Peptide–Receptor Interactions

International Edition: DOI: 10.1002/anie.201411688
German Edition: DOI: 10.1002/ange.201411688

Unwinding of the C-Terminal Residues of Neuropeptide Y is critical for Y_2 Receptor Binding and Activation**

*Anette Kaiser, Paul Müller, Tristan Zellmann, Holger A. Scheidt, Lars Thomas, Mathias Bosse, Rene Meier, Jens Meiler, Daniel Huster, Annette G. Beck-Sickinger, and Peter Schmidt**

Abstract: *Despite recent breakthroughs in the structural characterization of G-protein-coupled receptors (GPCRs), there is only sparse data on how GPCRs recognize larger peptide ligands. NMR spectroscopy, molecular modeling, and double-cycle mutagenesis studies were integrated to obtain a structural model of the peptide hormone neuropeptide Y (NPY) bound to its human G-protein-coupled Y_2 receptor (Y_2R). Solid-state NMR measurements of specific isotope-labeled NPY in complex with in vitro folded Y_2R reconstituted into phospholipid bicelles provided the bioactive structure of the peptide. Guided by solution NMR experiments, it could be shown that the ligand is tethered to the second extracellular loop by hydrophobic contacts. The C-terminal α-helix of NPY, which is formed in a membrane environment in the absence of the receptor, is unwound starting at T^{32} to provide optimal contacts in a deep binding pocket within the transmembrane bundle of the Y_2R.*

The interaction of GPCRs with their natural ligands plays a central role in numerous transmembrane signal transduction pathways. For small-molecule ligands, including peptides, a conserved binding cradle to class A (rhodopsin-like) GPCRs was recently proposed.[1] To date, structural models for the smaller GPCR-bound peptide ligands bradykinin,[2] a truncated six amino acid variant of neurotensin,[3] and the fatty acid leukotriene B_4[4] have been generated by NMR spectroscopy.

[*] P. Müller,[+] Dr. H. A. Scheidt, Dr. L. Thomas, M. Bosse, Prof. Dr. D. Huster, Dr. P. Schmidt
Institut für Medizinische Physik und Biophysik
Universität Leipzig, Härtelstraße 16–18, 04107 Leipzig (Germany)
E-mail: peter.schmidt@medizin.uni-leipzig.de
A. Kaiser,[+] T. Zellmann,[+] Dr. R. Meier, Prof. Dr. A. G. Beck-Sickinger
Institut für Biochemie, Universität Leipzig
Brüderstraße 34, 04103 Leipzig (Germany)
T. Zellmann,[+] Prof. Dr. J. Meiler
Center for Structural Biology, Vanderbilt University
465 21st Ave South, Nashville, TN 37203 (USA)
[+] These authors contributed equally to this work.

[**] This study was supported by the European Community, the Free State of Saxony (ESF 22117016 and 24127009) and the German Research Foundation (SFB 1052A3 and DFG BE1264/16). Work in the Meiler laboratory is supported through NIH (R01 DK097376) and NSF (CHE 1305874). We thank R. Reppich-Sacher and J. Schwesinger for expert technical assistance, and the ZIH Dresden for providing computational resources.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411688.

Herein, we report a model for the structure and binding mode of the 36 amino acid, C-terminally amidated NPY bound to the Y_2R. This interaction plays an essential role in the control of food intake and memory retention, and is involved in mood disorders and epilepsy.[5] The binding of NPY to its receptor is suggested to be a two-step process.[6] According to this model, NPY first binds to the lipid membrane to increase its effective concentration, and is then recognized by the Y_2R.[6]

