In silico analysis of Human and Zebrafish α-2 Adrenergic Receptors

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Abstract
α-2 adrenoceptors, belong to class of Rhodopsin-like G-protein coupled receptors. Proteins of the G-protein coupled receptor (GPCR) family are involved in many pathophysiological conditions and hence are targets for various drug discovery methods. The current information on the structure of GPCRs is limited to few structures like Rhodopsin, β adrenergic receptors, adenosine A2A receptors, Human Dopamine D3 and Chemokine receptor. In our study α-2 adrenergic receptors of Human and Zebrafish were modeled using MODELLER with Human Dopamine D3 receptor (PDB ID: 3PBL) as template. Through our modeling studies we have identified the critical role played by Proline residues (2.38, 2.59, 4.39, 4.59, 4.60, 7.50) of transmembrane helices and extracellular loop in stabilizing structural deviations in the transmembrane. Novel ligand binding residues S/T (6.56) and F (7.35) along with the positional significance of Y (3.28), Y (6.55) in regulating function were identified. Our models have shown that the Phenylalanine at 7.39 in TM7 can favourably interact with positively charged N-methyl group of the catecholamine ligands via hydrophobic contacts rather than 7.38 as reported previously. Furthermore, we are able to correctly show the orientation of Serine at 5.42 and 5.46 and discuss the relevance of residues at position 3.37 and 5.43 in the receptor regulation. We also demonstrate and propose that the orientation of V (2.61)/S should be taken into account in drug/ pharmacophore design specific for α-2 adrenergic receptors. We believe that these findings will open new lead for ligand/ pharmacophore design, *in silico* leading further to experimental validation using Zebrafish as experimental model.
Keywords: α-2 adrenergic receptor, Zebrafish, ligand binding, residue conservation, homology modeling, ionic lock, toggle switch.

1. Introduction

Alpha (α) 2 adrenoceptors are a family of aminergic proteins that belong to Class A Rhodopsin- like G-protein coupled receptors. These proteins have an extracellular N-terminus, seven transmembrane α helices and a cytoplasmic C-terminus. The TM helices have water accessible binding sites which forms a ligand binding pocket within the TM bundle. Agonist ligands which activate downstream signaling bind to this region and activate the receptor protein (Nyrönen *et al*., 2001). α-2 adrenoreceptors, through interactions with natural and synthetic ligands, are involved in many physiological signaling processes, making them relevant pharmacological targets in many diseases of the cardiovascular and nervous systems. Earlier studies have identified three α2 -adrenoreceptor subtypes in mammals, α-2A, α-2B, and α-2C, encoded by distinct genes and expressed in a wide variety of tissues (Regan *et al*., 1988). In addition, a fourth subtype with no orthologs in mammals and fish-specific duplicates of the four α-2-adrenoceptors subtypes, have been identified in zebra fish (Ruuskanen *et al*., 2004). The true fourth paralogous α-2 adrenoceptor subtype was named as α-2D and the two duplicates of this receptor as α-2DA and α-2DB (Ruuskanen *et al*., 2005 a). Studies have reported conservation in localization of Zebrafish α-2A, α-2B and α-2C adrenoceptors when compared with mammals. It was also reported that the distribution pattern of Zebrafish α-2A, α-2DA and α-2DB receptors is similar to the mammalian α-2A adrenerergic receptors (Ruuskanen *et al*., 2005 b).

Zebrafish (*Danio rerio*), a teleost fish, is widely accepted as experimental model organism in developmental biology, genetics and behavioral studies (Peitsaro *et al*., 2003; Anichtchik *et al*., 2004). Thus structural, functional and pharmacological correlation of Zebrafish and Human adrenoceptors would be useful in validating Zebrafish as a model for *in vivo* research on α-2 adrenergic system. Earlier studies have reported models of Human and Zebrafish α-2 adrenoreceptors based on then available crystal structure of Bovine Rhodopsin (PDB ID:1HZX) (Xhaard *et al*., 2005). Rhodopsin is unusual in that it is highly abundant from natural sources and is structurally stabilized by the covalently bound ligand 11-cis-retinal, which maintains the receptor in a dark-adapted, non-signaling conformation (Palczewski *et al*., 2000; Kobilka and Schertler, 2008). G-protein-coupled receptors (GPCRs) constitute a large family of structurally similar proteins that respond to a chemically diverse array of physiological and environmental stimulants. Until recently, high-resolution structural

information was limited to rhodopsin, a naturally abundant GPCR that is highly specialized for the detection of light. Non-rhodopsin GPCRs for diffusible hormones and neurotransmitters have proven more resistant to crystallography approaches, possibly because of their inherent structural flexibility and the need for recombinant expression. Recently, crystal structures of the human β2 adrenoceptor have been obtained using two different approaches to stabilize receptor protein and increase polar surface area. These structures, together with the existing structures of rhodopsin, represent an important first step in understanding how GPCRs work at a molecular level. Much more high-resolution information is needed for this important family of membrane proteins, however: for example, the structures of GPCRs from different families, structures bound to ligands having different efficacies, and structures of GPCRs in complex with G proteins and other signaling molecules. Methods to characterize the dynamic aspects of the GPCR architecture at high resolution will also be important (Kobilka and Schertler, 2008).

The lack of stability and higher-order symmetry of membrane proteins in the native state effectively hindered the development of diffraction methods for other GPCRs for a long time. In the past few years, very significant progress in technology has made crystallization and high resolution X-ray diffraction analysis possible for some other GPCRs, and the crystal structures of the Human β2-adrenoceptor (PDB ID: 2RH1), the Turkey β1- adrenoceptor (2Y04), and the Human A2A adenosine (PDB ID: 3EML), Dopamine D3 (PDB ID: 3PBL) and CXCR4 Chemokine (3OE9) receptors have now been characterized (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008; Chien *et al.*, 2010; Wu *et al.*, 2010).

α-2 adrenergic receptors belong to Class A Rhodopsin-like Receptors and their structure would be closer to X-ray structure of Human D3 Dopamine structure than to Rhodopsin, as they both belong to the Amine family of GPCRs and recognize naturally occurring catecholamines like adrenaline, noradrenaline and their precursor dopamine. In the present study we have modeled the three Human and five Zebrafish α-2 adrenoceptor subtypes based on the X-ray structure of Human D3 Receptor in complex with a D2/D3 Selective Antagonist Eticlopride (PDB ID: 3PBL) (Chien *et al.*, 2010). Our study has identified the role of different residues in the transmembrane regions, loop regions and residues lining ligand binding cavity leading to identification of new target residues for mutational analysis, structure based drug design and pharmacophore based virtual screening.

