HEPES-NaOH (pH 7.6), 100 mM NaCl, 1 mM EDTA, and 5% (w/v) glycerol, flash frozen in liquid nitrogen and stored at -80°C until purification. The receptor was solubilized in 1% *n*-dodecyl β -D-maltoside (DDM) and 0.1% cholesteryl hemisuccinate (CHS) for 3 h at 4°C after dilution of the membranes in buffer A (50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 5% (w/v) glycerol, 200 nM ^{15}N - ^{13}C -labeled DAKD or DALK). Nonsolubilized material was removed by ultracentrifugation (210,000g for 45 min at 4°C) and cleared solubilizates were loaded onto HisTrap HP columns (GE Healthcare, Munich, Germany) using Äkta systems (GE Healthcare). Receptors were washed with buffer A1 (buffer A supplemented with 20 mM imidazole, 200 nM ^{15}N - ^{13}C -labeled DAKD or DALK, 0.07% DDM, 0.007% CHS) and buffer A2 (buffer A supplemented with 50 mM imidazole, 200 nM ^{15}N - ^{13}C -labeled DAKD or DALK, 0.07% DDM, 0.007% CHS) and eluted in buffer B (buffer A supplemented with 400 mM imidazole, 0.07% DDM, 0.007% CHS). Receptors were concentrated in 50 kDa molecular weight cut-off concentrators (Amicon, Merck Millipore, Darmstadt, Germany), and buffer was exchanged to 50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 5% (w/v) [^{12}C - ^2H]glycerol (Euriso-Top, Saint-Aubin, France) in 76% $\text{D}_2\text{O}/18\%$ H_2O . The labeled peptide was added in molar excess during concentration and omitted in the final concentration steps to reduce nonspecific binding. 300–400 μg receptor–ligand complex (equivalent to 285–340 μM) were used per sample and were supplemented with 10 mM AMUPol, mixed in a 1:1 ratio [^{12}C - ^2H]glycerol, and transferred to 3.2 mm sapphire or zirconium oxide rotors. Samples were frozen *in situ* in the cryo-gas flow of the spectrometer or in liquid nitrogen.

Reference samples contained 10 μg ^{15}N - ^{13}C -labeled DAKD or DALK in 50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, 5% (w/v) [^{12}C - ^2H]glycerol (76% $\text{D}_2\text{O}/18\%$ H_2O), 10 mM AMUPol and 4% DDM/0.4% CHS to mimic detergent increase during receptor–ligand complex concentration.

Heterologous expression of B_1R s in HEK293T cells. Wild-type and mutant B_1R sequences were synthesized (GenScript) and cloned into pcDNA3.1 for mammalian expression. Sequence integrity was verified by sequencing. HEK293T cells were cultured in DMEM medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37°C . Cells were passaged once in 3 d until they reached 100% confluence. At 24 h before transfection

cells were seeded into a 10-cm culture dish (Thermo Fisher Scientific, Waltham, MA, USA) at a cell density of 2.2×10^6 cells per dish (five dishes per construct). Transfection was performed with 10 μg DNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. At 48 h post transfection, growth medium was removed, cells were washed with PBS and detached using a cell scraper. Cells were collected by centrifuging at 130g for 5 min and snap frozen in liquid nitrogen until further use.

Cells were resuspended in breaking buffer (25 mM HEPES-NaOH pH 7.5; 1 mM EDTA) and protease inhibitors (Complete EDTA free, Roche Applied Science; 1 mM PMSF) at a cell density of 4×10^6 cells/ml. Cells were disrupted by nitrogen decompression in a pressurized vessel (Parr, Moline, USA). Intact cells and cell debris were removed by centrifugation (1000g for 10 min at 4°C). Membranes were then pelleted using an ultracentrifuge (100,000g for 60 min at 4°C) and resuspended in membrane buffer (25 mM HEPES-NaOH pH 7.5; 150 mM NaCl; 10% glycerol) using a glass dounce homogenizer. Total membrane protein was quantified using the bicinchoninic acid (BCA) method (Pierce Biotechnology, Rockford, USA), with bovine serum albumin as a standard. Membranes were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C until further use. After resuspension in lysis buffer (25 mM HEPES-NaOH, pH 7.5, 10 mM EDTA and protease inhibitors (Complete EDTA free, Roche Applied Science; 1 mM PMSF (Carl Roth))) cells were lysed by nitrogen decompression (Parr Instrument Company, Moline, IL, USA). Nonlysed cells and debris were removed by centrifugation (1,000g for 10 min at 4°C), and membranes were subsequently pelleted by ultracentrifugation (180,000g for 60 min at 4°C). Membranes were homogenized in membrane buffer (25 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5% (w/v) glycerol), aliquoted, flash frozen and stored at -80°C until use. Total membrane protein content was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard.