We applied solid-state and solution NMR spectroscopy to derive a set of structural restraints for molecular modeling and targeted docking, which was complemented by double-cycle mutagenesis to verify NPY–Y_2R interactions. For the NMR measurements, ten NPY variants with $^{15}N/^{13}C$-labeled amino acids in different positions were synthesized, covering 30 of the 36 NPY residues (Table S1). Milligram amounts of a cysteine-deficient variant of the Y_2R[7] were prepared through recombinant *E. coli* inclusion body expression and purified in sodium dodecyl sulfate (SDS), as described previously.[8] Receptor functionality was achieved in a two-step in vitro folding process (see the Supporting Information). Briefly, in the first step, the SDS concentration was reduced below its critical micelle concentration by dialysis[7] and the native disulfide bridge between the two remaining cysteine residues was formed by using glutathione. In the second step, the Y_2R was functionally reconstituted into 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC-c7) bicelles by using heat cycling[9] at Y_2R/DMPC ratios of 1:200 or 1:600. The size of the bicelles was adjusted by varying the q-value (molar DMPC/DHPC-c7 ratio) from isotropically tumbling bicelles ($q < 0.25$) to large membrane structures with little residual detergent ($q > 20$) applicable to solution and solid-state NMR, respectively.[10] Finally, for NMR experiments, the reconstituted Y_2R was concentrated by either pelleting (in case of large membrane structure with $q > 20$) or dialyzing against polyethylene glycol 20000 (in case of small bicelles with $q < 0.25$) to remove water. Functionality of the Y_2R preparations was verified through NPY binding assays (Figure S1 in the Supporting Information), which indicated 89 ± 9% functional receptor molecules.

Solid-state NMR ^{13}C double quantum/single quantum (DQSQ) correlation spectra (shown in Figure S2a and S3) for all NPY variants in the Y_2R-bound state were recorded at −30 °C to reduce the uniaxial rotational motion of the receptor about the membrane normal.[9] The assigned carbon chemical shifts of Y_2R-bound NPY (listed in Table S2 in the Supporting Information) and the resulting chemical-shift indices[11] (Figure 1a) were used to model the

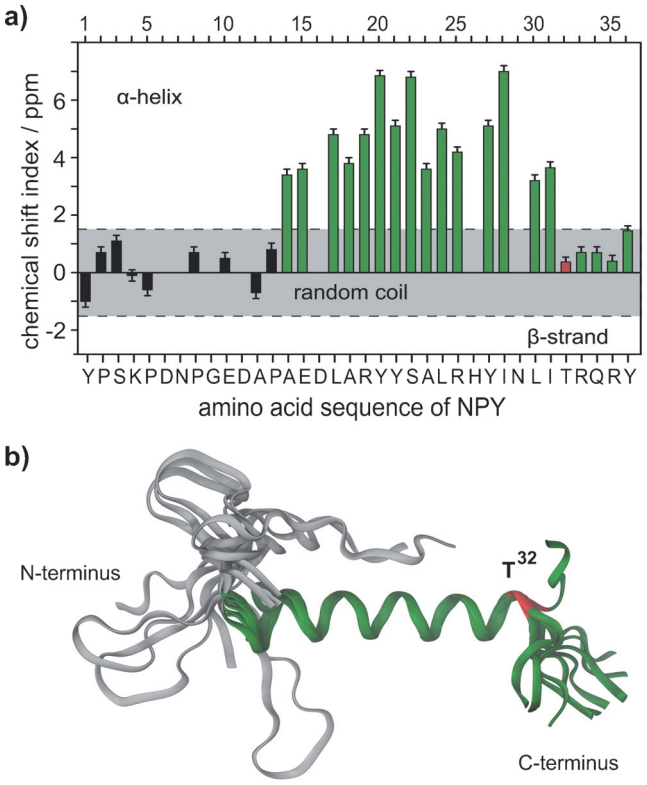

Figure 1. a) ^{13}C chemical-shift index of Y_2R-bound NPY (measured [(C_α-C_β)–random coil (C_α-C_β)]. b) Superposition of the ten best-scoring NPY models derived from solid-state NMR restraints. The N-terminus (gray) was excluded from scoring. The C-terminal α-helical structure of NPY (green) unwinds starting at T^{32} (highlighted in red) upon receptor binding.

peptide structures by comparing predicted chemical shifts from an ensemble of 400000 de novo folded[12] NPY molecules with the experimental data. The ten best-scoring models, shown in Figure 1b, surprisingly revealed a C-terminal random coil structure from T^{32} to Y^{36}. This clearly deviates from the NPY structures determined in solution[13] and in the presence of micelles,[14] where the regular α-helix structure continues up to the amidated C-terminus. However, at very low physiological concentration, NPY is putatively monomeric in solution and the C-terminus might not be entirely folded in this form.[13]