2. Methods

2.1 Homology Modeling

The sequences of Human α-2 adrenergic receptors and Zebrafish α-2 adrenoreceptors, Human Dopamine D3 Receptor and Bovine Rhodopsin were retrieved from Swiss-Prot databank (http://www.uniprot.org/). Homology models of three Human (α-2A (PO8913) , α-2B(P18089), α-2C(P18825) and five Zebrafish (α-2A (Q90WY4), α-2B (Q90WY5), α-2C (Q90WY6), α-2DA (Q8JG70) α-2DB (Q8JG69)) adrenergic receptors were generated based on the X-ray crystal structure of Human Dopamine D3 receptor in complex with a selective antagonist Eticlopride. The coordinates of X-ray crystal structures of Human Dopamine D3 Receptor (3PBL, 2.89A^0) was obtained from Protein Data Bank (http://www.rcsb.org/pdb). The models of α-2 adrenoceptor sub-types in Human and Zebrafish were based on multiple sequence alignment of α-2 adrenoreceptor sequences with Human Dopamine D3 receptor sequence, generated by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin *et al.*, 2007). The construction of structural models was done using MODELLER 9v2 (Sali and Blundell, 1993). In the crystal structure of 3PBL, the N-terminal 31 residues were not included as they do not have interpretable density. So, approximately 30 residues at the N-terminal sequence of adrenergic receptors were not included in the modeling studies. We have used UCSF Chimera software for structure visualization (Pettersen *et al.*, 2004). The position of the residues was designated according to the Ballesteros and Weinstein system (Ballesteros *et al.*, 1995). Procheck (Laskowski *et al*, 1993) was used to assess the stereochemical quality of the structures obtained through MODELLER.

3. Results

The sequences of Human and Zebrafish α-2 adrenergic receptors, Human Dopamine D3 receptor and Bovine Rhodopsin were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Figure S1) (Larkin *et al.*, 2007).

Comparative modeling was used to model structures of α-2 adrenergic receptors of Human and Zebrafish based on X-Ray crystal structure of Human Dopamine D3 receptor (PDB ID: 3PBL) as they share a sequence identity of more than 30%. The sequence identity with other already solved crystal structures was less than 30% and hence our modeling studies were based on Human Dopamine D3 receptor (Table S1). The sequence identity in the TM region was more than 50% with Human Dopamine D3 receptor which was comparatively higher than with Bovine Rhodopsin which is around 25%, which was used as template for modeling till recently (Palczewski

et al., 2000), and β- adrenergic receptor (35%) (Table S2).

In order to verify the structural identity of the Human Dopamine D3 receptor with α-2 adrenergic receptors of Human and Zebrafish, Fold and Profile Assessment was done using FFASO3 server (http://ffas.ljcrf.edu/ffas-cgi/cgi/pair_aln.pl?ses=&rv=&lv=) (Jaroszewski *et al*., 2005) (Table S1).

The observation of sequence and structural similarity of Human and Zebrafish α-2 adrenergic Receptors with Human Dopamine D3 receptor (DRD3_HUMAN), Bovine Rhodopsin (OPSD_BOVIN) and Human β2 adrenergic receptor (ADRB2) led us to choose Human Dopamine D3 Receptor (3PBL) as appropriate template when compared to Bovine Rhodopsin. Hence our modeling studies were done using 3PBL as template. Our conclusion is also based on earlier reports on Rhodopsin, where they have documented rigidity in Rhodopsin structure in comparison to flexible GPCRs (Kobilka and Schertler, 2008). The N-terminal and C-Terminal residues of Human α-2 A/B/C were not included in modeling. The residues between TM5 and TM6 were not analyzed.

3.1 Transmembrane region

The overall topology of all the models of α-2 adrenergic receptors from Human and Zebrafish was conserved as reported earlier (Ruuskanen *et al*., 2005 a). Based on the model comparison certain conserved residues were identified for each TM. In Helix-I, G (1.49), N (1.50), L (1.52), V (1.53), A (1.56), V (1.57) and T (1.59) were conserved among all adrenergic receptors. These residues occupied TM portion of the receptor and are positioned towards cytoplasmic ends of Helix-I. The orientation of Helix-I in all the adrenergic receptors was similar to the template (3PBL) with the major differences restricted to the length of Helix-I, which was primarily due to the difference in the number of residues. A shorter Helix-I was observed in α-2C models of Human and Zebrafish. All other receptors were of approximately equal length with about 20-24 residues. The residues P(2.38), Q(2.39), N(2.40), L(2.41), F(2.42), L(2.43), V(2.44), S(2.45), L(2.46), A(2.47), A(2.49), D(2.50), I(2.51), L(2.52), V(2.53), A(2.54), T(2.55), L(2.56), P(2.59), F(2.60), S(2.61), L(2.62), A(2.63), N(2.64), E(2.65) of Helix-II were conserved in all Human and Zebrafish α-2 adrenergic receptors (FigureS1, Figure S2). The position of the last two residues was shifted to ECL-1 (XL-1) in Zebrafish 2B adrenoceptor. The presence of conserved Proline (2.38) in the beginning of Helix-II may dictate the overall fold of adrenoceptors and the second Proline of Helix –II (2.59) was responsible for the similar kink observed in all the Human and Zebrafish α-2 adrenoceptors, leading to slight bending of Helix-II towards the center of the TM Bundle at the extracellular side of the structures (Figure S3).

The conserved residues in Helix-III were W(3.24), C(3.25), Y(3.28), L(3.29), A(3.30), L(3.31), D(3.32), V(3.33), L(3.34), F(3.35), C(3.36), T(3.37), S(3.39), I(3.40), H(3.42) L(3.43), C(3.44), A(3.45), I(3.46), S(3.47), L(3.48), D(3.49), R(3.50), Y(3.51) (Figure S1, Figure S1). The enclosing space between Helix-II and Helix-III was same in α-2DA and 2DB of Zebra fish and α-2B of Human. In Zebrafish α-2B the enclosing space or the orientation of XL-1 was much more towards periphery exposing more of ligand binding space when compared to Human and Zebrafish α-2A and 2C adrenoceptors. In Helix- IV, P(4.39), R(4.41), K(4.43), I(4.46), V(4.49), W(4.50), I(4.52), A(4.54), V(4.55), I(4.56), S(4.57), P(4.59), P(4.60), L(4.61) residues were conserved. Of these, the residues P, R in Human α-2A, 2B and Zebrafish α-2A were located in ICL-2 region. In α-2C Zebrafish P, P, L residues were located in XL-2 region. Helix-III is inserted diagonally into the membrane with extracellular end close to Helix-II and the cytoplasmic end close to Helix-V. Helix- III and IV were oriented in similar fashion in α-2DA, α-2DB, α-2C of Zebrafish and α-2C of Human, accompanied by a shorter Helix-IV in the above mentioned model structures. Proline was located at the beginning of Helix IV in α-2DA, 2DB, 2B, 2C of Zebrafish and 2C of Human, whereas in α2A and 2B of Human and α-2A of Zebrafish it was present in the loop resulting in an extra turn in the ICL2 region of the receptors. Because of Proline there was a break in the continuity of the helix in α-2DA, 2DB and 2C of Zebrafish and the helix was distorted.