For western blotting, membrane suspensions equivalent to 150 μg total membrane protein were treated with 4 U benzonase endonuclease in benzonase buffer (50 mM Tris-HCl pH 8.0, 1 mM MgCl_2) and protease inhibitors (Complete EDTA free, Roche Applied Science; 1 mM PMSF) for 20 min at 4°C , resolved on a 4–12% Bis-Tris NuPAGE gel (Thermo Fisher Waltham, USA), and transferred onto PVDF membranes. The PVDF membrane was blocked with 5% nonfat milk at room temperature (20 – 22°C) for 1 h in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl; 0.05% Tween-20) and incubated with a monoclonal alkaline phosphatase-coupled anti-FLAG M2 antibody (Sigma-Aldrich, A8592 Steinheim, Germany) for 1 h at room temperature. The PVDF membrane was washed five times for 5 min with TBST and developed in alkaline phosphate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl_2) containing 0.33 mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidinium (BCIP) and 0.165 mg/ml nitro-blue tetrazolium chloride (NBT).

Binding assays. B_1R binding assays in Sf9 membranes were executed by incubation of 10–50 μg total membrane protein with increasing concentrations of radiolabeled DAKD (^3H Lys[Des-Arg⁹]bradykinin (^3H]DAKD, PerkinElmer, Boston, MA, USA)) (for determination of dissociation constants (K_D)) or unlabeled DAKD variants (for determination of inhibition constants) in 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 , 1 \times SyntheChol (Sigma-Aldrich Steinheim, Germany) for 60 min at room temperature. Binding was terminated by rapid filtration over GF/B glass fiber filters, wash steps were executed with 50 mM HEPES-NaOH (pH 7.6), and remaining radioactivity was analyzed by liquid scintillation counting. Competition binding assays using receptors expressed in HEK293T cells were performed accordingly, using 100 μg total membrane protein in 50 mM Tris-HCl (pH 7.4), 5 mM MgCl_2 and 1 nM [^3H]DAKD. GF/B filters were washed with ice-cold water. The affinity state of solubilized B_1R was analyzed by immobilizing the receptor via the C-terminal StrepII tag on StrepTactin beads (Qiagen, Hilden, Germany) and incubation with increasing concentrations of radiolabeled DAKD in buffer A. Beads were washed with buffer A and analyzed by liquid scintillation counting. Data were evaluated with Prism6 (GraphPad Software, La Jolla, USA) and determined dissociation constants were used to convert half maximal inhibitory concentrations (IC_{50}) to inhibition constants (K_I) via the Cheng–Prusoff equation. Data are

represented as means \pm s.e.m. from two or three independent experiments each performed in triplicate ($n = 3$).

DNP-enhanced solid-state NMR experiments. All DNP-enhanced MAS ssNMR experiments were carried out on a Bruker Avance II DNP ssNMR spectrometer operating at 400.197 MHz (9.40 T). The spectrometer is equipped with a Bruker 3.2 mm HCN cryo-MAS probehead. The dry nitrogen gas for cryo-MAS and temperature control is pre-cooled in a low temperature heat exchanger maintained by continuous liquid nitrogen supply. High-power continuous-wave (CW) microwave irradiation was generated in a CPI gyrotron (Communications and Power Industries) and transmitted to sample location in the probehead via corrugated waveguides. For approaching the optimized DNP enhancement, the microwave frequency was adjusted to 263.580 GHz by setting the cavity temperature. The microwave output power was about 35 W as calibrated using an external water load. The microwave power attenuation of the corrugated waveguide was about 4.8 dB, which permits about 1/3 of the gyrotron output power to reach the sample. The stability of the microwave power was monitored by an external thermometer coupled to a mirror-load device.

