

Supplementary S1

Non-canonical G_q Activation by Orexin Receptor Type 2 and Lemborexant Observed in Microsecond Molecular Dynamics Simulations

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CLUSTAL O (1.2.4) multiple sequence alignment

6CMO RHO_chimera	FADYKDDDDAKLQTMHHHHHHHHHENLYFQGGTADLEDNWETLNDNLKVIEKADNAAQV	60
6OSA NTSR1 (NC)	--DYKDDDDAMGQP-----	12
6OS9 NTSR1 (C)	--DYKDDDDAMGQP-----	12
sp O43613 OX1R_HUMAN	---MEFSAT-PAQA-----	10
sp O43614 OX2R_HUMAN	-----M-SGTK-----	5
7L1V OX2R	--DYKDDDA-MGTK-----	11
6CMO RHO_chimera	KDALTKMRAAALDAQKATPPKLEDKSPDSEPMKDFRHGFDILVGQIDDAKLANEGKVKE	120
6OSA NTSR1 (NC)	-----GNGSA-----FLLAP-----NRS--HA	27
6OS9 NTSR1 (C)	-----GNGSA-----FLLAP-----NRS--HA	27
sp O43613 OX1R_HUMAN	-----	10
sp O43614 OX2R_HUMAN	-----LEDSP-----P-----CRN----	14
7L1V OX2R	-----LEDSP-----P-----CRN----	20
6CMO RHO_chimera	AQAAAEQLKTRNAYIQ-KYLMCGTEGPNFYVPPSNATGVVRSPFEYPQYYLAEPWQ---	176
6OSA NTSR1 (NC)	P-----DHDVENLYFQGGRAQAGLEEALLAPGFGNASGNAS-----ERVLAAPSSSELD	75
6OS9 NTSR1 (C)	P-----DHDVENLYFQGGRAQAGLEEALLAPGFGNASGNAS-----ERVLAAPSSSELD	75
sp O43613 OX1R_HUMAN	-----MGVPPGSRPSPVP-----PDYE-----DEFRLRYLWRD-Y	39
sp O43614 OX2R_HUMAN	-----WSSASELNETQEPFLNP-----TDYDD-----EEFLRYLWRE-Y	47
7L1V OX2R	-----WSSASELNETQEPFLNP-----TDYDD-----EEFLRYLWRE-Y	53
6CMO RHO_chimera	-----FSMLAAYM--FLLIVLGFPI ^{1.50} NFLTLTYVTQHKKL--RTPLNYILLNLAVADLFM ^{2.50}	226
6OSA NTSR1 (NC)	VNTDIYSKVLVTAVYLALFVVGTVGNTVTTLFTLARKKSLQSLQSTVHYHLGSLALSDLLT	135
6OS9 NTSR1 (C)	VNTDIYSKVLVTAVYLALFVVGTVGNTVTTLFTLARKKSLQSLQSTVHYHLGSLALSDLLT	135
sp O43613 OX1R_HUMAN	LYPKQYE-WVLIAAYVAVFVVALVGN ^{1.50} TLVCLAVWRNHHM--RTVTNYFIVNLSLADVLV	95
sp O43614 OX2R_HUMAN	LHPKEYE-WVLIAGYIIVFVVALIG ^{1.50} NVLVCVAVWKNHHM--RTVTNYFIVNLSLADVLV	103
7L1V OX2R	LHPKEYE-WVLIAGYIIVFVVALIG ^{1.50} NVLVCVAVWKNHHM--RTVTNYFIVNLSLADVLV	109
6CMO RHO_chimera	VLGGFTSTLY--TSLHGYPVFGPTGCNLQGGFATLGGELALWSLVVLAIE ^{3.50} RYVVVCKPMS	284
6OSA NTSR1 (NC)	LLLAMPVELYNFIWVHHPWAFGDAGCRGYFLRDACTYATALNVASLSVER ^{3.50} RYLAICHFFK	195
6OS9 NTSR1 (C)	LLLAMPVELYNFIWVHHPWAFGDAGCRGYFLRDACTYATALNVASLSVER ^{3.50} RYLAICHFFK	195
sp O43613 OX1R_HUMAN	TAICLPASLL--VDITESWLFGHALCKVPIYLQAVSVSVAVITLSFIALDR ^{3.50} WYAICHPLL	153
sp O43614 OX2R_HUMAN	TITCLPATLV--VDITETWFFGQSCKVPIYLQAVSVSVSVLTLSFIALDR ^{3.50} WYAICHPLM	161
7L1V OX2R	TITCLPATLV--VDITETWFFGQSCKVPIYLQAVSVSVSVLTLSFIALDR ^{3.50} WYAICHPLM	167
6CMO RHO_chimera	NFRFGENHA-IMGVAF ^{4.50} TWMLACAAAPLA--GWSRYIEG-----LQCCSGDIYYTL	334
6OSA NTSR1 (NC)	AKTLMRSRRTKKFISAI ^{4.50} WLASALLAVPMLFTMGEQNRSDGQHAG--GLVCTPTI----	248
6OS9 NTSR1 (C)	AKTLMRSRRTKKFISAI ^{4.50} WLASALLAVPMLFTMGEQNRSDGQHAG--GLVCTPTI----	248
sp O43613 OX1R_HUMAN	FKSTARRAR--GSILGI ^{4.50} WAVSLAIMVPQAAMVECSSVLPELANRTRLFVSVCDERW----	206
sp O43614 OX2R_HUMAN	FKSTAKRAR--NSIVII ^{4.50} WIVSCIIMI ^{4.50} PQAIVMCSTVFPGLANKTTLFTVCDERW----	214
7L1V OX2R	FKSTAKRAR--NSIVII ^{4.50} WIVSCIIMI ^{4.50} PQAIVMCSTVFPGLANKTTLFTVCDERW----	220
6CMO RHO_chimera	KPEVNNEFVIYMFVVHFTI ^{5.50} PMIIIFCYGQLVF-----TVKEAAA-----	375
6OSA NTSR1 (NC)	HTATVKVVIQVNT-FMSFIF ^{5.50} PMVVISV-----LN-----TIANKLTVMVRQAA-----	291
6OS9 NTSR1 (C)	HTATVKVVIQVNT-FMSFIF ^{5.50} PMVVISV-----LN-----TIANKLTVMVRQAA-----	291
sp O43613 OX1R_HUMAN	ADDLYPKIYHSCFFIVTYLA ^{5.50} PLGLMAMAYFQIFRKLWGRQIPGTTLSALVRNWKRPSPDQLG	266
sp O43614 OX2R_HUMAN	GGEIYPKMYHICFFLVTYMA ^{5.50} PLCLMVLAYLQIFRKLWCRQIPGTSSVQKWKPLQPVSQ	274
7L1V OX2R	GGEIYPKMYHICFFLVTYMA ^{5.50} PLCLMVLAYLQIFRKLWCRQIPGTSSVQKWKPLQPVSQ	266
6CMO RHO_chimera	---QQQ-----ESATTQKAEKEVTRMVIYVIAFLICW ^{6.50} VPYASVAFYI----	415
6OSA NTSR1 (NC)	--EQGQ-----VCTVGGPGRVQALRHGVRVLRRAVIAFVVCW ^{6.50} LPYHVRRLMFC---Y	338
6OS9 NTSR1 (C)	--EQGQ-----VCTVGGPGRVQALRHGVRVLRRAVIAFVVCW ^{6.50} LPYHVRRLMFC---Y	338
sp O43613 OX1R_HUMAN	DLEQGLSGEPQPRARAFLAEVKQMRARRKTAKMLMVLLVFAICYL ^{6.50} PIISVLNVLKRVFGM	326
sp O43614 OX2R_HUMAN	--PRGPGQPTKSRMSAVAAEIKQIRARRKTARMLMIVLLVFAICYL ^{6.50} PIISVLNVLKRVFGM	332
7L1V OX2R	-----EIKQIRARRKTARMLMIVLLVFAICYL ^{6.50} PIISVLNVLKRVFGM	307
6CMO RHO_chimera	FTHQG-----SCFGPIFMTIPAFFAKSAAIYN ^{7.50} PVIYIMMNKQFRNCMLTTICC-----	463
6OSA NTSR1 (NC)	ISDEQWTPFLYDFYHY-FYMTNALFYVSSTIN ^{7.50} PIYLNLSANFRHIFLATLACLPVWR	397
6OS9 NTSR1 (C)	ISDEQWTPFLYDFYHY-FYMTNALFYVSSTIN ^{7.50} PIYLNLSANFRHIFLATLACLPVWR	397
sp O43613 OX1R_HUMAN	FRQASDREAVYAC-----FTFSHWLVYANSAAN ^{7.50} PIIYNFLSGKFREFKAAFSCCCLPGLG	381
sp O43614 OX2R_HUMAN	FAHTEDRETVEYAW-----FTFSHWLVYANSAAN ^{7.50} PIIYNFLSGKFREFKAAFSCCCLGVH	387
7L1V OX2R	FAHTEDRETVEYAW-----FTFSHWLVYANSAAN ^{7.50} PIIYNFLSGKFREFKAAFSCCCLGVH	362
6CMO RHO_chimera	-----	463
6OSA NTSR1 (NC)	RRRKRPAFSRKADSVSSNHTL-----SSNATRETLYLEVLFGQ-----	435
6OS9 NTSR1 (C)	RRRKRPAFSRKADSVSSNHTL-----SSNATRETLYLEVLFGQ-----	435
sp O43613 OX1R_HUMAN	PCGSLKAPSRSS--ASHKSL--SLQSRCSISKISEHVVLTSVTTVLV-----	425
sp O43614 OX2R_HUMAN	HRQEDRLTRGRTST-ESRKSLLTQISNFDNISKLSQVVLTSISTLPAANGAGPLQNW	444

Figure S1. Multiple sequence alignment of OX₁, OX₂ (UniProt ID: O43613, O43614, respectively), NTSR1 (6OSA, 6OS9), and rhodopsin (6CMO). OX₁ and OX₂ sequences were derived from UniProt [1, 2], while the remaining sequences were extracted from PDB structures [3, 4, 5]. The most conserved residues were marked in red and numbered according to the Ballesteros-Weinstein notation [6]. The alignment was performed using Clustal Omega v. 1.2.4.[7].