In Helix-V the residues W(5.37), Y(5.38), S(5.42), S(5.46), F(5.47), F(5.48), P(5.50), I(5.53), M(5.54), V(5.57), Y(5.58), I(5.61), Y(5.62), A(5.65), K(5.66) were totally conserved in all the receptors. Of these, W and Y were located in XL-2 in 2D Zebra fish and W is in XL-2 in α-2A of Zebrafish. There seems to be conservation in the orientation of Helix-V between Human and Zebrafish receptors. In Helix-VI the residues E(6.30), K(6.31), R(6.32), F(6.33), T(6.34), F(6.35), V(6.36), L(6.37), A(6.38), V(6.39), V(6.40), G(6.42), V(6.43), F(6.44), V(6.45), C(6.47), W(6.48), F(6.49), P(6.50), F(6.51), F(6.52), F(6.53), Y(6.55) were conserved in all the Human and Zebrafish adrenoceptor models. In Helix VII the residues L(7.34), F(7.35), F(7.38), F(7.39), W(7.40), G(7.42), Y(7.43), C(7.44), N(7.45), S(7.46), N(7.49), P(7.50), I(7.52), Y(7.53), T(7.54), F(7.56) were conserved (Figure 1). The difference in the number of Prolines did not result in significant difference in overall shape of Helix-VI and VII of α-2 adrenoceptors. In Helix- VIII comparatively lesser residues were conserved in all the adrenoceptor subtypes of Human and Zebrafish. The residues conserved were D, F, R and F in Helix VIII. Helix VIII was aligned parallel to the membrane.

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3.2 Extracellular region

Regions in the extracellular domain of Rhodopsin (NH2-terminal and interhelical loops E-I, E-II, and E-III) associate to form a compact structure. The ligand binding site of the receptor is defined by the ECLs and N termini of GPCRs and the extracellular regions of the transmembrane helices (Angers *et al*., 2000). Thus, the ECLs could contribute significantly in the pharmacology of the receptors. Studies suggest that small-molecule ligands may bind deep within the space created by the transmembrane domain helices, whereas larger ligands (e.g.: peptides) bind close to the membrane surface near the ECLs (Ji *et al*., 1998; Gether, 2000). Mutagenesis studies suggest that the β2 adrenergic receptor binds its ligand deep within the transmembrane helix bundle, which may be related to the observation that the extracellular regions have a rather simple structure with short loops connecting transmembrane helices II and III with helices VI and VII (Cherezov *et al*., 2007). In contrast to the buried β-sheet structure of this loop in Rhodopsin, ECL2 in β2 Adrenergic receptor is much more exposed to the solvent and contains an extra helical segment (Cherezov *et al*., 2007), whereas the extracellular loop of the Human Dopamine D3 receptor is shorter and lacks beta sheet like Rhodopsin and helical structure like β2 adrenergic receptor.. On comparing the Human adrenoceptors models with each other, we observed that the ECL2 loop in Human 2A adrenoceptor was kinked due to the presence of three Prolines (186, 181, 183). In comparison, Human 2B adrenoceptor (162, 156, 158) was more kinked, which may be due lesser number of residues in ECL-2 loop of this receptor (Table S7). In Human α-2C ECL-2 loop has only two Proline residues at positions 194 and 200, resulting in a distorted and much folded architecture. Zebrafish α-2 adrenoceptors, on the other hand, are not Proline rich at extracellular sites with 2DA, 2DB and 2A with no Prolines and one Proline at position 193 and 184 in ECL2 loop of 2B and 2C respectively. The lack of interactions between the N-terminus and ECL2 in structural models of α-2 adrenergic receptors further enables diffusible ligand access to the binding site (Cherezov *et al*., 2007). However, a completely disordered N-terminus may be an artifact induced by the presence of the N-terminal Flag tag, which carries an overall positive charge and may disrupt N-terminal interactions. The presence of Proline may increase the strength of loop in a rigid conformation that may help to stabilize the core of the receptor and lock ECL2 in a conformation that does not hinder access to the binding pocket (Table S3).

The conserved residue in ECL1 region of Human and Zebrafish α-2 adrenoceptors was W, in ECL2 the residues were N (xl2.53), C (xl2.50) and in ECL3 the residues were C and P (Figure S1). We observed that the Tryptophan residue in ECL1 of Human and Zebrafish α-2 adrenoceptors was pulled towards the helix allowing more space for ligand entry.

3.3 Intracellular Loops

The residues constituting ICL2 Loop, between TM3 and TM4, showed variation, in terms of length, between 3PBL (13 residues) and 1HZX (9 residues), which may have important implications in recognition of G-proteins. Our models of Human and Zebrafish showed similar length of ICL2 loops comparable to 3PBL with around 12-15 residues. The conserved residues in Human and Zebrafish α-2 adrenoceptors in ICL1 were S, R, L, residues A, Y, K, R, T in ICL2 and N in ICL4. In the receptors studied, the residues Q, A were conserved in Human and Zebrafish α-2A and Human and Zebrafish α-2C. It was replaced by R, A in α-2B and K, A in α-2DA and 2DB of Zebrafish. The sequence motif YNLKRTPR/Q was conserved in Zebrafish 2DA, 2DB, 2C and Human 2A and 2C. In α-2A Zebrafish the last residue R was replaced by Q. The conformation of ICL2 loop in α-2A Human and Zebrafish revealed similar orientation towards the cytoplasmic region. α-2C adrenoreceptors of Human and Zebrafish showed different conformation even though most of the residues were conserved when compared to Human and Zebrafish α-2A receptors, which may influence their interaction with the G-proteins. The orientation of ICL2 in α-2B Zebrafish was extended, with a small turn, when compared to much more helical loop in α-2C Zebrafish. The structural significance of this has to be established.

3.4 Disulfide bridges

The Cysteine residues which are involved in the formation of disulphide bridge (C(xl2.50), C(3.25)) were conserved among Human D3 Dopamine receptor, Bovine Rhodopsin and all adrenergic receptors of Human and Zebrafish respectively. The other disulphide bridge was observed between Cysteines in ECL3 region only in Human α-2B, 2C and Zebrafish α-2B, 2C, 2DA and 2DB adrenergic receptors. In Human α-2A one of the Cysteine residues, in ECL3, was replaced by Glycine and hence disulphide bridge was not established. We relate substitution of Cysteine by Glycine in Human α-2A receptor a part of creating specificity to receptor, thus giving more space for the entry of ligands. In Zebrafish α-2A even though Cysteines are present in the ECL3 region, disulphide bride was not observed. As a result Helix VI and VII were held rigidly in place enclosing more space in the extracellular region between TM helices. The lack of disulphide bridge at ECL3 could help distinguish

subtype α-2A receptors in Human and Zebrafish from other subtypes namely α-2B, 2C of Human and α-2B, 2C, 2DA and 2DB of Zebrafish. The functional correlation of this structural feature needs further evaluation. There was no disulphide bridge observed at the same position in Bovine Rhodopsin. The residues corresponding to Cysteine were replaced by Threonine and Serine in Rhodopsin (Table S4).