To reveal NPY residue-specific alterations upon Y_2R binding, two-dimensional ^1H-^{15}N HSQC spectra were recorded in the presence of bicelles containing the Y_2R and empty bicelles for all of the NPY variants (Figure S2b). The weighted chemical-shift differences and signal broadening, caused by local altered exchange processes (e.g. exchange processes within the binding pocket) or reduced overall tumbling, are displayed in Figure 2a. Line broadening thresholds of more than 100 and 300 Hz were arbitrarily chosen to illustrate this effect (line widths are given in Table S2). Significant alterations were observed for the six C-terminal residues, which have been identified to be critical for the binding of NPY to the Y_2R.[15] Furthermore, at the hydrophobic face of the α-helix of NPY (L^{17}/A^{18}, Y^{20}/Y^{21}, L^{24}, Y^{27}/I^{28}, I^{31}), chemical-shift changes and/or signal broadening were detected, thus suggesting an additional binding site based on

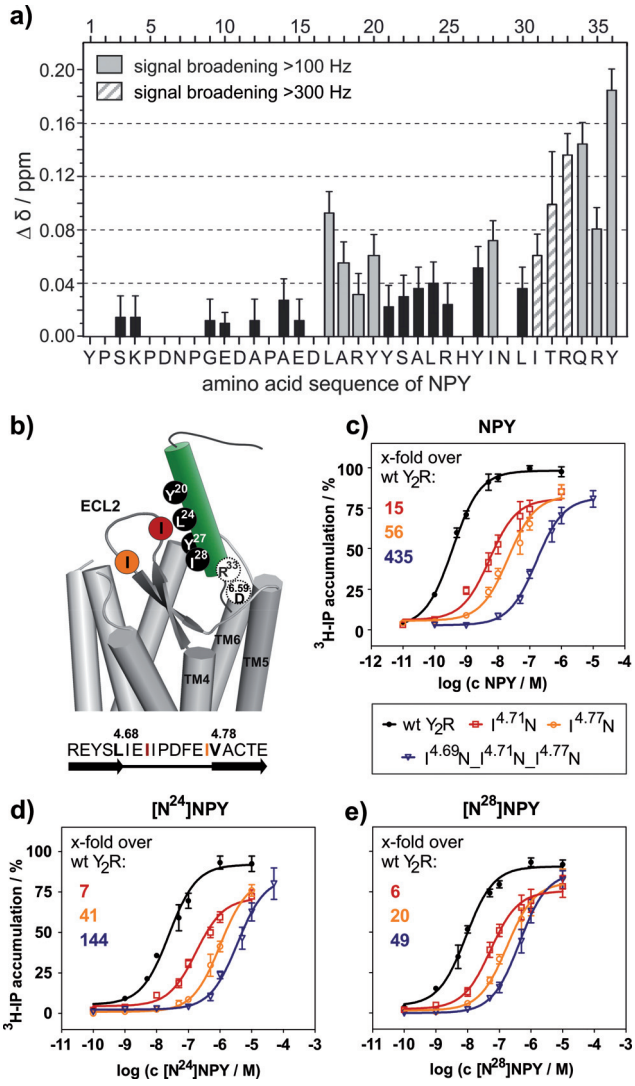

Figure 2. a) Weighted chemical-shift changes ($\Delta\delta = [(\Delta\delta^1H)^2 + (0.2\,\Delta\delta^{15}N)^2]^{1/2}$) for membrane- and receptor-bound NPY, and ^1H NMR signal broadening upon binding of >100 Hz (gray) and >300 Hz (shaded) obtained from HSQC solution NMR. b) Schematic representation of the initial docking of NPY (green) into a hydrophobic groove of Y_2R. c–e) Double-cycle mutagenesis to identify the interacting residues between ECL2 and NPY. Y_2R mutants $I^{4.71}N$ (red), $I^{4.77}N$ (orange) and the combination variant $I^{4.69}N\_I^{4.71}N\_I^{4.77}N$ (blue) were tested against [$N^{24/28}$]NPY variants to identify the interacting residues. Numbers in the upper left represent EC_{50} shifts relative to the wildtype Y_2R curve, with reduced shifts indicating direct interaction of receptor and peptide at the modified positions.