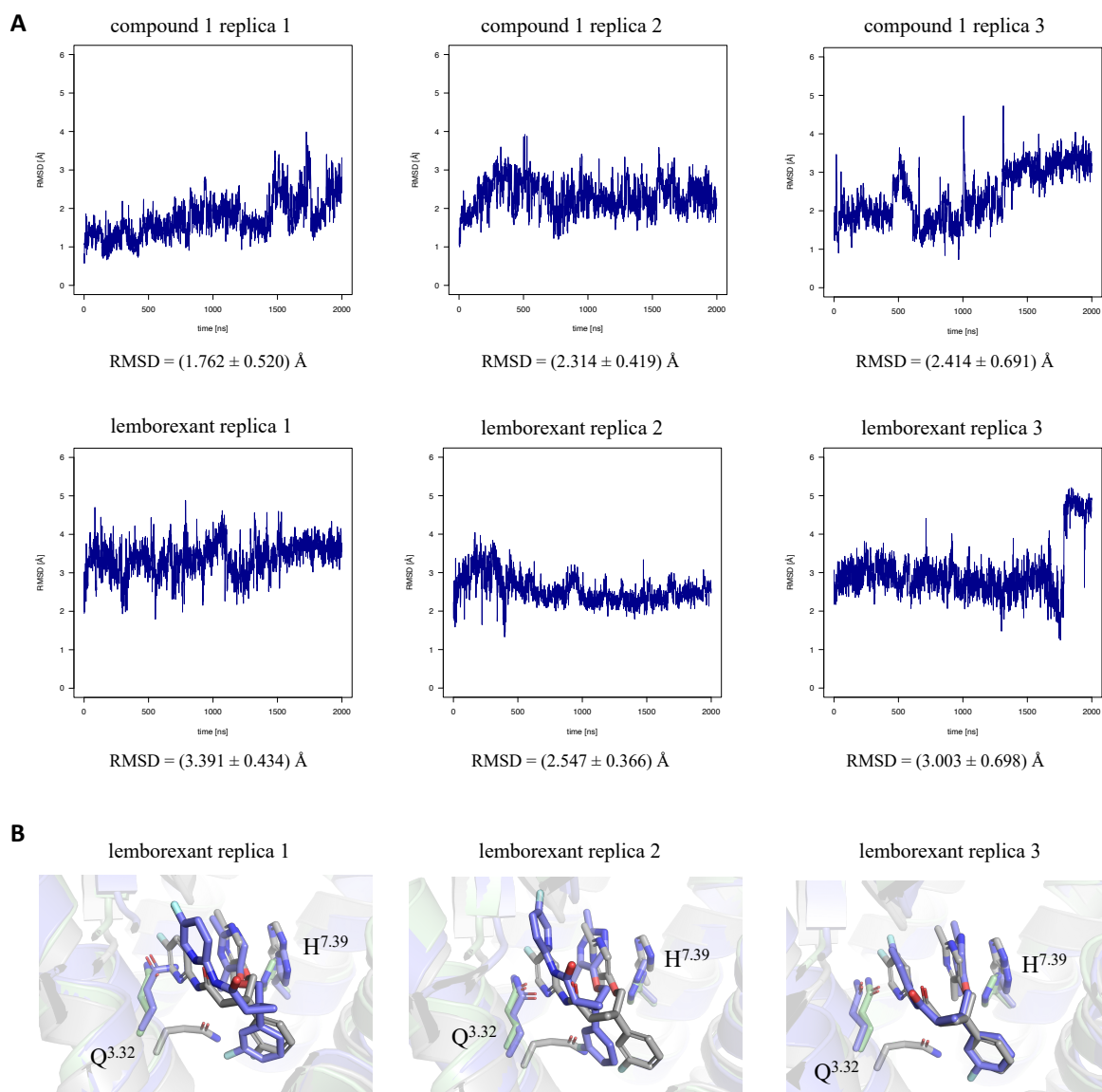


Figure S2. Fluctuations in the binding site area and comparison with PDB structures. (A) The RMSD plots and average RMSD fluctuations obtained for compound 1 and lemborexant-including simulation systems. (B) A superposition of the active-state 7L1V PDB structure (green), the inactive-state 7XRR structure (gray), and the first frame of the lemborexant-including simulations (blue). Residues Q^{3.32} and H^{7.39} are shown as sticks.

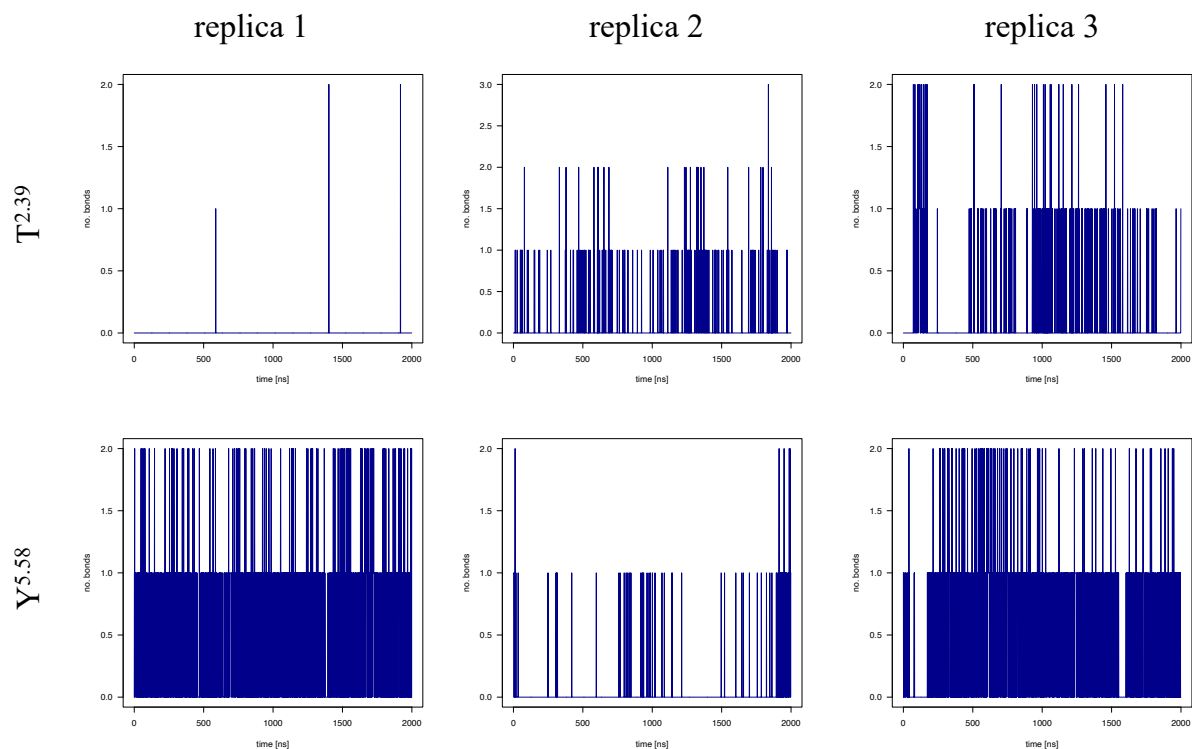


Figure S3. The formation of hydrogen bonds between R^{3.50} of the 'DRW' motif and residues T^{2.39} and Y^{5.58} in the compound 1-including simulations. The 'DRW' motif corresponds to the well-known 'DRY' motif in GPCRs. The maximum hydrogen bond distance was set to 4 Å and the angle cut-off was set to 20° (VMD settings). In orexin receptors, the inactive-state interactions of R^{3.50} are visible in 7XRR (OX₂ bound to lemborexant), while the active-state interactions are visible in 7L1U (OX₂ bound to OxB) and 7L1V (OX₂ bound to compound 1).