3.5 Ligand binding cavity

The residues predicted to line the binding cavity of Human and Zebrafish α-2 adrenergic receptors were nearly identical, reflecting their close evolutionary relationship. Of seventeen ligand binding residues predicted (Table 1), occupying various positions in transmembrane region, six residues were different. V (2.61) is replaced by Serine. S (5.43) was replaced by Cysteine in Human α-2A, 2C, Zebrafish α-2A, 2DA, 2DB and by Threonine in α-2B Zebrafish. H (6.55) was replaced by Tyrosine. V (6.56) was replaced by Threonine in α-2A Human and Zebrafish. Whereas it was replaced by Serine in α-2B, 2C of Human and α-2B, 2C, 2DA, 2DB of Zebrafish. Y (7.35) and T (7.39) were replaced by Phenylalanine. Our structural models based on 3PBL have led to the identification of two residues, S/T (6.56), F (7.35) in α- 2 adrenergic receptors of Human and Zebrafish respectively. These residues are located on the cavity lining the binding surface and may play an important role in drug design (Table 1, Figure 1).

3.6 Comparison of orientation of predicted ligand binding residues

Based on the predicted ligand binding residues in the models with Bovine Rhodopsin as template (Xhaard *et al*., 2005) and our models using Human Dopamine D3 receptor structure as template, we have done a comparative study on the orientation of these key residues in transmembrane helices and at the same time observed the relative positions of these residues in our models (Figure 1) *Vs.* schematic representation given by previous studies (Xhaard *et al*., 2005). Crystal structures of Bovine Rhodopsin and Human D3 receptor have not identified any common conserved residues in TM1 and TM2 as important for ligand binding. The residues lining the ligand binding cavity were mostly present in TM3, TM5, TM6 and TM7. In TM3 the first common residue identified was Y (3.28). We believe that substitution of Phenylalanine in Dopamine D3 receptor in place of E 3.28 in 1HZX was part of evolutionary process leading to stability of the protein structure. Further, replacement of Phenylalanine with tyrosine appears to be an evolutionary phenomenon in stabilizing structures with retention of functional groups. The second residue, D110 (3.32) was already predicted to establish a double salt bridge from the amide nitrogen as well as the promoted nitrogen to D (3.32) in the crystal structure of Dopamine D3 receptor (Chien *et al*., 2010). Our structural models predicted the position of D (3.32) in a corresponding position relative to that of 3PBL and its position was completely buried as opposed to partially buried status depicted in the schematic representation by previous studies based on 1HZX as template (Xhaard *et al*., 2005). The third residue V(3.33) was buried, facing ligand binding cavity in our structural models of Human and Zebrafish in contrast to partially buried position given in the analysis of models based on 1HZX (Xhaard *et al*., 2005). The positional difference of this residue has to be taken care of while designing antagonist for these receptors.

The Cysteine at position 3.36 was conserved in the sequence of Human and Zebrafish adrenergic receptors. In agreement to the earlier studies it faced towards ligand binding cavity. Further the role of Cysteine in structure and function has to be proved by *in vivo* studies as it is conserved in all α-2 adrenergic receptors of Human and Zebrafish. Our structural models were in agreement with earlier predictions of conserved hydrophobic region around the beginning of TM5. In TM5, the residues predicted to line the binding cavity were located at positions 5.39, 5.42, 5.43 and 5.46. The first residue, Valine (5.39), was exposed in our models, in agreement with previous studies using models based on 1HZX (Xhaard *et al*., 2005). The second residue, occupying position 5.42, was Serine in our models and was observed to be partially buried in contrast to earlier studies using models based on 1HZX, where it was reported to be fully exposed. The next residue, Cysteine/Serine (5.43) was partially buried, in agreement with previous models based on 1HZX (Xhaard *et al*., 2005). The fourth residue, Serine (5.46) was fully buried in our models in contrast to earlier models based on 1HZX, where it was reported to be fully exposed (Xhaard *et al*., 2005).

In TM6, the first residue identified, Tryptophan (6.48) was observed to be conserved between Human and Zebrafish α-2 adrenergic receptors, Human Dopamine D3 receptor and Bovine Rhodopsin. It was partially buried in our models, in agreement with the earlier models based on 1HZX (Xhaard *et al*., 2005). The second residue, Phenylalanine (6.51), was completely buried in our models in contrast to earlier models based on 1HZX where it was reported to be partially buried (Xhaard *et al*., 2005). This residue was observed to be positioned towards the ligand binding cavity. We propose that this Phenylalanine will make favourable contacts with the ligands and the position of this residue should be considered in designing new ligands specific to adrenergic receptors. The next residue Phenylalanine (6.52) was observed to be fully buried in our models, facing the ligand binding cavity, in contrast to earlier studies on models based on 1HZX where it was reported to be partially

buried (Xhaard *et al*., 2005). The fourth residue, Tyrosine (6.55), was fully buried in our models, in contrast to earlier models based on 1HZX where it was reported to be fully exposed (Xhaard *et al*., 2005). The orientation of the residue is towards the ligand binding cavity. The position 6.55 occupied by Alanine in Rhodopsin and Histidine in 3PBL was substituted by Tyrosine in the models. The ligand binding cavity appeared to be partially covered by Tyrosine at 3.28 and 6.55 positions in the models of Human and Zebrafish adrenergic receptors and may play a key role in the entry of ligand to its cavity. The replacement of Phenylalanine at 3.28 and Histdine at 6.55 with Tyrosine could play a major role in the designing of ligands specific to the binding pocket of adrenergic receptors and may play a key role in the regulation of receptors. The ligand binding residues were predominantly aromatic in nature with Tyrosine in 6.55 and Phenylalanine at 6.51 and 6.52 and Tryptophan at 6.48 and there seems to be positional conservation relating to functional property of these residues in Human and Zebrafish α-2 adrenergic receptors and Human Dopamine D3 receptor. Whereas in Rhodopsin structure, of the four amino acids, only at positions 6.48 and 6.51 aromatic residues were observed, with the other two aromatic amino acids at positional 6.52, 6.55 replaced by aliphatic residues namely Alanine. Trytophan (6.48) seems to be highly conserved in TM6 and appears to play a key role in the extent of penetration of ligand into the binding cavity. Thus the length of binding cavity within the receptor appears to be more or less conserved with major differences concentrated around the beginning of ligand binding cavity.

Ligand binding residues in Helix VII, based on the structural models, at positions 7.39 and 7.43 are Phenylalanine and Tyrosine. The orientation of Phenylalanine is parallel to proposed ligand binding cavity below which Tyrosine penetrates deep into the binding cavity. These aromatic amino acids and their possible interactions should be considered while designing inhibitors. In the template, 3PBL, the corresponding residues are Threonine and Tyrosine. Furthermore it appears that the drugs specific to adrenergic receptors can be modeled considering the space enclosed due to presence of Threonine and Tyrosine in Dopamine D3 receptor compared to that of adrenergic receptors. This difference can be exploited in designing drugs specific to Dopamine and Adrenergic receptors.