All the samples were loaded into the 3.2 mm rotors (sapphire or ZrO₂ material) and sealed with Vespel caps. The rotors were snap-frozen in liquid nitrogen before NMR experiments or directly in the low temperature cryo-MAS gas flow. All samples were transferred on dry ice/in liquid nitrogen and preserved at -80°C between measurements. The MAS frequency was stabilized at 8,000 \pm 6 Hz at about 110 K in all measurements.

¹H-¹³C cross-polarization (CP) was achieved using a ramped spin-lock (SL) pulse (80–100) for protons and a constant amplitude SL pulse for carbons. The average SL power for ¹H was about 48 kHz, and the field strength for ¹³C SL was about 40 kHz. The contact time was set to 800 μs . ¹³C-¹³C and ¹⁵N-¹³C 2D spectra were constructed using DQ-SQ and out-and-back TEDOR approaches. These two methods were chosen based on their good efficiency and robustness under DNP conditions. DQ-SQ ¹³C-¹³C 2D spectra were recorded using the largest possible F1 window³⁸. The double quantum coherence was excited by POST-C7 pulses³⁹ at 56 kHz with 100 kHz CW heterodecoupling. Both DQ excitation and reconnection times were set to 500 μs (four rotor periods), corresponding to 14 POST-C7 units at 8,000 kHz MAS. The DQ efficiency was about 20–25%. Typically, 512 to 1,024 scans were accumulated for each t1 point of DQ-SQ spectra of peptide-GPCR samples. The DQ-SQ ¹³C-¹³C 2D spectra were acquired with 1,536 (F2) by 32 or 48 (F1) points for spectral windows of 296 (SQ) and 560 (DQ) p.p.m., respectively, and were processed with a 4,094 (F2) by 1,024 (F1) matrix. An exponential window function with Lorentzian broadening factor of 100 Hz was applied on both direct and indirect dimension. The TEDOR ¹⁵N-¹³C 2D experiments were conducted using a scheme shown in ref. 40. Briefly, each train of recoupling pulses before and after the central ¹³C π pulse is composed of four ¹⁵N π pulses (10 μs). This corresponds to 1,000 μs (eight rotor period) total recoupling time in whole pulse sequence. CW heterodecoupling at 100 kHz were applied during TEDOR recoupling. Typically, 2,048 scans were accumulated for each t1 point of TEDOR experiments on peptide-GPCR samples. The TEDOR ¹⁵N-¹³C 2D spectra were acquired with 1,024 (F2) by 32 (F1) points for spectral windows of 296 (¹³C) and 49 (¹⁵N) p.p.m., respectively, and were processed with a 4,094 (F2) by 1,024 (F1) matrix. A Gaussian window function with Lorentzian broadening factor of 20 Hz and a Gaussian broadening factor 0.05 was applied on direct dimension. Indirect dimension was processed using a pure cosine window function.

The DQ and REDOR doubly filtered 1D ¹³C spectrum (Fig. 2f) was acquired using the pulse sequence shown in Supplementary Figure 6. The DQF step was set as mentioned above. The “REDOR” dephasing is achieved by two sets of dipolar recoupling pulses, each containing four ¹⁵N π pulses (10 μs), tethered symmetrically by a rotor synchronized ¹³C π pulse (8 μs). CW heterodecoupling at 100 kHz were applied during the DQF and REDOR dephasing periods. For all experiments 100 kHz decoupling using SPINAL64 (ref. 41) was applied during acquisition.

All ¹³C chemical shifts reported in Supplementary Tables 2 and 3 were referenced indirectly to TSP via alanine (LT) and adamantane (RT) signals. ¹⁵N chemical shifts were referenced indirectly via the gyromagnetic ratios to liquid ammonia.

Peptide structure calculation. Backbone structures were calculated with torsion angle restraints derived by a ‘backward’ approach based on chemical shifts. These structures were examined using a ‘forward’ method based on chemical shifts predicted from a library of random conformations. In addition, the robustness of this procedure was tested on the structure of free DAKD in solution, determined by liquid-state NMR based on additional distance restraints.

