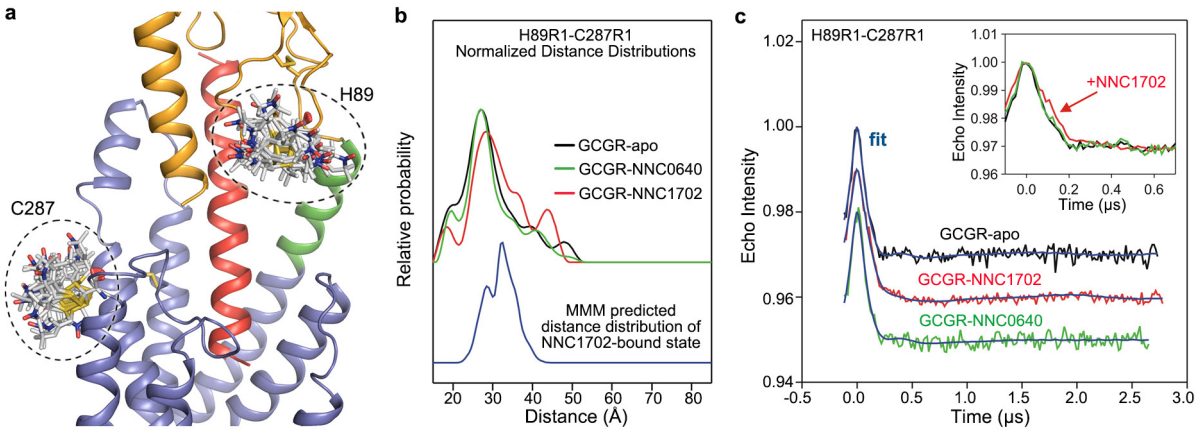

Extended Data Figure 5 | DEER spectroscopy of assembly of GCGR–ligand complex. a, The GCGR–NNC1702 assembly showing modelled R1 spin labels at the ECD site H89R1 and the TMD site C287R1 on the basis of the GCGR–NNC1702 crystal structure. The nitroxide rotameric models were generated with the MMM software package[44]. **b**, Experimental distance distributions between the nitroxide spin-labelled R1 pair of H89R1 and C287R1 in the apo state or in the presence of NNC0640 or NNC1702. The experimental distributions were normalized by area under the curves for comparison purposes. A predicted distance distribution based on the GCGR–NNC1702 structure that was derived from the MMM software (offset blue trace) is also shown. This prediction can be directly compared to the experimentally measured distributions, though rotameric weighting may be different in the prediction. **c**, Background-corrected dipolar evolution functions (DEFs) and their fits for each of the GCGR samples. The DEF functions were scaled to compare traces. The traces of the apo receptor and the GCGR–NNC0640 and GCGR–NNC1702 complexes are offset in the main plot to show the quality of the fits. The inset shows the overlaid portion of the DEFs. The DEER data demonstrate that all protein samples exhibit multiple peaks, and the addition of the peptide NNC1702 populates longer distances (32–43 Å), which match the distance distribution predicted by the MMM software using the GCGR–NNC1702 structure as a template (**b**). The main DEER distance that the apo GCGR and the NNC0640-bound receptor showed is around 26 Å. The conformation possibilities of this distance include the inactive conformation observed in the GCGR–NNC0640–mAb1 structure in which the H89R1–C287R1 distance is about 26 Å between nitroxide N–O bonds when using common R1 rotamers[42] for modelling, and the different inactive conformational states of the apo receptor that display close contacts between the ECD and TMD, as suggested by previous molecular dynamics simulation studies[16,17] , with H89R1–C287R1 distances of 23–29 Å between the nitroxide N–O bonds when R1 side chains are modelled. These results suggest that the ECD in the apo GCGR or the NNC0640-bound receptor may adopt one conformation or multiple conformations, with a H89R1–C287R1 distance of about 26 Å between nitroxide N–O bonds. The longer distance upon binding to the peptide ligand NNC1702 indicates that the receptor ECD undergoes a conformational change to accommodate the peptide. Equilibrium between these conformational states may potentially exist. NNC1702 probably shifts it towards the conformation favourable for peptide binding, in contrast to the small-molecule NAM NNC0640 that has a weak effect on the ECD conformation. This equilibrium between peptide-free and peptide-bound receptors may help explain the fact that more than one peak was observed for the GCGR–NNC1702 complex in this study, although the concentration of NNC1702 used during protein purification and DEER measurements is 50 μM, which is much higher than the binding affinity of the peptide. G-protein binding may further shift the equilibrium to the peptide-bound conformation, although specific experimental data regarding the G-protein-bound receptor are required to validate this point. Our findings support the flexibility of the ECD conformation and further highlight that the conformational change of the ECD is required for peptide binding.