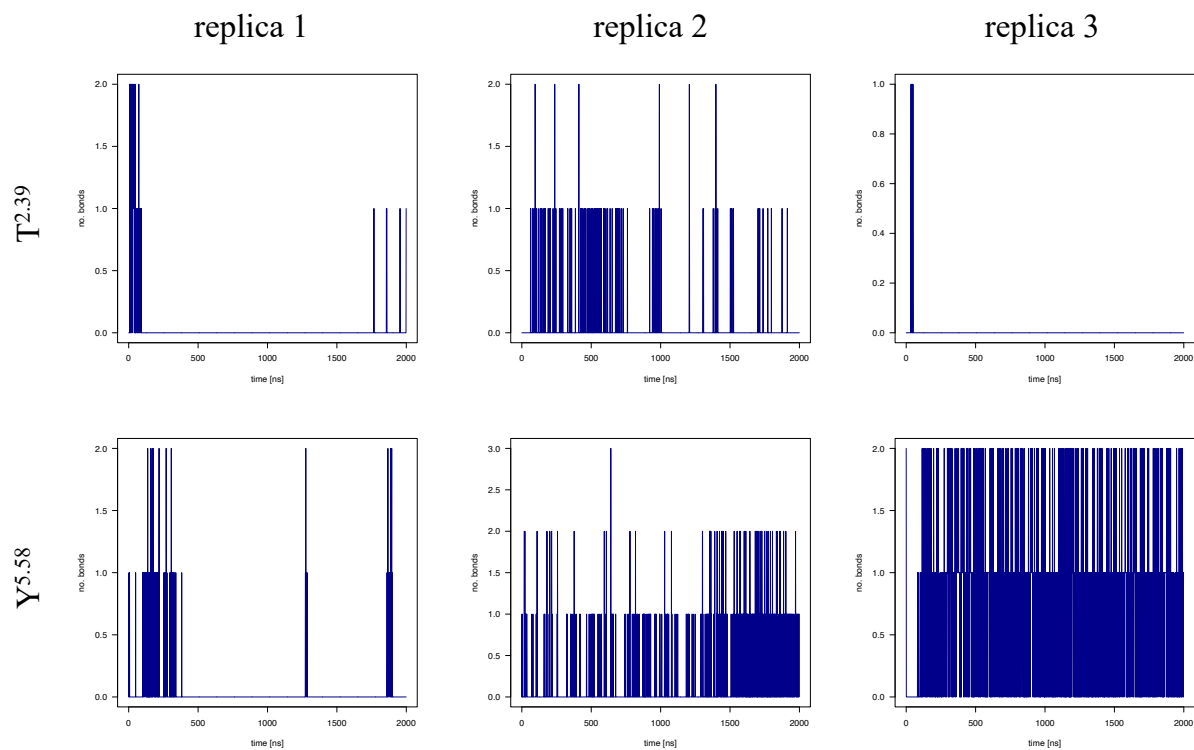


Figure S4. The formation of hydrogen bonds between R^{3.50} of the 'DRW' motif and residues T^{2.39} and Y^{5.58} in the lemborexant-including simulations. The maximum hydrogen bond distance was set to 4 Å and the angle cut-off was set to 20° (VMD typical settings).

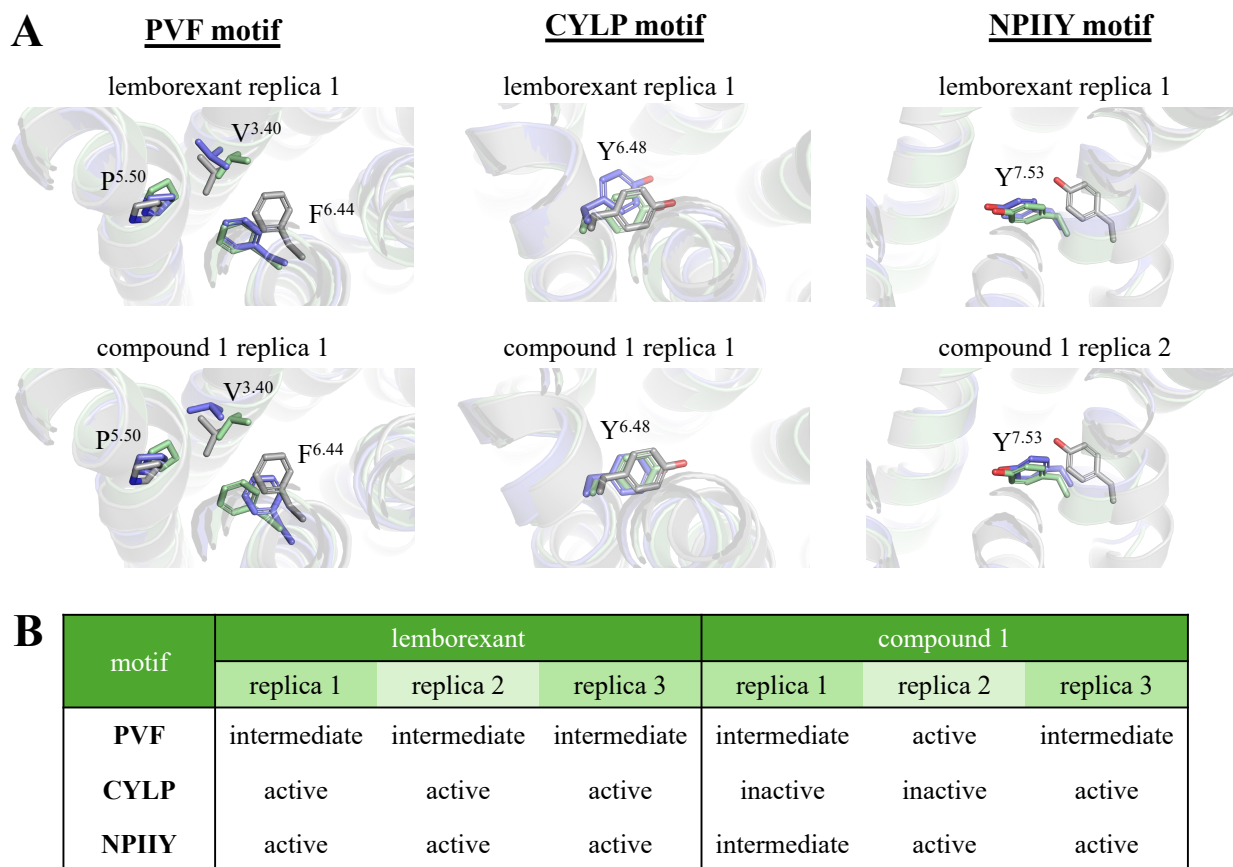


Figure S5. A comparison of conformations of sequence motifs in simulations systems and in PDB structures. (A) A comparison of the PIF (PVF in OX₂), CWxP (CYLP in OX₂), and NPxxY (NPIIY in OX₂) microswitches in the active-state 7L1V structure (green), inactive-state 7XRR structure (gray), and the final frame of the simulation replicas (blue). (B) A table describing the conformations of the microswitches in the final frame for each replica.