hydrophobic contacts. From a Y_2R comparative model, we suspected hydrophobic residues in extracellular loop 2 (ECL2) to be interaction partners and exchanged them to asparagine (similarly sized, hydrophilic). $I^{4.71}$ and $I^{4.77}$ (receptor nomenclature[16]) were susceptible to mutation, and double-cycle mutagenesis[17] with modified ligands confirmed direct contacts to L^{24} and I^{28}, as shown in Figure 2b–e (see the Supporting Information and Table S3).

Using these contacts together with the previously described salt bridge between R^{33} of NPY and $D^{6.59}$ on top of transmembrane helix 6 (TM6)[17] as restraints, the C-terminal part (NPY^{13-36}) of the top-scoring NPY models (Figure 1) was

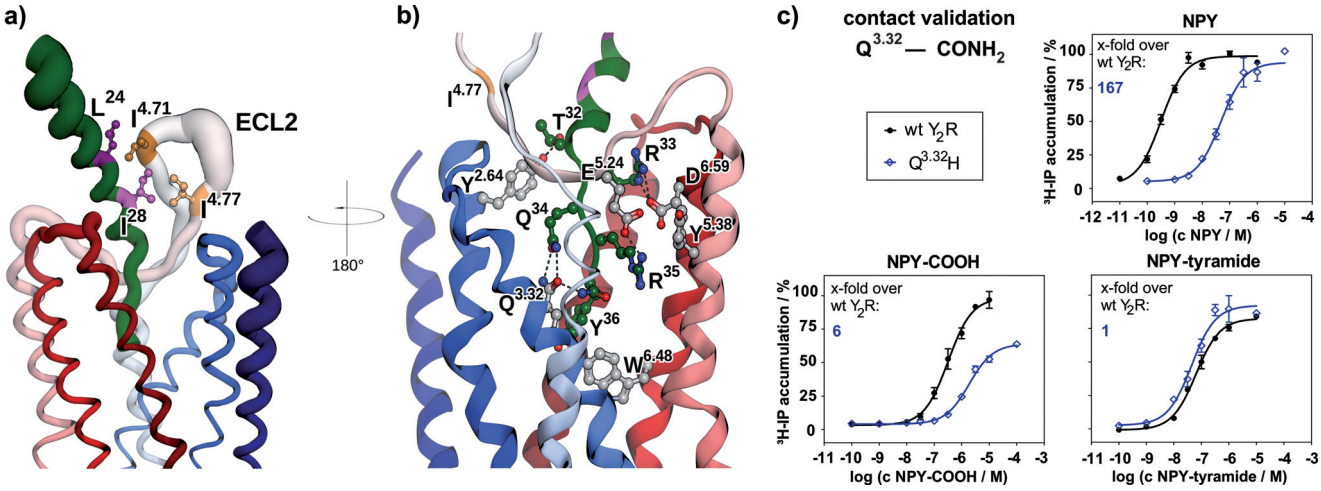

Figure 3. a) Model of NPY (green) docked into Y_2R (N to C terminus shown in blue to red). Structural diversity of the ensemble is indicated by the thickness of the ribbons. NPY is tethered to ECL2 through the interaction of L^{24} (purple) and I^{28} (pink) with $I^{4.71}$ and $I^{4.77}$ (orange). b) Representative view of the C-terminus of NPY. Polar interactions are indicated by dashed lines. c) The interaction of $Q^{3.32}$ (light blue) with the C-terminal amide group of NPY was verified by double-cycle mutagenesis through the use of a slightly basic interaction partner for the free acid form of NPY or a nondiscriminating analogue completely lacking the C-terminal $CONH_2$ (NPY-tyramide).