3.7 Ionic lock

Ionic lock is thought to play an important role in receptor activation in many class A GPCRs. The ionic lock is constituted by a salt bridge between the charged Arg (3.50) in the conserved "D[E]RY" motif and Asp/Glu (6.30) and is at the cytoplasmic side of helices III and VI. This interaction was observed in the inactive Rhodopsin crystal structure (Palczewski *et al*., 2000; Okada *et al*., 2004) and has been implicated through mutagenesis as a major factor in stabilizing the receptors in the inactive conformation (Ballesteros *et al*., 2001 a; Vogel *et al*., 2008). In the Human Dopamine D3 receptor structure the salt bridge is formed between R128 (3.50) and E324 (6.30). In addition, the side chain of Y138 in the ICL2 helix of the D3R is inserted into the seven-TM bundle forming hydrogen bonds with T64 (2.39), R128 (3.50), and D127 (3.49) (distances of 3.0, 3.2, and 3.2 Å, respectively), potentially stabilizing the ionic lock. In Human α-2A structure, salt bridge between R 131 (3.50) and E 369 (6.30) was observed, whereas Y 141 in ICL2 α Helix formed Hydrogen bonds with only Asp 130 (3.49). The remaining Hydrogen bonds seen in 3PBL were absent as the residue corresponding to position 64 in 3PBL was replaced by E (2.39). The salt bridges were located at positions E369 (6.30) - R131 (3.50), E366 (6.30) - R110 (3.50), E377 (6.30) - R149 (3.50) in Human α-2A, 2B, 2C and at residue positions E306 (6.30) - R120 (3.50), E426 (6.30) - R139 (3.50), E333 (6.30)-R131 (3.50) in Zebrafish α-2A, 2B and 2DB respectively. Zebrafish α-2C receptor salt bridges were absent, whereas in α-2DA there were two salt bridges at E326 (6.30)-R128 (3.50), D127 (3.49)-R128 (3.50) (Figure S4).

3.8 Comparison of the structural models of the Human and Zebrafish α-2 adrenoceptors

In the structural models of the receptor proteins, the amino acids that were predicted to constitute the membrane-embedded ligand-accessible surface of the receptors are located in TM2-TM7 and in XL2, and ICL2 and most of them were found to be highly conserved. Only three residues (at positions 5.39, 5.43 and 6.56) of 17 ligand accessible amino acids were found to differ within the TM domains in the structural models. Of these, the role of Serine/Cysteine variation at position 5.43 in the α-2 adrenoceptors has been studied extensively with regard to catecholamine binding (Nyrönen *et al*., 2001; Rudlin *et al*., 1999; Peltonen *et al*., 2003). Other amino acids known to be important for the binding of small catecholic ligands in TM3-TM6, D(3.32), V(3.33), C(3.36), S(5.42), S(5.46), W(6.48), F(6.51), F(6.52) and Y(6.55), F(7.35), F(7.39), F(7.43) are conserved among all of the Human and Zebrafish α-2 adrenoceptor subtypes. Apart from these similarities we have identified sequence motifs conserved between Human and Zebrafish shown as boxes (Figure S1). Table S6 shows the Transmembrane identities of Zebrafish α-2 adrenoceptors with Human α2-Adrenoceptors. The sequence motif YNLKRTPR/Q was conserved in Zebrafish 2DA, 2DB, 2C and Human 2A and 2C. In α-2A Zebrafish the last residue R was replaced by Q. The conformation of ICL2 loop in α-2A Human and Zebrafish revealed similar

orientation towards the cytoplasmic region. We observed that Y of this sequence motif has a critical role in regulating ionic lock conformation which in turn is involved in determining active and inactive state conformations of the receptor. We also observed that the orientation of Y in ICL2 in Zebrafish α-2C was parallel to the membrane and hence was not in a position to interact with residues involved in ionic lock formation.

Ramachandran Plot showed that 91.3% of the residues for Zebrafish α-2DA model were in the most favored region. In Zebrafish α-2DB, 91.6% of the residues were in the most favoured region. In Zebrafish α-2A, 90.9% of the residues were located in the most favoured region. In Zebrafish α-2B and 2C 91.4% and 91.9% of the residues were located in the most favoured regions. In Human α-2A, 90.9%, α-2B 91.4%, α-2C 91% of the residues were in the most favoured region.

4. Discussion

Adrenergic receptors are mediators of physiological effects of the neurotransmitters adrenaline and noradrenaline. Earlier work on α-2 adrenergic receptor structural models (Xhaard *et al*., 2005) revealed important residues related to function in the adrenergic receptors of Human and Zebrafish structural models, but their study was based on then available crystal structure of Rhodopsin, as Rhodopsin happens to be the first protein in GPCR clan whose structural details were available (PDB ID: 1HZX (Palczewski *et al*., 2000) and thus was a choice for most of the homology modeling based predictions. Dopamine receptor was closer to query (>40%) (α-2 adrenergic receptors) among available crystal structures of Bovine Rhodopsin, Human Dopamine D3 Receptor and Human β2 Adrenergic receptor (Table S1).

In our analysis of the structural models of all the α-2 adrenergic receptors from Human and Zebrafish we observed that the overall topology was conserved. Comparison of the modeled structures revealed that 123 residues in the TM helices, 5 residues in extracellular loops and 9 residues in the intracellular loop regions were conserved between Human and Zebrafish α-2 adrenergic receptors indicating conservation of function in orthologs and paralogs. The conserved residues in the transmembrane region appear to play important role in the orientation of helices among the subtype of receptors studied. Thus, it indicates that the different amino acids can support similar α helical structure, exhibiting structural mimicry by which a common ancestor could diverge sufficiently to develop selectivity necessary to interact with diverse signals while maintaining a similar overall fold. Through this process, the core function of signaling activation through a conformational change in the TM segments that alters conformation of cytoplasmic surface and subsequent interaction with G-proteins is shared by entire Class A family of receptors, despite their selectivity for diverse group of ligands (Ballesteros *et al*., 2001 b). The orientation of the transmembrane helices was same except for the differences related to the corresponding number of residues or to Prolines which induce local distortion/kinks (Table S3). The presence of conserved Proline (2.38) in the beginning of Helix-II may dictate the overall fold of adrenoceptors and the second Proline of Helix –II (2.59) is responsible for the similar kink observed in all the Human and Zebrafish α-2 adrenoceptors, leading to slight bending of Helix-II towards the center of the TM Bundle at the extracellular side of the structures. Proline is located at the beginning of Helix IV in α2-DA, 2DB, 2B, 2C of Zebrafish and 2C of Human, whereas in α-2A and 2B of Human and α-2A of Zebrafish it is present in the loop resulting in an extra turn in the ICL2 region of the receptors.