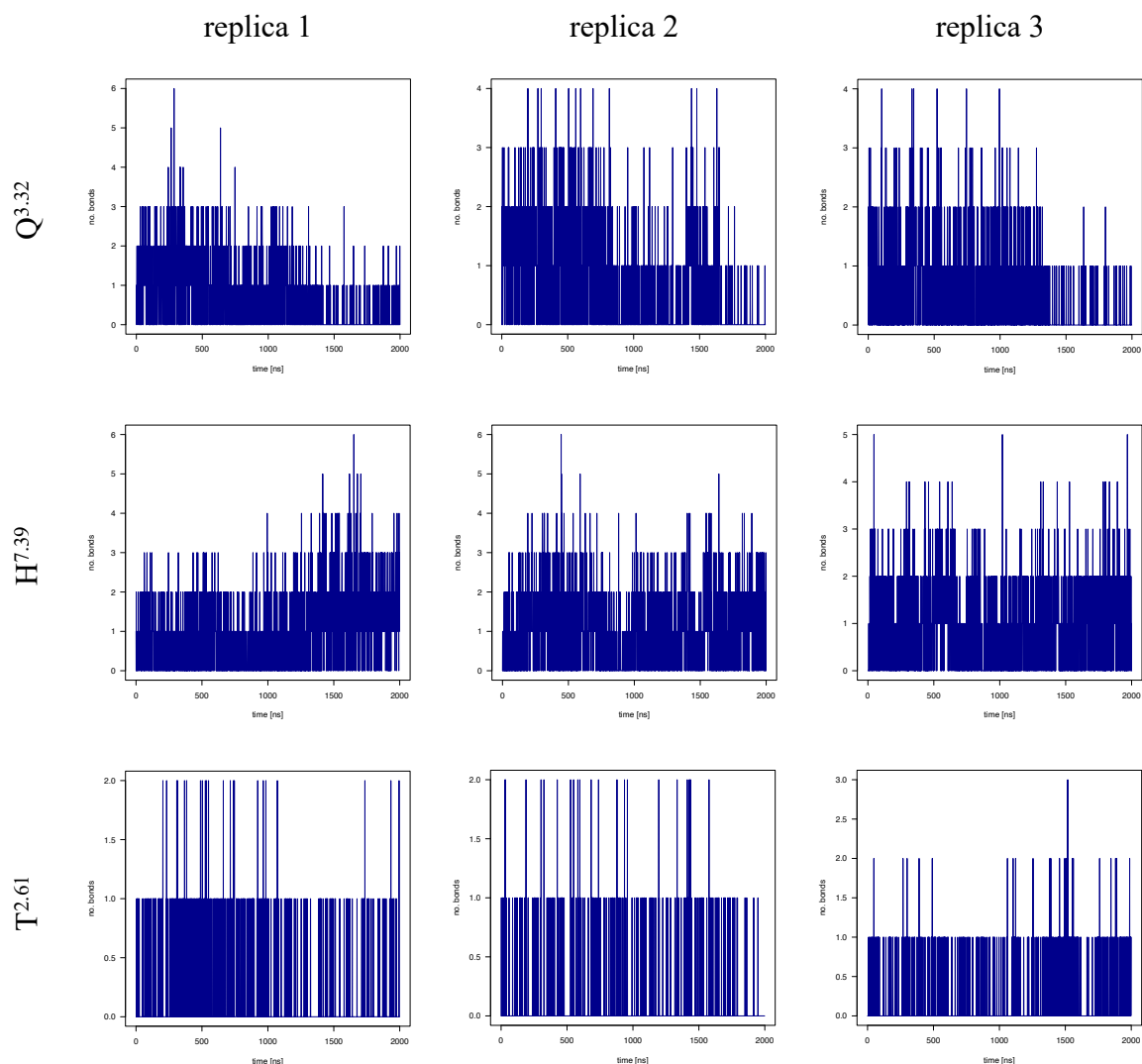


Figure S6. The formation of hydrogen bonds between compound 1 and the OX₂ receptor residues Q^{3.32}, H^{7.39}, and T^{2.61}. The maximum hydrogen bond distance was set to 4 Å, and the angle cut-off was set 20° (VMD settings). According to Hong et al., a hydrogen bond should be present between H^{7.39} and the amide carbonyl linker of compound 1, and further contacts with this residue stabilized the bent conformation of the ligand [3].

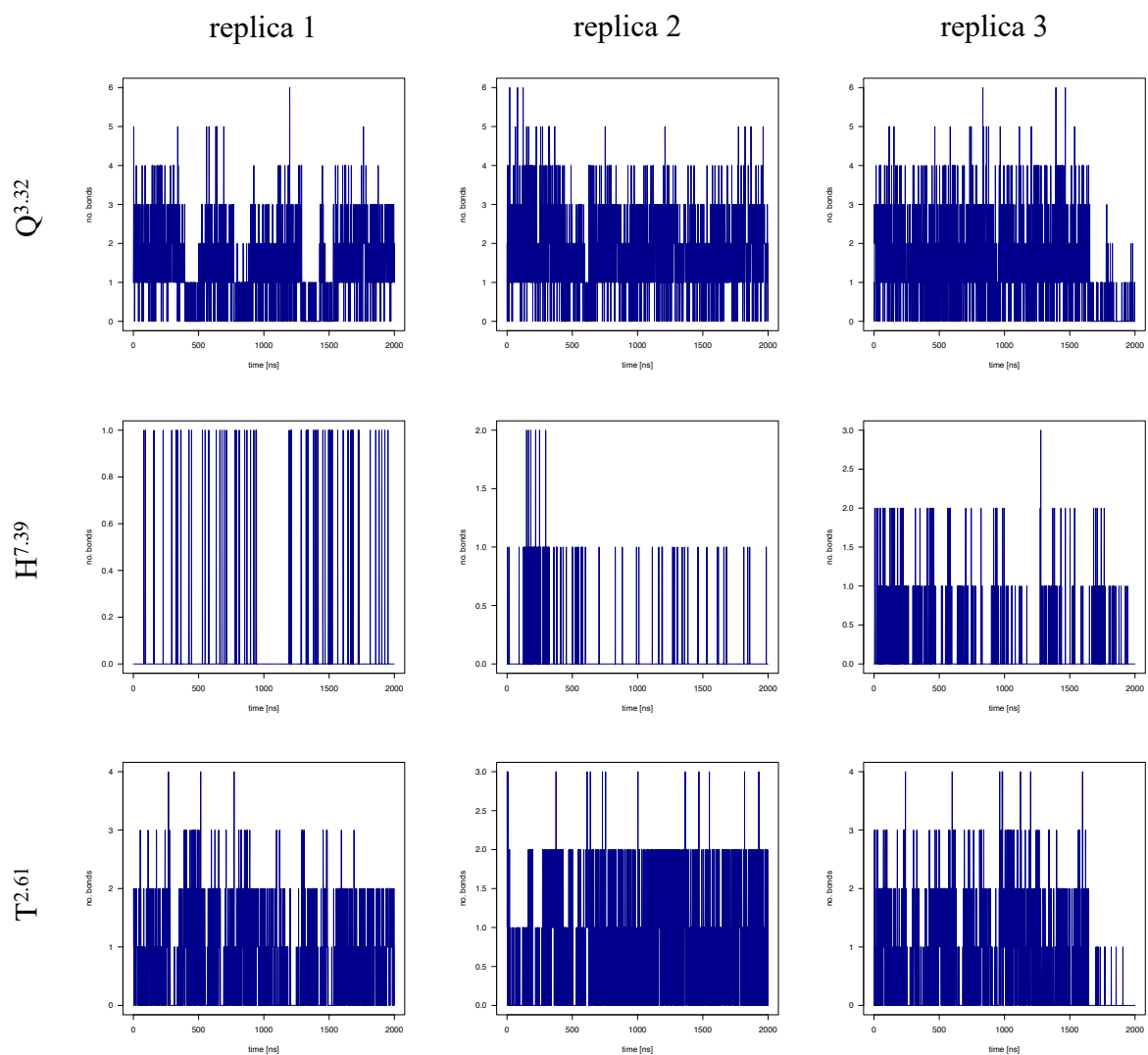


Figure S7. The formation of hydrogen bonds between lemborexant and the OX₂ receptor residues Q^{3.32}, H^{7.39}, and T^{2.61}. The maximum hydrogen bond distance was set to 4 Å, and the angle cut-off was set 20° (VMD typical settings).

CLUSTAL O(1.2.4) multiple sequence alignment

		HN	S1	P-loop	H1				
sp P50148 GNAQ_HUMAN	MTLESIMACCLSEEAKEARRINDEIERQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR					60			
7L1V mini-Gq	-----GTLSAEDKAAVERSKMIEKQLQKDKQVYRRTLRLLLLGLADNSGKSTIVKQMR					52			
7SQ2 Gq	-----GADARRELKLLLLGTGESGKSTFIKQMR					28			
			** *:*****:;*****:*****						
		HA		HB					
sp P50148 GNAQ_HUMAN	IIHGSGYSDEDKRGFTKLVIYQNIIFTAMQAMIRAMDTLKI PYKYEHNKAHAQLVREVDVEK					120			
7L1V mini-Gq	ILHGGSGGSG-----					62			
7SQ2 Gq	IIHGSGYSDEDKRGFTKLVIYQNIIFTAMQAMIRAMDTLKI PYKYEHNKAHAQLVREVDVEK					88			
		*:***. .							
		HC	HD	HE	HF				
sp P50148 GNAQ_HUMAN	VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYLLNLDLRVADPAYLP TQQDVL					180			
7L1V mini-Gq	-----					62			
7SQ2 Gq	VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYLLNLDLRVADPSYLP TQQDVL					148			
		switch I	S2	S3	switch II	H2	S4	switch III	
sp P50148 GNAQ_HUMAN	RVRVPTTGTIIIEYPFDLQSVIFRMVDVGGQSRERRKWIHCFENVTSIMFLVALSEYDQVLV								240
7L1V mini-Gq	----GTSGIFETKFQVDKVNFMFDVGGQDRERRKWIQCFNDVTAIIFVVDSSDYNR---								115
7SQ2 Gq	RVRVPTTGTIIIEYPFDLQSVIFRMVDVGGQSRERRKWIHCFENVTSIMFLVALSEYDQVLV								208
		*:***: *	*:***: *	*:***: *	*****:*****:*****:*****	*****:*****	*:***: *	*:***: *	
		H3	S5	HG					
sp P50148 GNAQ_HUMAN	ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMY--SHLVDYFPEYDGP								298
7L1V mini-Gq	-----LQEALNDFKSIWNNRWLRTISVILFLNKQDLLAEKVLAKGSKIEDYFPEFARY								168
7SQ2 Gq	ESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMY--SHLVDYFPEYDGP								266
		:*: *:*** .	*:***: *****:*****:*****	:*: *****:					
		H4		S6	H5				
sp P50148 GNAQ_HUMAN	QR-----DAQAAREFILKMFVDLN---PDSDKIYSHFTCATDTENIRFVFAA								343
7L1V mini-Gq	TTPEDATPEPGEDPRVTRAKYFIRKEFVDISTASGDRHICYPHFTCAVDTENARRIFND								228
7SQ2 Gq	QR-----DAQAAREFILKMFVDLN---PDSDKIYSHFTCATDTENIRFVFAA								311
		. *: ** * ***:.	*:***: *****:*****	*:***: *					
		H4							
sp P50148 GNAQ_HUMAN	VKDTILQLNLKEYNLV								359
7L1V mini-Gq	CKDIILQMNLREYNLV								244
7SQ2 Gq	VKDTILQLNLKEYNLV								327
		** ***:*****							

Figure S8. Multiple sequence alignment of $G\alpha_q$ (UniProt ID: P50148) and $G\alpha$ subunits in the 7L1V and 7SQ2 PDB structures. The first $G\alpha_q$ sequence was derived from UniProt [8] and following $G\alpha$ sequences from 7L1V [3] and 7SQ2 [9] PDB structures, respectively. Residues were assigned a secondary structure based on the GproteinDb [10] numbering for $G\alpha_q$ and the topology of the $G\alpha$ subunit described by Calebiro et al. [11] The multiple sequence alignment was performed using Clustal Omega v. 1.2.4. [12].