(a) *B₁R-bound DAKD backbone structure calculation from chemical shifts (‘backward’ approach).* Backbone torsion angle restraints were generated from chemical shifts (Supplementary Table 2) using TALOS+⁴², TALOS-N⁴³ or PREDITOR⁴⁴. They were used by CYANA2.1 (ref. 45) for structure calculations in torsion angle space. 500 initial models were annealed through 20,000 steps under torsion angle restraints with a weight set to 0.10. A bundle size of ten output structures was chosen. The structure refinement statistics was analyzed by CYANA and iCING⁴⁶ (Supplementary Table 7).

(b) *B₁R-bound DAKD backbone structure calculation via a ‘forward’ approach based on Flexible-Meccano/SHIFTX predictions.* As a control, an unrestrained backbone ensemble containing 300,000 conformations was generated by Flexible-Meccano⁴⁷. The backbone chemical shifts of each conformation in this collection were predicted using SHIFTX⁴⁸, and the deviations between the predicted and experimental values were calculated. A simple search for the best matching sets of chemical shifts returned ambiguous conformational ensemble, and additional conditions for selecting the correct structures had to be introduced.

Therefore, a conformational test ensemble containing 500 DAKD structures was calculated by CYANA starting from 12,500 initial models using torsion angle restraints generated from our experimental chemical shifts by TALOS+ ‘backward’ calculations. For each structure in this ensemble, ¹³C chemical shifts were recalculated by SHIFTX. These predicted chemical shifts were compared to the experimental input values for each site. Those showing significantly large deviations (R2^{DAKD} C α , F6^{DAKD} C, S7^{DAKD} C) were excluded from the following analysis to reduce a biasing introduced by the intrinsic deviation between the SHIFTX and TALOS+ algorithms. The intrinsic deviation of SHIFTX-predicted chemical shifts within the CYANA test ensemble (‘cutoff’) is represented by the sum of the absolute values of the largest backbone ¹³C chemical shift differences.

$$(\text{cutoff} = \sum_{\text{nuclei}} \max_{\substack{i,j \\ i < j}}^{500} |C_i^{\text{nuclei}} - C_j^{\text{nuclei}}| = 8.9 \text{ p.p.m.})$$

where C_i^{nuclei} and C_j^{nuclei} are the predicted ¹³C chemical shifts of a certain nuclei in the i th and j th peptide structure in the CYANA ensemble, respectively.

For each of the 300,000 members of the Flexible-Meccano ensemble, absolute values of the differences between SHIFTX-predicted and experimental backbone ¹³C chemical shifts were calculated and summed up

$$(\Sigma \Delta \text{CS}_i = \sum_{\text{nuclei}} |C_{\text{SHIFTX}}^{\text{nuclei},i} - C_{\text{exp}}^{\text{nuclei}}|)$$

where $C_{\text{SHIFTX}}^{\text{nuclei},i}$ is the predicted ¹³C chemical shift of certain nuclei in the i th peptide structure in the Flexible-Meccano ensemble, and $C_{\text{exp}}^{\text{nuclei}}$ is the experimental ¹³C chemical shift of this nuclei.

Within the Flexible-Meccano ensemble, $\Sigma \Delta \text{CS}$ assumes a Gaussian-like distribution with a minimum value at 6.61 p.p.m., a maximum at 13 p.p.m. and a width of 12 p.p.m. The value of the cutoff parameter was now used to select the best candidates with minimal $\Sigma \Delta \text{CS}$. A set of 1,490 structures was found between the global minimal $\Sigma \Delta \text{CS}$ of 6.61 p.p.m. and the cutoff value. The use of such a “large” set of conformations selected by defined cutoff improves structural clustering compared to previous protocols⁹.

To derive restraints for further structure refinements, the backbone torsion angles (ϕ , ψ) from the selected set of structures were extracted and fitted by a Gaussian distribution (Supplementary Fig. 15a,b). Its mean and s.d. values were taken as the mean torsion angle and the allowed deviation. Three of the torsion angles (G5 ϕ , F6 ψ , and S7 ψ) showed a double distribution. The independence of these ambiguous torsion angles was examined using a ‘Ramachandran’ plot correlating these pairs of torsion angles extracted from each individual in the top 1,490 structures (Supplementary Fig. 15c).