The Disulfide linkages, which play important role in dictating strength and conformation of a protein, were observed between C (xl2.50) and C (3.25), which appears to be present in all the GPCR class of receptors (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Jaakola *et al*., 2008; Warne *et al*., 2008; Chien *et al*., 2010; Wu *et al*., 2010). An additional disulphide bridge was observed between two Cysteines in the extracellular loop 3 of all the α-2 adrenergic receptor and Human Dopamine D3 crystal structure except subtype α-2A in Human and Zebrafish (Table S4). The structural significance of this needs further analysis, but it might contribute to constraining the position of ECL3 (Chien *et al*., 2010). Both disulfide bonds at the extracellular side of CXCR4 are critical for ligand binding (Zhou and Tai, 2000).

Our models have identified the important structural constraint posed by the conserved Tyrosine residue in ICL2 loop which interacts with residues involved in ionic lock formation. Ionic lock conformations in the receptors are thought to contribute to different conformational state of receptor proteins, which in turn might influence basal signaling mechanisms and is thought to play a role in activating the receptor (Chien *et al*., 2010). Of the receptor structures modeled in our study, ionic lock conformations, due to formation of salt bridge between E(6.30) in the sixth helix and R(3.50) in the third helix, were observed in Human α-2A, 2B, 2C receptors and Zebrafish α-2A, 2B, 2DA and 2DB receptor whereas it was absent in 2C receptor of Zebrafish. The interaction between Tyrosine in ICL2 and residues involved in ionic lock formation is observed in Zebrafish α-2DA, α-2DB, α-2A, α-2C and Human α-2A, α-2B, α-2C. In Zebrafish α-2C, the Tyrosine residue (Y143) is oriented away from the TM bundle and is thus not unable to interact with D (3.49), R (3.50) and E (6.30) residues. The functional relevance of this difference in orientation needs further investigation. We have observed

that Y (7.43) penetrates into the ligand binding cavity and may play a critical role in pharmacophore design. We believe that Y (7.43) interacts with the residues F (6.51), W (6.48), F (6.52), F (3.36) (Ji *et al*., 1998; Zhou and Tai, 2000) of ligand binding cavity. Thus, we propose that Y (7.43) along with residue at position at 7.42 (Okada *et al*., 2002) plays an important role in the interaction between TM3 and TM6. We also demonstrate that the residues at position 3.35/6 (McAllister *et al*., 2004) and 7.41 may not be important for regulating toggle switch as they are oriented away from ligand banding cavity. In Zebrafish α-2A F (6.52) is tilted slightly away from W (6.48) and this might affect its interaction with W (6.48) and thus alter toggle switch mechanism. We observed conservation in orientation and position of W6.48 in all the Human and Zebrafish α-2 adrenoceptors along with conservation in residues F (6.51), F (6.52) and F (5.47).

Our models have shown that the Phenylalanine at 7.39 in TM7 will be able to interact with positively charged *N*-methyl group of the catecholamine ligands *via* hydrophobic contacts rather than 7.38 as reported previously. Two Serine residues in TM5 were shown to provide hydrogen bonding sites for the two catecholic hydroxyl groups of the catecholamines with the *meta*-OH probably interacting with the Serine in position 5.42 and the *para*-OH with the Serine in position 5.46 of the Human α-2A adrenoceptor (Peltonen *et al*., 2003; Okada *et al*., 2004; Marjamäki *et al*., 1992; Salminen *et al*., 1999). We have been able to prove earlier findings that residues at 3.37 and 5.43 have no role in receptor activation (Nyrönen *et al*., 2001; Peltonen *et al*., 2003) through our modeling studies as the orientation of these residues is not favorable to interact with any residue involved in the activation of receptor.

Of the seventeen ligand binding residues identified, nine residues are conserved between 3PBL and Human and Zebrafish α-2 adrenoceptors and eight residues substitutions (Table 1) were observed between the template and the Adrenoceptors. Of these eight, five residues in the adrenoceptors possessed almost similar chemical properties as those in 3PBL while the chemical properties of the other three residues, V (2.61)/S, V (6.56)/S/T. T (7.39)/ F, differed. Even though the importance of serine at position 2.61 in TM2, involved in the stereoselectivity of the α-2A-adrenoceptor for the (-)- or *R* enantiomers of catecholamine agonists, and in addition to play a role in the attachment of the α-hydroxyl groups of the catecholamines to α-2A adrenoceptors (Hehr *et al*., 1997; Hieble *et al*., 1998) was reported, there was no direct evidence. We have shown the critical position that this residue occupies through our models. Apart from the residues identified earlier we have identified two other residues S/T (6.56) and F (7.35) which would play key role in ligand binding, which were not identified by previous researcher studies (Xhaard *et al*., 2005). There was swapping of hydrophobic group with hydrophilic residue on one side (V/ T, S (6.56)) and vice versa on other side (Y/F (7.35)) when compared to 3PBL. This difference would help in designing drugs specific to α2-adrenergic receptors. Comparison of orientation of ligand binding residues between our structural models and those predicted based on 1HZX (Xhaard *et al*., 2005) revealed significant differences in the orientation of residues. We also found that in the structural models of Human and Zebrafish α-2 adrenoceptors, modeled based on 3PBL, Y (3.28) and Y (6.55) form a cover over the ligand binding cavity and this covering might influence ligand entry into the cavity. Conservation in ligand binding residues in the TM region between Human and Zebrafish α-2 adrenoceptors might indicate similarities in the mechanism of ligand binding between them and might also contribute to similar ligand binding profile. *In silico* docking studies and *in vivo* experiments would help in validating whether the two species share similarities in ligand binding interactions and mechanisms. The electrostatic nature of residues in the beginning of cavity may play a key role in designing drugs specific to the different classes of GPCRs.

5. Conclusions

α-2 adrenergic receptors are involved in mediating diverse biological effects of the endogenous catecholamines epinephrine and norepinephrine and are target molecules of several clinically important drugs. Structural models using Dopamine as template resulted in the identification of novel residues- Y (ICL2) in ionic lock, Y (7.42) in toggle switch, S (6.56) and F (7.35, 7.39) in ligand binding and the models could correctly show the orientation of serine at 5.42 and 5.46. We believe that the residues at 3.37 and 5.43 may dictate the geometry of ligand interacting with the binding cavity and hence the activity of the receptor. We propose that this will open new lead for ligand design *in silico* and *in vivo* analysis, using Zebrafish as a model organism.