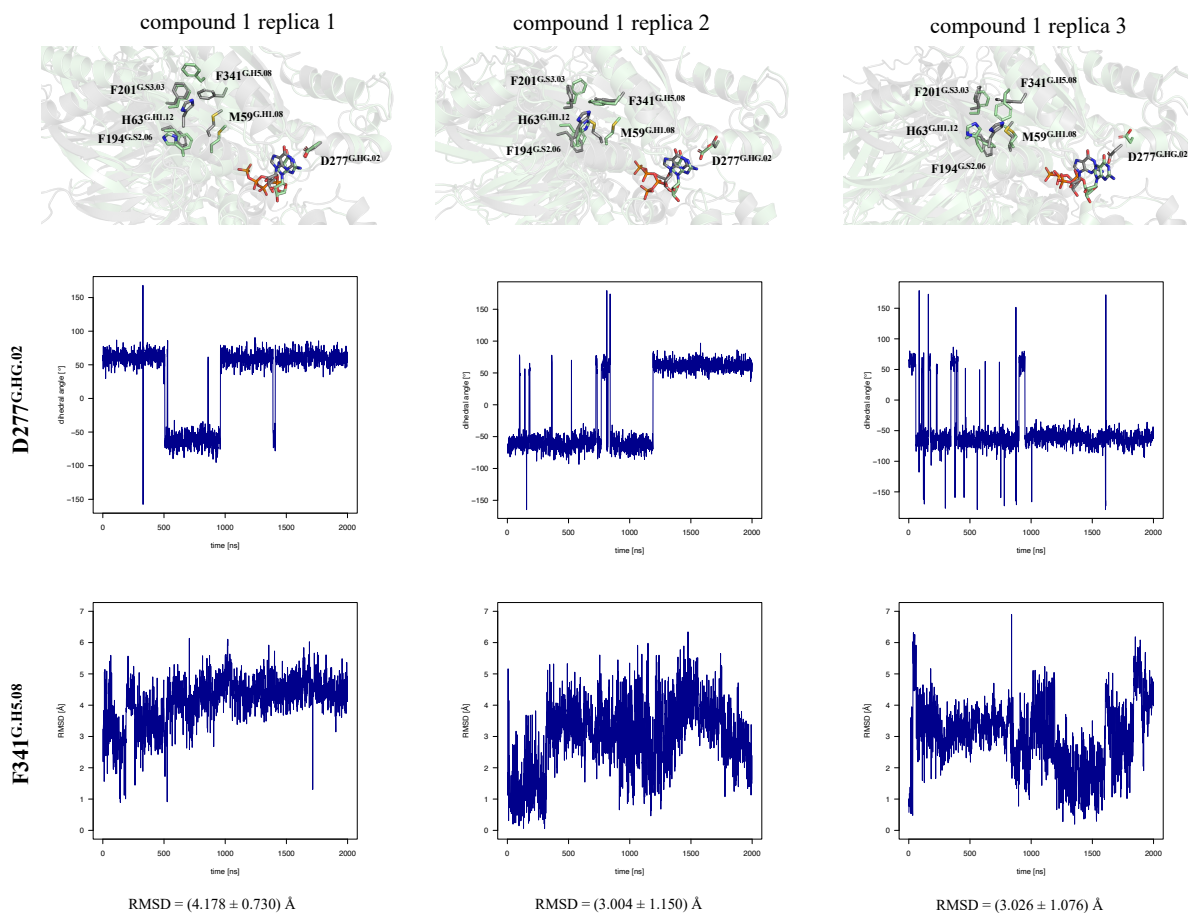


Figure S9. Changes of the D277 (D272) and F341 (F336) microswitches conformations observed in the compound 1-including simulations. (Top) Changes in the conformation of the microswitches between the first (gray) and the last (green) frame of the compound 1-including simulations. (Middle) The χ_1 dihedral angle ($\text{N}-\text{C}_\alpha-\text{C}_\beta-\text{C}_\gamma$) computed for microswitch $\text{D277}^{\text{G.HG.02}}$ (ca. -60° —on; 60° —off). (Bottom) The RMSD graphs and mean RMSD values computed for a single carbon atom of the phenyl group of microswitch $\text{F341}^{\text{G.H5.08}}$. $\text{D277}^{\text{G.HG.02}}$ and $\text{F341}^{\text{G.H5.08}}$ refer to D272 and F336 as labeled by Ham et al. [13].

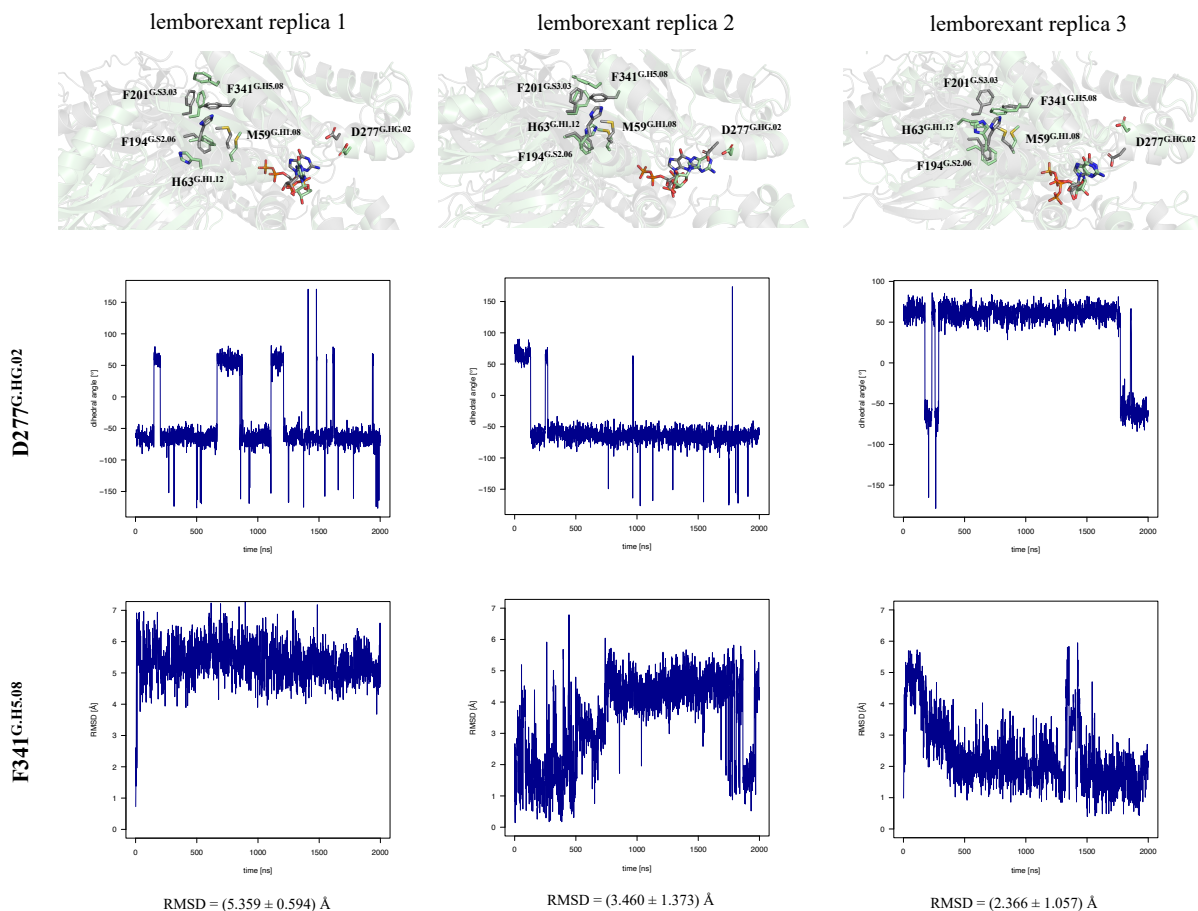


Figure S10. Changes of the D227 (D272) and F341 (F336) microswitches conformations observed in the lemborexant-including simulations. (Top) The changes in the conformations of the microswitches between the first (gray) and the last (green) frame of the lemborexant-including simulations. **(Middle)** The dihedral angle between the nitrogen and carbon atoms of the main chain and the two carbon atoms of the side chain computed for microswitch D277^{G.HG.02} (ca. -60°—on; 60°—off). **(Bottom)** The RMSD graphs and mean RMSD values computed for a single carbon atom of the phenyl group of microswitch F341^{G.H5.08}. D277^{G.HG.02} and F341^{G.H5.08} refer to D272 and F336 as labeled by Ham et al. [13].