Eventually, we obtained eight ($2 \times (G5) \times 2 \times (F6) \times 2 \times (S7)$) sets of backbone torsion angle restraints extracted from the Flexible-Meccano/SHIFTX ensemble. These eight sets of restraints were used as inputs in CYANA calculations. Eight distinct clusters (labeled A-H) with ten structures each were generated.

(c) *Comparison of DAKD structures calculated from forward, backward and Rosetta/docking approaches.* Using approaches (a) and (b), 11 structural ensembles were generated in total. To elucidate the relationships of these ensembles, a systematic analysis was conducted. In addition, the structural ensemble derived from the Rosetta/Docking approach was included for comparison. The 120 structures within the 12 clusters were compared by computing 6,600 pairwise backbone heavy atom r.m.s. deviation values. They were used to visualize the structural similarities in a string plot (**Supplementary Fig. 16**): TALOS+/TALOS-N/PREDITOR ensembles and the cluster A and E from the forward approaches are closely related to each other and agree well with the Rosetta/docking solution. The clusters B, C, D, F, G, and H could be immediately excluded. The clusters A and E mainly differ from the 'backward' solutions in their S7 ψ angle but only A matches the other solutions.

Based on the string plot in **Supplementary Figure 16**, the ensemble calculated by CYANA using TALOS+ restraints agrees best with the Rosetta/Docking solution. In addition, as shown in **Supplementary Table 7**, the TALOS+/CYANA approach yielded better structures compared to TALOS-N/CYANA and PREDITOR/CYANA in terms of violations of restraints and backbone torsion angle distributions on Ramachandran plots. Therefore, the combination of TALOS+/CYANA was selected as the method of choice for all further calculations.

(d) *Determination of DAKD (free) and B₂R-bound and free BK peptide conformations using TALOS+/CYANA.* The torsion angle restraints of DAKD without receptor and BK peptide with and without receptor were generated by TALOS+, and the structures were calculated using the same backward CYANA protocol as described in section (a) above. The chemical shifts of DAKD without receptor are listed in **Supplementary Table 3**. The chemical shift values used for BK structure determination were previously reported in Lopez *et al.*¹⁰ The statistics of structure determination of DAKD and BK peptides in receptor-bound and free states were summarized in **Supplementary Table 8**.

For further validation, the structure of free DAKD was determined by solution-state NMR based on chemical shift as well as distance restraints (see Section (e) below and **Supplementary Tables 5 and 6**). For comparison, the same procedure as described in (a) was applied using the experimental solution-state NMR chemical shifts to derive backbone torsion angle restraints. In addition, a 'forward' calculation approach as described in section (b) was applied. Briefly, we started from the same random conformation library of DAKD (300,000 conformations) and re-ranked all the individuals according to the level of matching with experimental solution NMR chemical shifts of free DAKD. The P2 C α and P8 C chemical shifts were excluded due to the large deviations among different sets of programs. The first round of selection with a Δ CSC cutoff at 7.10 p.p.m. yielded a sublibrary containing 4,745 conformations (1.58% of full library). The backbone torsion angle distributions within this sublibrary were extracted and used to build the representative bundles as CYANA restraints. The final result contained four bundles as a result of two independent ambiguities in G5 ϕ and S7 ψ angles.

We then ran a comparative analysis of all the backward and forward solutions of free DAKD based on backbone r.m.s. deviation. As shown in the string plot (**Supplementary Fig. 17**), the TALOS-based backward calculations, as well as two of the forward clusters, resemble the solution NMR structure in terms of backbone r.m.s. deviation. A second round of selection based on S7 ψ angle led to the convergence via identification of a unique solution from all forward bundles. In general, the bundles passing the selection steps correctly depicted the V-shaped folding of DAKD in solution, which appears similar to the bound state. These analyses demonstrate that our computational pipeline could reliably determine the conformations of the peptides.