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Table 1. Active site residues in Human and Zebrafish α-2 adrenoceptors predicted based on Human Dopamine D3 Receptor

3PBL_A	ADA2A_HUMAN	ADA2B_HUMAN	ADA2C_HUMAN	ADA2A_DANRE	ADA2B_DANRE	ADA2C_DANRE	AA2DA_DANRE	AA2DB_DANRE
V86 (2.61)	S90 (2.61)	S 69 (2.61)	S 108 (2.61)	S 79 (2.61)	S 98 (2.61)	S 92 (2.61)	S 87 (2.61)	S 90 (2.61)
F 106 (3.28)	Y 109 (3.28)	Y 88 (3.28)	Y 127 (3.28)	Y 98 (3.28)	Y 117 (3.28)	Y111 (3.28)	Y 106 (3.28)	Y 109 (3.28)
D 110 (3.32)	D 113 (3.32)	D 92 (3.32)	D 131 (3.32)	D 102 (3.32)	D 121 (3.32)	D 115 (3.32)	D 110 (3.32)	D 113 (3.32)
V 111 (3.33)	V 114 (3.33)	V 93 (3.33)	V 132 (3.33)	V 103 (3.33)	V 122 (3.33)	V 116 (3.33)	V111 (3.33)	V 114 (3.33)
C 114 (3.36)	C 117 (3.36)	C 96 (3.36)	C 135 (3.36)	C 106 (3.36)	C 125 (3.36)	C 119 (3.36)	C 114 (3.36)	C117 (3.36)
V 189 (5.39)	V 197 (5.39)	I 173 (5.39)	I 211 (5.39)	I 178 (5.39)	I 204 (5.39)	I 195 (5.39)	I 185 (5.39)	I 188 (5.39)
S 192 (5.42)	S 200 (5.42)	S 176 (5.42)	S 214 (5.42)	S 181 (5.42)	S 207 (5.42)	S198 (5.42)	S 188 (5.42)	S 191 (5.42)
S193 (5.43)	C 201 (5.43)	S 177 (5.43)	C 215 (5.43)	C 182 (5.43)	T 208 (5.43)	S 199 (5.43)	C 189 (5.43)	C 192 (5.43)

S 196 (5.46)	S 204 (5.46)	S 180 (5.46)	S 218 (5.46)	S 185 (5.46)	S 211 (5.46)	S 202 (5.46)	S 192 (5.46)	S 195 (5.46)
W 342 (6.48)	W 387 (6.48)	W 384 (6.48)	W 395 (6.48)	W 324 (6.48)	W 444 (6.48)	W 367 (6.48)	W 344 (6.48)	W 351 (6.48)
F 345 (6.51)	F 390 (6.51)	F 387 (6.51)	F 398 (6.51)	F 327 (6.51)	F 447 (6.51)	F 370 (6.51)	F 347 (6.51)	F 354 (6.51)
F 346 (6.52)	F 391 (6.52)	F 388 (6.52)	F 399 (6.52)	F 328 (6.52)	F 448 (6.52)	F 371 (6.52)	F 348 (6.52)	F 355 (6.52)
H 349 (6.55)	Y 394 (6.55)	Y 391 (6.55)	Y 402 (6.55)	Y 331 (6.55)	Y 451 (6.55)	Y 374 (6.55)	Y 351 (6.55)	Y 358 (6.55)
V 350 (6.56)	T 395 (6.56)	S 392 (6.56)	S 403 (6.56)	T 332 (6.56)	S 452 (6.56)	S 375 (6.56)	S 352 (6.56)	S 359 (6.56)
Y 365 (7.35)	F 408 (7.35)	F 408 (7.35)	F 419 (7.35)	F 346 (7.35)	F 468 (7.35)	F 391 (7.35)	F 368 (7.35)	F 375 (7.35)
T 369 (7.39)	F 412 (7.39)	F 412 (7.39)	F 423 (7.39)	F 350 (7.39)	F 472 (7.39)	F 395 (7.39)	F 372 (7.39)	F 379 (7.39)
Y 373 (7.43)	Y 416 (7.43)	Y 416 (7.43)	Y 427 (7.43)	Y 354 (7.43)	Y 476 (7.43)	Y 399 (7.43)	Y 376 (7.43)	Y 383 (7.43)