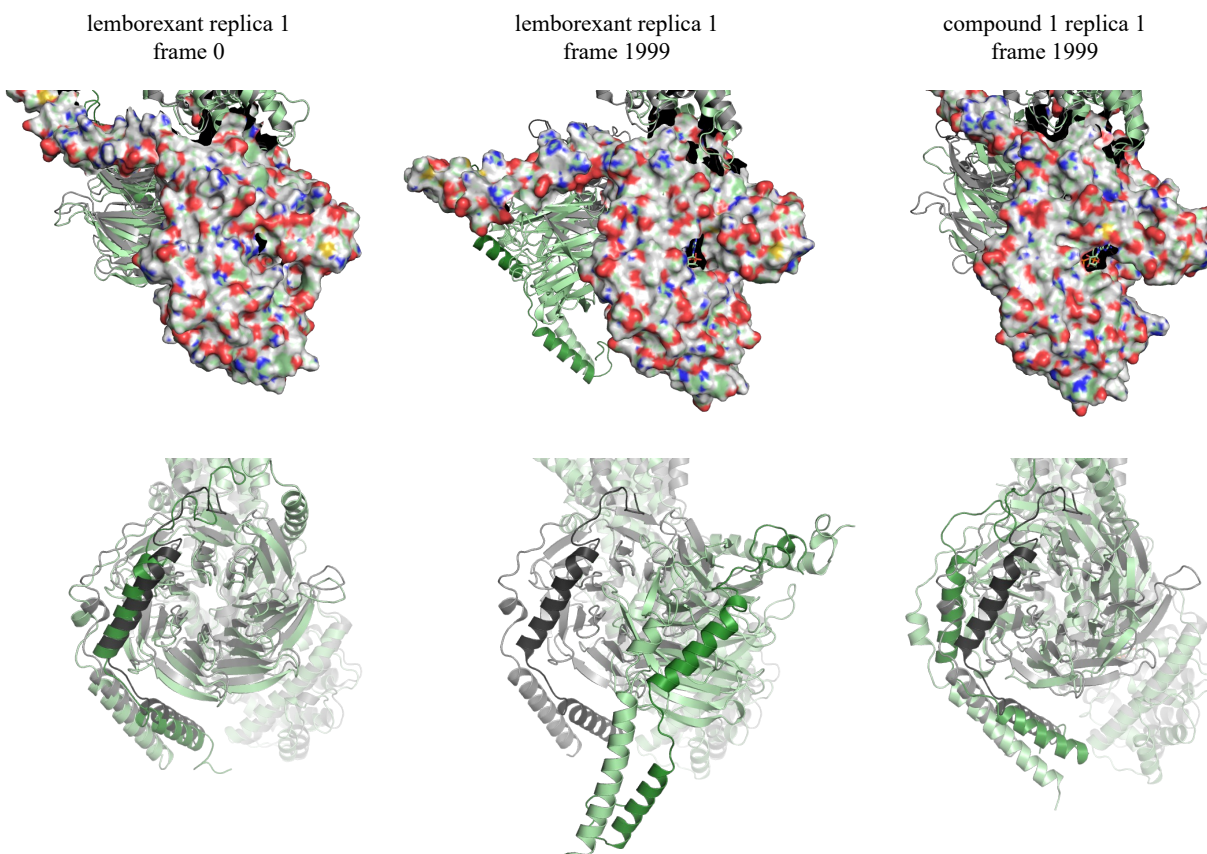


Figure S11. Changes in conformations of G protein subunits observed in MD simulations.

(**Top**) A surface view of the full-length $G\alpha$ subunit model (green) for the first frame of lemborexant replica 3 (**left**), the last frame of lemborexant replica 1 (**center**), and the last frame of compound 1 replica 1 (**right**) superposed on the templates ($G\alpha$ —the 7SQ2 crystal structure; $G\beta$, $G\gamma$ and the receptor—the 7L1V cryo-EM structure) used for the homology modeling (gray). $G\gamma$ was marked in dark gray in the template structure and in dark green in the simulation-derived conformations. (**Bottom**) The side view from the $G\beta$ and $G\gamma$ subunits. The movement of $G\gamma$ and the adjacent helical regions of $G\beta$ during the $G\alpha$ opening was observed in comparison to the template structure.

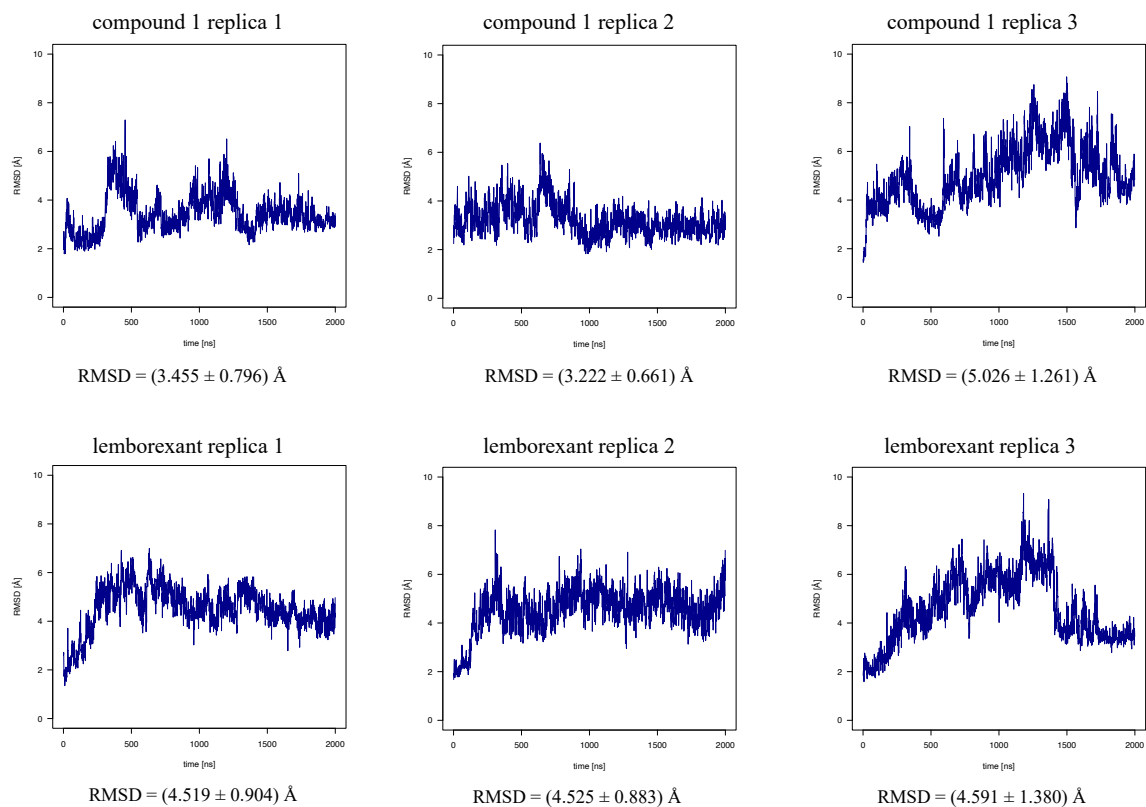


Figure S12. Fluctuations of the AHD domain in simulations. The RMSD plots and average RMSD fluctuations observed for AHD in compound 1 and lemborexant-including replicas.