(e) *Determination of solution NMR structure of free DAKD using CS and distance-restraints.* As further validation, the structure of free DAKD was determined by solution-state NMR based on chemical shift as well as distance restraints. The NMR experiments of the free peptide (3 mM sample in 50 mM MES buffer, pH 5.6, 100 mM NaCl and 10% D₂O) were conducted

at a temperature of 295 K on a Bruker Avance III HD 600 MHz spectrometer, equipped with Prodigy cryogenic triple-resonance probes. NMR spectra were acquired and processed using TopSpin version 3.5 (Bruker BioSpin 2017). For the chemical shift assignment, the following experiments were conducted: homonuclear 2D ¹H-¹H-ROESY (100, 200, 300, and 500 ms mixing time) and 2D ¹H-¹H-TOCSY (20 and 80 ms mixing time), and heteronuclear 2D ¹H-¹³C-edited-HSQC, 2D ¹H-¹³C-HMBC and 2D ¹H-¹⁵N-sofast-HMQC at natural abundance. The solution-NMR structure calculations of free DAKD were performed using ARIA 1.2 with CNS 1.1 (refs. 49,50). The standard simulated annealing (SA) protocols were used, including ROE distance calibration and spin-diffusion correction. The three 2D ¹H,¹H-ROESY spectra (100, 200, and 300 ms mixing time) used for the structure calculation were manually peak picked and assigned using Sparky 3.114 (T.D. Goddard and D.G. Kneller, University of California, San Francisco). Backbone dihedral angle restraints have been included based on TALOS-N⁴³ predictions when in agreement with prior calculations for which only ROEs were used. Fifty starting structures were generated based on a linear template molecule. For each iteration (0–7), in which 50 structures were calculated, the ROE distance restraints were recalibrated by ARIA based on the 20 lowest energy structures. The violation tolerance was progressively reduced to 0.1 Å in the last iteration (8) in which 200 structures were calculated. For the structure calculations, a four-stage SA protocol was used using torsion angle dynamics. The high temperature stage consisted of 10,000 steps at 10,000 K. This step was followed by refinement and cooling down stages: 8,000 steps at 2,000 K, 5,000 steps to 1,000 K and 10,000 steps to 50 K. During the SA protocol the force constant for the distance restraints was set to 0, 10, 10, and 50 kcalmol⁻¹Å⁻² for the successive stages. The final 20 lowest energy structures were further refined in explicit water. The solution-state NMR chemical shifts are provided in **Supplementary Table 5**. The structure calculation refinement statistics are summarized in **Supplementary Table 6**. The DAKD structure in solution is displayed in **Supplementary Figure 9c**.

Homology modeling and docking. The overall workflow for receptor modeling and peptide docking is summarized in **Supplementary Figure 18**. Structural models of the B₁R-DAKD and B₂R-bradykinin complexes were generated using the protein structure prediction software package Rosetta, version 3.5 (ref. 51). Comparative models of B₁R and B₂R were built based on 24 experimentally determined class A GPCR structures as possible templates (**Supplementary Table 9**). These structures were aligned with MUSTANG⁵², and the resulting multiple-sequence alignment was aligned with the B₁R and B₂R sequence using ClustalW⁵³. The N- and C-terminal sequence termini were truncated by 31 and 22 residues for B₁R and 51 and 39 residues for B₂R because of a lack of coordinates for the aligned residues in most of the template structures. The sequence alignment was manually adjusted to remove gaps within transmembrane helix regions and to ensure that highly conserved residues and helix endings remain aligned (**Supplementary Table 10**). Transmembrane helix regions were predicted by programs PSPRED⁵⁴ and OCTOPUS⁵⁵. The B₁R or B₂R residues were threaded onto the atomic coordinates of the aligned residues within each of the 24 template GPCRs. 4,000 B₁R and B₂R models were assembled by Rosetta comparative modeling⁵⁶ using segments of the threaded structures and sequence-based fragments. All models underwent all-atom refinement in internal and Cartesian coordinate space with gradient minimization. The models were clustered based on backbone r.m.s. deviation with automatic radius detection, and the top-scoring models from the ten largest clusters were selected and checked for incorrect structural features such as helix-helix clashes or unlikely helix kinks. To sample loop conformations more thoroughly, these receptor models were subjected to another round of comparative modeling in which the extracellular loop regions were individually reconstructed using only seven peptide-binding GPCRs as templates: angiotensin II type-1 receptor (PDB code 4YAY), C-C chemokine receptor type 5 (PDB code 4MBS), κ -opioid receptor (PDB code 4DJH), δ -opioid receptor (PDB code 4N6H), μ -opioid receptor (PDB code 4DKL) and orexin receptor type 2 (PDB code 4S0V). An atom pair constraint between B₁R residues C110^{3.25} and C189^{ECL2} and between B₂R residues C130^{3.25} and C211^{ECL2} was included to account for the expected, highly conserved disulfide bond. Furthermore, a β -strand pairing constraint was applied to B₁R