docked into a comparative model of the Y_2R by using ROSETTA (see the Supporting Information). The NPY/Y_2R model with the best agreement to experimental data and NPY structure–activity relationships is depicted in Figure 3. Semi-quantitative energetic analysis of this complex (Figure 4) underscores the increasing binding contributions towards the C-terminus of NPY and the results are in good qualitative agreement with important receptor positions identified by mutagenesis.[15,17]

The hydrophobic contacts to ECL2 constrain NPY at an angle of approximately 45° relative to the membrane normal. Taking into account the highly dynamic features of the Y_2R,[9] the position of NPY in the binding pocket is probably not static. Rather, the peptide ligand might follow the motions of the ECL2, which was modelled simultaneously with NPY docking to account for its high flexibility, thereby resulting in a cone-like distribution with the C-terminal part as the receptor-anchoring point and increasing amplitude motions towards the N-terminus (Figure 3a).

It is also tempting to speculate that hydrophobic contacts to the extracellular domains of the receptor might pick up the ligand from the membrane-bound or soluble state and pre-orient it in the binding pocket. As a consequence of the increasing angle between the NPY α-helix and the membrane surface, important membrane binding residues (L^{17}, Y^{20}/Y^{21})[6] now become exposed to a rather polar environment, as supported by the solution NMR data (Figure 2a).

Concomitantly, L^{24}, I^{28}, and the unwound C-terminal pentapeptide change their membrane contacts[6] to form thermodynamically more favorable direct interactions with the Y_2R (Figure 4). In this manner, receptor contacts in the binding pocket can be maximized (Figure 3b), as previously proposed.[15] Given that the second high-affinity natural ligand of the Y_2R, PYY, shares the same sequence for the C-terminal pentapeptide and prefers a C-terminal extended structure even in the unbound state,[18] the thermodynamic barrier for such a transition is presumably rather small.

The first unwinding residue T^{32} is located in a narrow point on top of the binding pocket and could fulfil two important functions in the binding process: 1) By accepting a hydrogen bond from $Y^{2.64}$, it brings about closing of the binding pocket, thereby locking NPY into its final binding position. This is supported by the measured signal broadening of more than 300 Hz for T^{32} and the neighboring residues. 2) T^{32} could also reduce the thermodynamic cost of helix unwinding in this rather apolar environment by donating a hydrogen bond to the exposed carbonyl oxygen atom of N^{29}, thereby capping the helix. A similar phenomenon is seen in the C-terminal helix of $G\alpha_{i/o}$-proteins, where a cysteine residue (C^{351} in human $G\alpha_{i1}$) "catches" the unwinding α5 helix upon binding to the activated receptor.[19]

The critical importance of R^{33} and R^{35} for NPY activity[5] is also reflected by our model. While R^{33} makes narrow ionic contacts to $D^{6.59}$,[17] R^{35} is positioned in a mixed acidic–aromatic pocket formed by $W^{5.26}$ and $Y^{5.38}$ coordinated by $E^{5.24}$. This is in agreement with earlier studies that highlighted the requirement of aromatic properties at R^{35} [20] and explains the difficulties in identifying its interaction partner within the binding pocket.[15]

A particularly important position for NPY activity at the Y_2R is Q^{34}.[5] Owing to the deep binding mode of NPY, the side chain of Q^{34} is fairly restricted and is oriented towards a small polar patch within TM2/3. Our model suggests a prominent interaction with $Q^{3.32}$, which also participates in an extensive hydrogen-bond network involving the amidated C terminus ($CONH_2$). To validate the latter interaction, we created a (slightly) basic interaction partner for the otherwise low-affine free acid of NPY (NPY-COOH). As shown in Figure 3c, stimulation with NPY-COOH largely abrogated the potency deficits of $Q^{3.32}H$ compared to wildtype Y_2R, which likewise occurred upon stimulation with NPY-tyramide,[21] a nondiscriminating analogue lacking the $CONH_2$ functionality (see the Supporting Information).