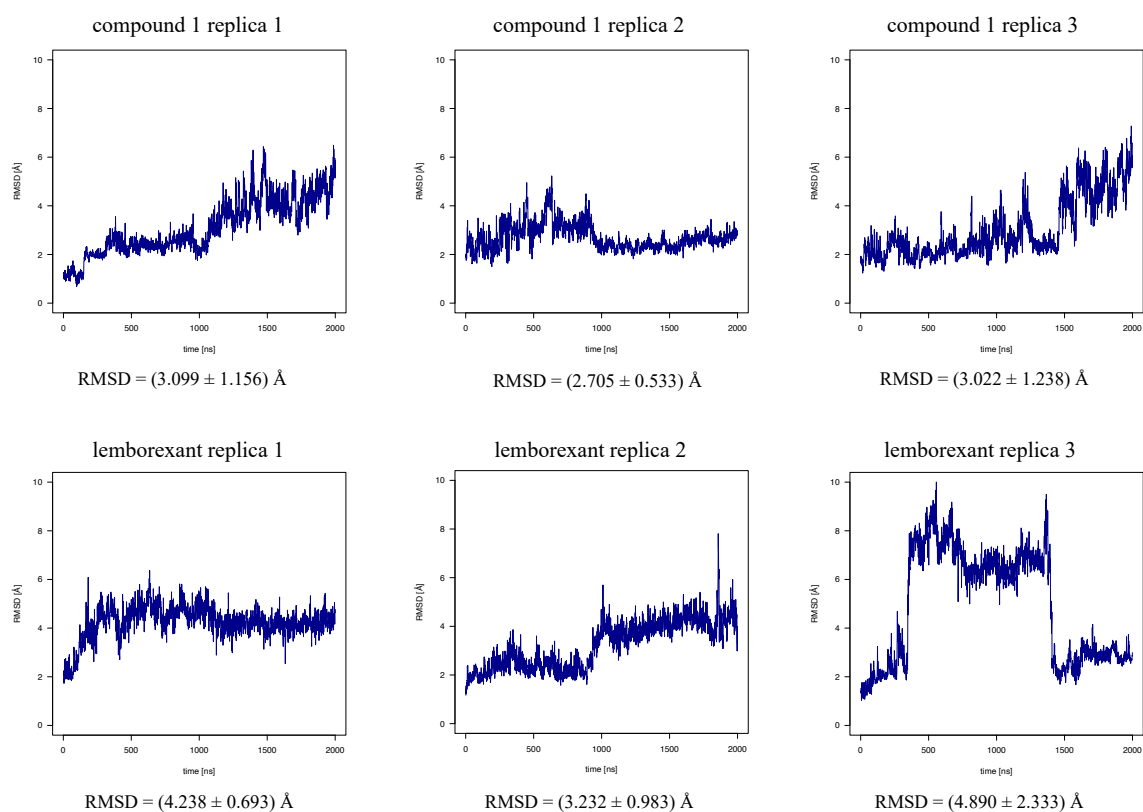


Figure S13. Fluctuations of GDP in simulations. The RMSD plots and average RMSD fluctuations observed for GDP in the compound 1 and lemborexant-including simulations.

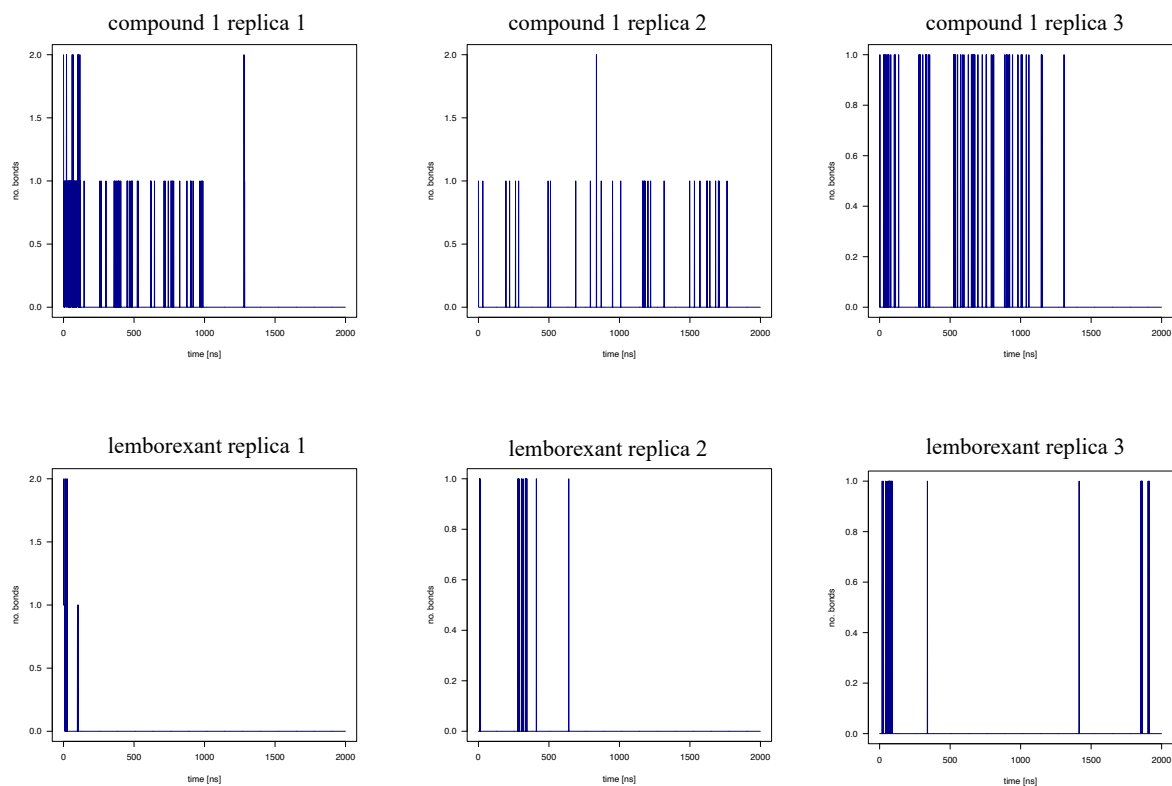


Figure S14. The formation of hydrogen bonds between GDP and R183^{G.hfs2.02} in the compound 1 and lemborexant-including simulations. The hydrogen bond distance was specified as 4 Å, and the angle cut-off was 20°.

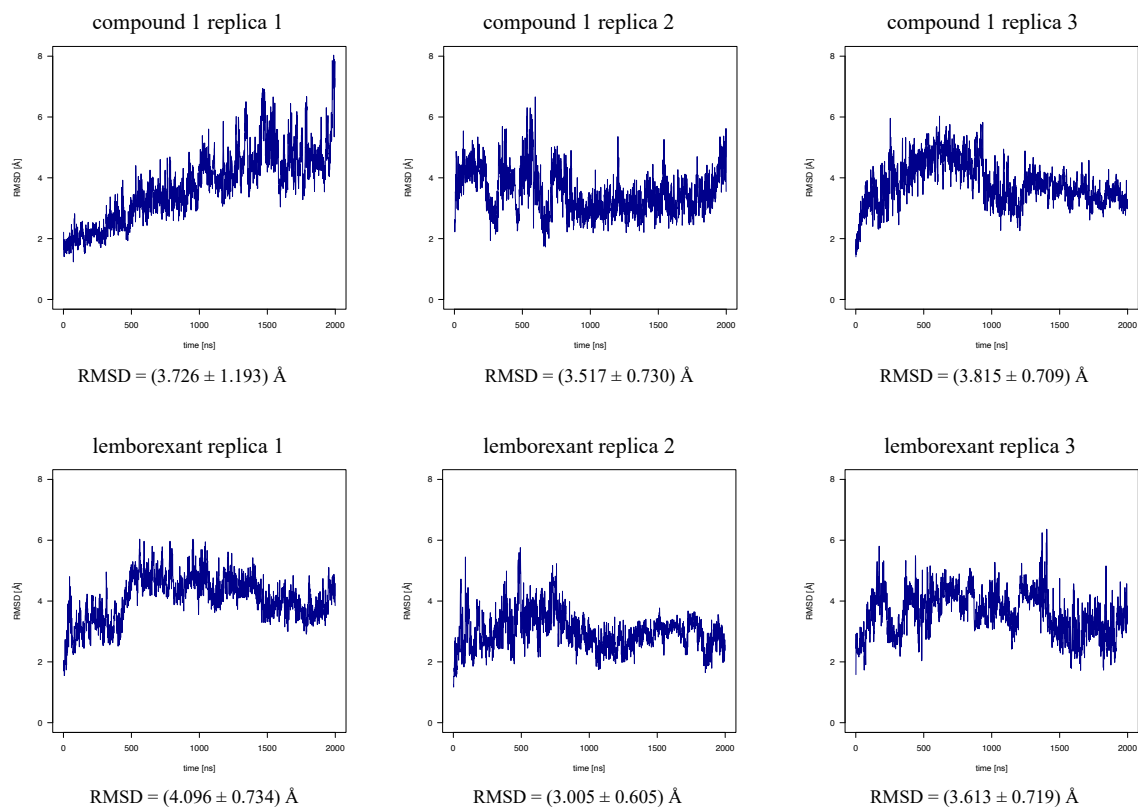


Figure S15. Fluctuations of the macroswitch I in simulations. The RMSD plots and average RMSD fluctuations observed for the macroswitch I in the compound 1 and lemborexant-including simulations.