residues R176^{ECL2} – L192^{ECL2} and B2R residues R196^{ECL2} – S214^{ECL2}. A β -sheet secondary structure formation of that region was predicted by PSIPRED⁵⁴ and observed in all template GPCR structures. In each step, 4,000 receptor models were created and 10–20 models with a high score and valid loop conformations were selected from the 10 largest clusters and used as input for the next round of loop modeling.

To avoid a collapse of the receptor loops into the receptor binding pocket during all-atom relaxation, and thus an occlusion of the ligand binding site, the peptide ligand was placed within the receptor pocket before and during loop modeling and re-docked afterwards. A set of 10 B₁R and 20 B₂R comparative models were used as input structures for the final ligand docking step. DAKD and bradykinin were simultaneously folded and docked into B₁R and B₂R using the Rosetta FlexPepDock application²⁷. This protocol combines Monte Carlo Metropolis-based rigid body moves and peptide backbone conformational sampling in Rosetta's low-resolution centroid mode with subsequent full-atom refinement and side chain optimization. Experimental information about putative ligand binding residues was used to derive restraints to guide ligand docking. Restraints were implemented as a set of ambiguous distance restraints between the C α atom of the proposed binding residue of the receptor and the C α atom of each ligand residue with a 10 Å distance cutoff. Only the distance giving the lowest energy was used to calculate the restraint energy of a specific receptor residue. For B₁R, restraints were derived for residues K118^{3,33} (ref. 29) and A270^{6,55}, E273^{6,58}, D291^{7,32}, L294^{7,35} and F302^{7,43} (all ref. 30). For B₂R, residues W113^{2,60} (ref. 57), S138^{3,33} (refs. 28,29), F286^{6,51}, T290^{6,5}, D293^{6,58}, D311^{7,32} and Q315^{7,36} (all ref. 31) were used to construct distance restraints for ligand docking. A total of 50,000 B₁R-DAKD and 72,000 B₂R-bradykinin models were generated. Models were selected by clustering of the 1,000 best models by combined Rosetta total, peptide and interface score (Supplementary Fig. 19). Putative ligand-binding residues of B₁R and B₂R were identified by a per-residue breakdown of the Rosetta interface energy and counting all interactions with a score lower than –1.0 Rosetta energy units within the 1,000 best models. The compliance of the structural models of DAKD and bradykinin with the experimental chemical shift data was checked by back-calculating chemical shifts from structure using the programs SPARTA⁵⁸ and SHIFTX2 (ref. 59) (Supplementary Fig. 20). A final set of 10 B₁R-DAKD and B₂R-bradykinin models that showed the smallest chemical shift r.m.s. deviation relative to the experimental data were selected as representative models.

Our used set of templates contained GPCR structures both in the active and the inactive states. It has previously been suggested⁶⁰ that the Rosetta comparative modeling protocol is insensitive to the state of the GPCR templates so that it would not affect modeling and docking. To further validate this assumption, we compared the similarity of active and inactive structures, as well as the similarity of our receptor models with each of the two subgroups by calculating r.m.s. deviations with the structure-based alignment tool MAMMOTH. The average r.m.s. deviation value of active structures (3.0 ± 0.5 Å) is not considerably different from inactive structures (3.3 ± 0.6 Å) and comparable to the average r.m.s. deviation when all templates were combined (3.4 ± 0.5 Å). No significant differences are found when calculating pairwise r.m.s. deviations of B₁R or B₂R with active (3.8 ± 0.1 Å / 3.5 ± 0.4 Å) and inactive structures (3.8 ± 0.1 Å / 3.6 ± 0.2 Å) which shows that the receptor models are indistinguishable with respect to the activation state and suggests that their modeling is indeed insensitive to the state of the template GPCRs.

Life sciences reporting summary. Further information on experimental design and reagents is available in the **Life Sciences Reporting Summary**.

Data availability. The authors declare that all data supporting the findings of this study are available within the article, its Supplementary Information file and from the corresponding authors upon reasonable request.

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