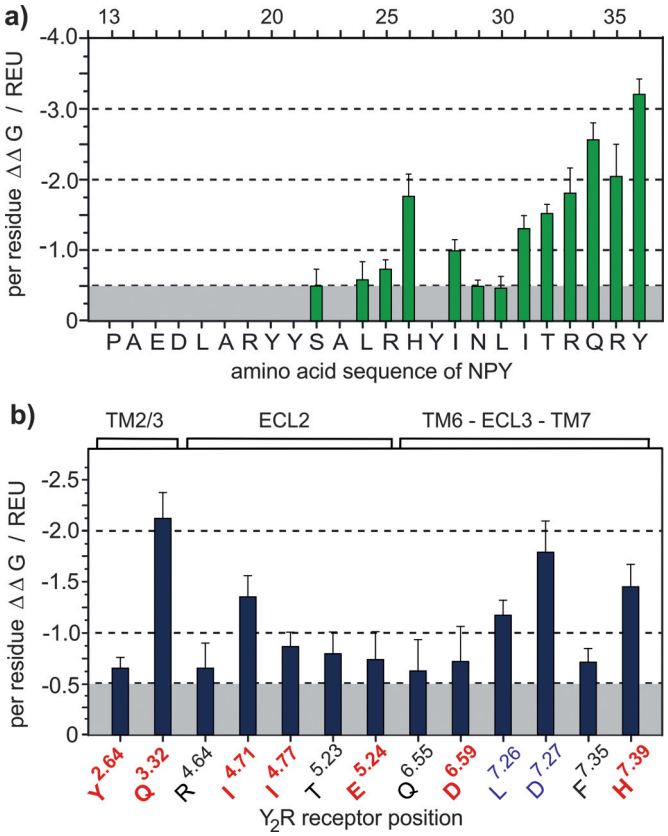

Figure 4. Energetic analysis of the NPY^{13-36}–Y_2R complex (see Figure 3b). a) The contribution of NPY residues to binding energy increases towards the C-terminus, in agreement with the solution NMR results (Figure 2a). b) For the Y_2R, significant binding energy is contributed by 13 residues, mostly at positions identified to be critical (red) earlier[15,17] and in the present study. $L^{7.26}$ and $D^{7.27}$ (light blue) are false positives triggered by supposed ionic contact of R^{25}/H^{26} with $D^{7.27}$ in many low-energy models and they are not sensitive to mutagenesis. The significance threshold was 0.5 ROSETTA energy units (REU; gray background).

The interaction network involving Q^{34}, $Q^{3.32}$, and the C-terminal amide also determines the position of the Y^{36} side chain. Mainly surrounded by the conserved hydrophobic amino acids of the receptor ($C^{2.47}$, $W^{6.48}$, $L^{6.51}$, $M^{7.43}$), it fills a long narrow pocket in the model. Of special interest is the proximity of Y^{36} to $W^{6.48}$, which has been discussed to act as a toggle switch triggering GPCR activation.[22] More recent investigations also support the hypothesis of direct interactions between $W^{6.48}$ and the ligand,[23] thus suggesting this to be a more general mechanism of GPCR activation.

In conclusion, we present a detailed structural model of NPY bound to its Y_2 GPCR. NMR measurements revealed NPY to undergo remarkable structural changes within the C-terminus, and the C-terminal pentapeptide takes part in an extensive but also fragile interaction network. Accordingly, changes in the C-terminal amino acids can easily disturb receptor binding or switch receptor selectivity as observed in numerous earlier structure–activity studies (reviewed in Ref. [5]). Moreover, our study indicates that larger peptide ligands also share the proposed common ligand binding cradle of rhodopsin-like GPCRs,[1] even though they are not a priori expected to bind deep in the transmembrane bundle. The binding mode of NPY might thus have more general implications for peptide-binding GPCR systems.