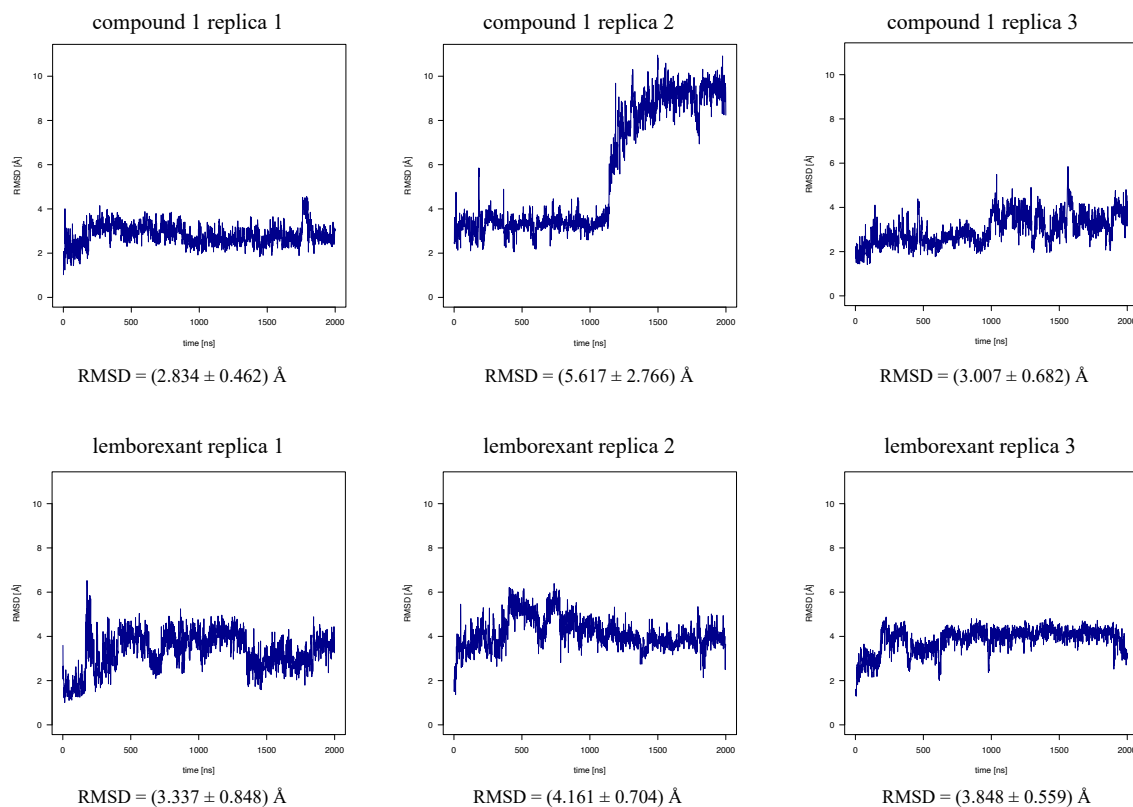


Figure S16. Fluctuations of the macroswitch III in simulations. The RMSD plots and average RMSD fluctuations observed for the macroswitch III in the compound 1 and lemborexant-including simulations.

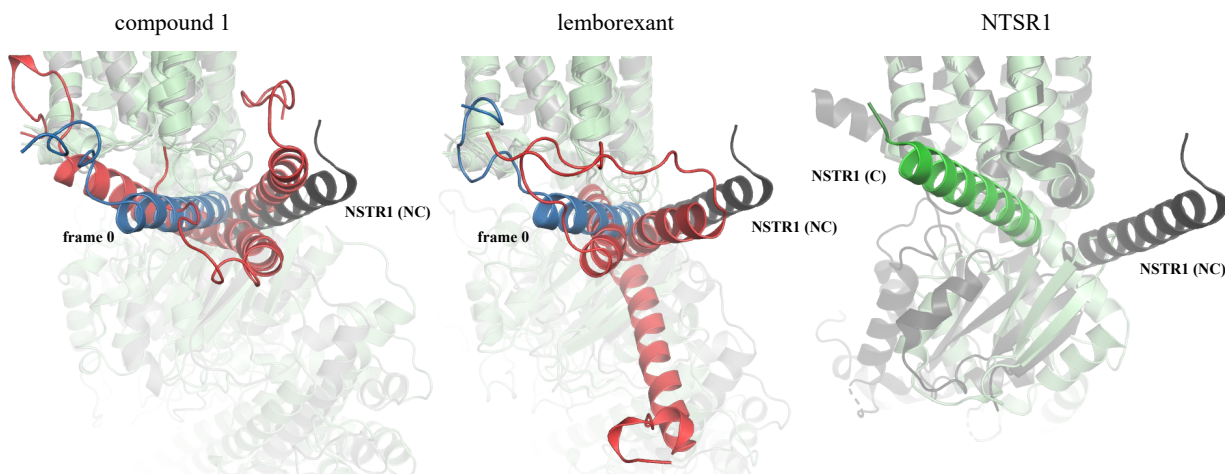


Figure S17. Changes in the orientation of N-terminal helices of $G\alpha$ in the compound 1 and lemborexant-including simulations in comparison to known active-state NTSR1 structures from PDB. The first frame of the simulation was shown in gray with a blue N-terminal helix, and the final frame was shown in green with a red N-terminal helix. The changes in the lemborexant-including complexes resembled the active-like direction of changes in NTSR1 [13]. **(Right)** The two active states of NTSR1: 6OSA—gray and 6OS9—green. In the case of NTSR1, one fully active state (C, PDB ID: 6OS9 [4]) was preceded by the other one (NC, PDB ID: 6OSA [4]), with the N-terminal helix of $G\alpha$ rotated by 45° counterclockwise with respect to the receptor main axis (right). The active-state structure of rhodopsin (6CMO) represented the fully active C state but with the N-terminal helix slightly moved to the intracellular side. The homology models used to generate the simulation systems all included the $G\alpha$ N-terminal helix in between the NC and C states, slightly closer to the C state. At the end of the simulations, the same direction of changes towards the C state was observed only for one compound 1-including replica 1, which also demonstrated the most similarities to pre-opening state of $G\alpha$. However, for compound 1-including replica 3, the N-terminal helix moved towards the NC state. In the remaining lemborexant-including replicas, the N-terminal helix of $G\alpha$ either remained as it was or moved to the bottom, not to the left as in 6OS9. This could suggest that the differences between the exerted effects of lemborexant and the classic agonist compound 1 on OX_2 could be due to the stabilization of slightly different conformational states of the OX_2 -G protein multicomplex during activation.

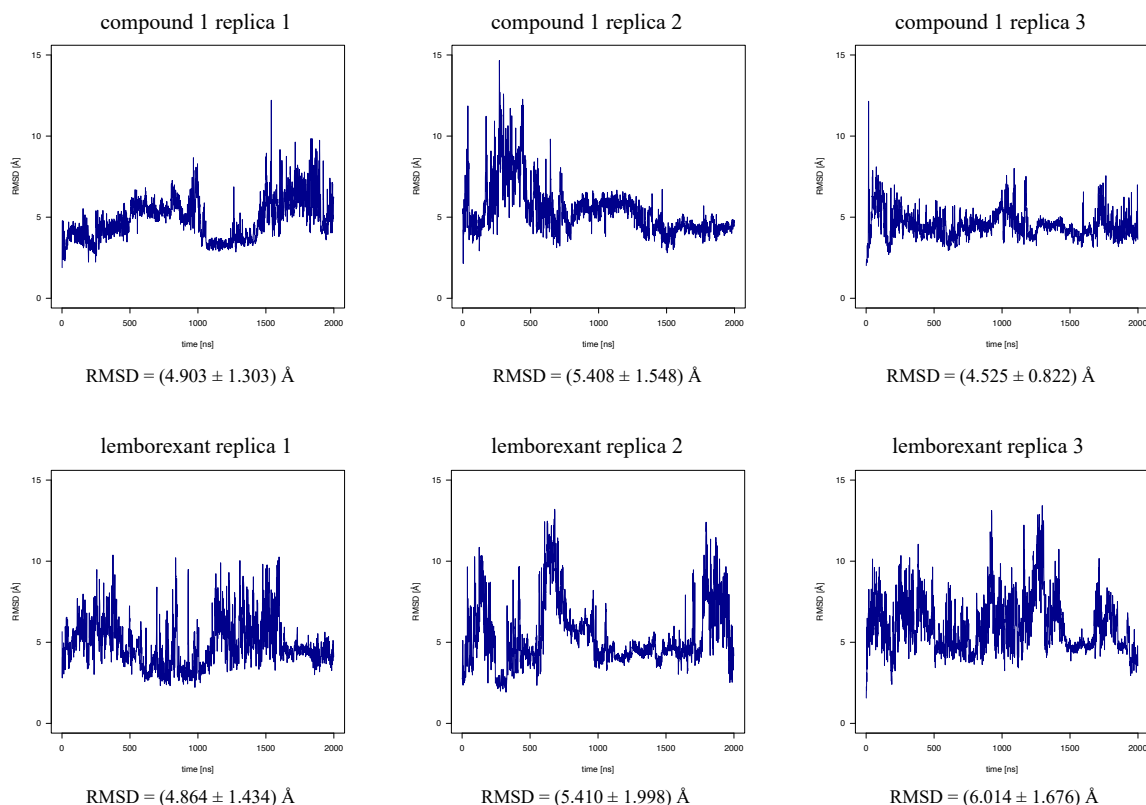


Figure S18. The RMSD plots and average RMSD fluctuations observed for ECL2 in the compound 1 and lemborexant-including simulations. The movement of ECL2 was also suggested when comparing the active-state (7L1V) and inactive-state (7XRR) structures, as it highly mobile in the former, and helical and arranged parallel to the lipid bilayer in the latter. The N-terminal helical region has been shown to be necessary for high-potency neuropeptide mediated activation of OX₂ [14]. In comparison to our previous MD simulations of OX₂ with mini-G_{sqi}, ECL2 closed even more quickly for the compound 1 simulations. More fluctuations were present in the lemborexant systems, and ECL2 closed more slowly when compared to compound 1.

References

1. *HCRT1—Orexin/Hypocretin receptor type 1—Homo sapiens (Human)* | UniProtKB | UniProt. (2022, August 3). <https://www.uniprot.org/uniprotkb/O43613/entry>
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