Keywords: GPCRs · neuropeptide Y · NMR spectroscopy · peptide structure · receptors

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 7446–7449
Angew. Chem. **2015**, *127*, 7554–7558

[1] A. J. Venkatakrishnan, X. Deupi, G. Lebon, C. G. Tate, G. F. Schertler, M. M. Babu, *Nature* **2013**, *494*, 185–194.
[2] J. J. Lopez, A. K. Shukla, C. Reinhart, H. Schwalbe, H. Michel, C. Glaubitz, *Angew. Chem. Int. Ed.* **2008**, *47*, 1668–1671; *Angew. Chem.* **2008**, *120*, 1692–1695.
[3] S. Luca, J. F. White, A. K. Sohal, D. V. Filippov, J. H. van Boom, R. Grisshammer, M. Baldus, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 10706–10711.
[4] L. J. Catoire, M. Damian, F. Giusti, A. Martin, H. C. van, J. L. Popot, E. Guittet, J. L. Baneres, *J. Am. Chem. Soc.* **2010**, *132*, 9049–9057.
[5] X. Pedragosa-Badia, J. Stichel, A. G. Beck-Sickinger, *Front. Endocrinol.* **2013**, *4*, 5.
[6] R. Bader, O. Zerbe, *ChemBioChem* **2005**, *6*, 1520–1534.
[7] K. Witte, A. Kaiser, P. Schmidt, V. Splith, L. Thomas, S. Berndt, D. Huster, A. G. Beck-Sickinger, *Biol. Chem.* **2013**, *394*, 1045–1056.
[8] P. Schmidt, C. Berger, H. A. Scheidt, S. Berndt, A. Bunge, A. G. Beck-Sickinger, D. Huster, *Biophys. Chem.* **2010**, *150*, 29–36.
[9] P. Schmidt, L. Thomas, P. Müller, H. A. Scheidt, D. Huster, *Chem. Eur. J.* **2014**, *20*, 4986–4992.
[10] W. S. Son, S. H. Park, H. J. Nothnagel, G. J. Lu, Y. Wang, H. Zhang, G. A. Cook, S. C. Howell, S. J. Opella, *J. Magn. Reson.* **2012**, *214*, 111–118.
[11] S. Spera, A. Bax, *J. Am. Chem. Soc.* **1991**, *113*, 5490–5492.
[12] D. E. Kim, D. Chivian, D. Baker, *Nucleic Acids Res.* **2004**, *32*, W526–W531.
[13] S. A. Monks, G. Karagianis, G. J. Howlett, R. S. Norton, *J. Biomol. NMR* **1996**, *8*, 379–390.
[14] R. Bader, A. Bettio, A. G. Beck-Sickinger, O. Zerbe, *J. Mol. Biol.* **2001**, *305*, 307–329.
[15] B. Xu, H. Fallmar, L. Boukharta, J. Pruner, I. Lundell, N. Mohell, H. Gutierrez-de-Teran, J. Aqvist, D. Larhammar, *Biochemistry* **2013**, *52*, 7987–7998.
[16] J. A. Ballesteros, H. Weinstein, *Methods in Neurosciences, Vol. 25*, Academic Press, New York, **1995**, pp. 366–428.
[17] N. Merten, D. Lindner, N. Rabe, H. Römpler, K. Mörl, T. Schöneberg, A. G. Beck-Sickinger, *J. Biol. Chem.* **2007**, *282*, 7543–7551.
[18] M. Lerch, M. Mayrhofer, O. Zerbe, *J. Mol. Biol.* **2004**, *339*, 1153–1168.
[19] N. S. Alexander, A. M. Preininger, A. I. Kaya, R. A. Stein, H. E. Hamm, J. Meiler, *Nat. Struct. Mol. Biol.* **2014**, *21*, 56–63.
[20] L. Albertsen, S. Ostergaard, J. F. Paulsson, J. C. Norrild, K. Stromgaard, *ChemMedChem* **2013**, *8*, 1505–1513, 1422.
[21] S. Hoffmann, B. Rist, G. Videnov, G. Jung, A. G. Beck-Sickinger, *Regul. Pept.* **1996**, *65*, 61–70.
[22] J. H. Park, P. Scheerer, K. P. Hofmann, H. W. Choe, O. P. Ernst, *Nature* **2008**, *454*, 183–187.
[23] L. A. Stoddart, B. Kellam, S. J. Briddon, S. J. Hill, *Br. J. Pharmacol.* **2014**, *171*, 3827–3844.

Received: December 4, 2014
Revised: February 24, 2015
Published online: April 29, 2015