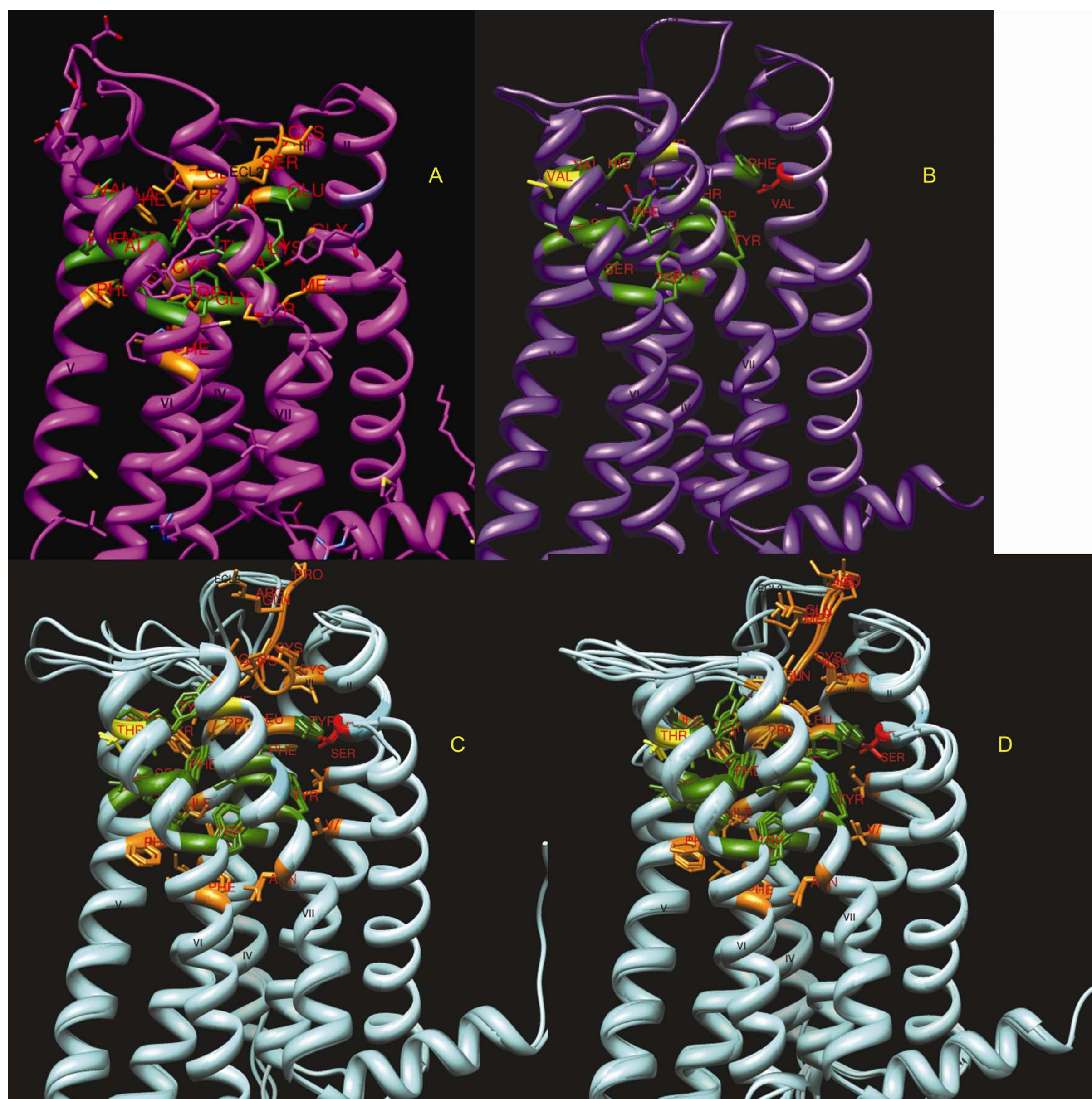

Figure 1. Ligand binding cavity residues. A.IHZX(Bovine Rhodopsin) B. 3PBL (Human Dopamine D3 Receptor) C. Superimposed structures of Human α-2A, 2B, 2C adrenergic receptors D. Superimposed structures of Zebrafish α-2A, 2B, 2C, 2DA, 2DB Adrenergic receptors. Novel residues identified on models with 3PBL as template are coloured in yellow, residues identified with 1HZX as template through earlier studies [8] are coloured in orange, residues common to models with 3PBL and 1HZX as template are coloured in green, Valine (2.61) in 3PBL and Serine (2.61) in Human and Zebrafish α-2 adrenoceptors is coloured in red.

Supplementary Files

Table S1. Sequence and Structural identity of Human and Zebrafish α-2 adrenergic Receptors with Human Dopamine D3 receptor (DRD3_HUMAN), Bovine Rhodopsin (OPSD_BOVIN) and Human β2 adrenergic receptor (ADRB2)

Sequence	Sequence Identity with DRD3_Human	Structure Identity with DRD3_Human	Sequence Identity with OPSD_Bovin	Structure Identity with OPSD_Bovin	Sequence Identity with ADRB2	Structure Identity with ADRB2
ADA2A_HUMAN	34%	28%	17%	13%	27%	22%
ADA2B_HUMAN	32%	27%	13%	12%	23%	21%
ADA2C_HUMAN	34%	28%	13%	13%	24%	22%
ADA2A_DANRE	34%	30%	18.56%	15%	29%	26%
ADA2B_DANRE	26%	22%	11.76%	10%	21%	19%
ADA2C_DANRE	34%	30%	16.90%	13%	27%	22%
AA2DA_DANRE	35%	29%	18.87%	14%	26%	23%
AA2DB_DANRE	36%	28%	16.87%	13%	28%	23%

Table S2. Transmembrane identity of Human and Zebrafish α-2 adrenoceptors with Human Dopamine D3 Receptor and Bovine Rhodopsin

	D3DR_HUMAN	OPSD_BOVIN
ADA2A_HUMAN	49%	26%
ADA2B_HUMAN	49%	27%
ADA2C_HUMAN	49%	19%
ADA2A_DANRE	46%	25%
ADA2B_DANRE	45%	24%
ADA2C_DANRE	49%	20%
AA2DA_DANRE	46%	19%
AA2DB_DANRE	47%	19%
OPSD_BOVIN	29%	

Table S3. Conserved Proline residues in Human and Zebrafish α-2 adrenoceptors

	HUMAN			ZEBRAFISH				
	ADA2A	ADA2B	ADA2C	ADA 2A	ADA2B	ADA2C	AA2DA	AA2DB
TM1	-	-	-	-	-	-	-	-
ICL 1	-	-	-	-	-	-	-	-
TM2	P(2.38), P(2.59)	P(2.38), P(2.59)	P(2.38), P(2.59)	P(2.3 8), P(2.5 9)	P(2.38), P(2.59)	P(2.38), P(2.59)	P(2.38), P(2.59)	P(2.38), P(2.59)
ECL 1	-	-	-	-	-	-	-	-
TM3	-	-	-	-	-	-	-	-
ICL 2	P(4.39)	P(4.39)	-	P(4.3 9)	-	-	-	-
TM4	P(4.59), P(4.60)	P(4.59), P(4.60)	P(4.39), P(4.59), P(4.60)	P(4.5 9), P(4.6 0)	P(4.39),P(4.59), P(4.60)	P(4.39)	P(4.39), P(4.59), P(4.60)	P(4.39), P(4.59), P(4.60)
ECL 2	-	-	-	-	-	P(4.59),P(4.60)	-	-
TM5	P(5.50)	P(5.50)	P(5.50)	P(5.5 0)	P(5.50)	P(5.50)	P(5.50)	P(5.50)
ICL 3								
TM6	P(6.50)	P(6.50)	P(6.50)	P(6.5 0)	P(6.50)	P(6.50)	P(6.50)	P(6.50)
ECL 3	P	P	P	P	P	P	P	P
TM7	P(7.50)	P(7.50)	P(7.50)	P(7.5 0)	P(7.50)	P(7.50)	P(7.50)	P(7.50)
ICL 4	-	-	-	-	-	-	-	-
TM8	-	-	-	-	-	-	-	-

Table S4. Disulphide bridges in Human and Zebrafish α-2 adrenoceptors, Human Dopamine D3 Receptor and Bovine Rhodopsin

	ADA2A_ HUMAN	ADA2B_ HUMAN	ADA2C_ HUMAN	ADA2A_ DANRE	ADA2B_ DANRE	ADA2C_ DANRE	AA2DA_ DANRE	AA2DB_ DANRE	3PBL	1HZ X
CYS	106	85	124	95	114	108	103	106	103	110
CYS	188	164	202	169	195	186	176	179	181	185
CYS		397	408		457	380	357	364	355	
CYS		401	412		461	384	361	368	358	

Table S5. Conserved signatures between Human and Zebrafish α-2 adrenoceptors, Human Dopamine D3 Receptor (3PBL) and Bovine Rhodopsin (1HZX)

	ADA2A_HUMAN	ADA2B_HUMAN	ADA2C_HUMAN	ADA2A_DANRE	ADA2B_DANRE	ADA2C_DANRE	AA2DA_DANRE	AA2DB_DANRE	3PBL	1HZX
V	73	52	91	62	81	75	70	73	69	
S	74	53	92	63	82	76	71	74	70	
L	75	54	93	64	83	77	72	75	71	
A	76	55	94	65	84	78	73	76	72	
L	81	60	99	70	89	83	78	81	77	
V	82	61	100	71	90	84	79	82	78	
A	83	62	101	72	91	85	80	83	79	
T	84	63	102	73	92	86	81	84	80	
L	85	64	103	74	93	87	82	85	81	
L	124	103	142	113	132	126	121	124	121	
C	125	104	143	114	133	127	122	125	122	
A	126	105	144	115	134	128	123	126	123	
I	127	106	145	116	135	129	124	127	124	
S	128	107	146	117	136	130	125	128	125	
P	167	146	185	156	175	169	164	167		170
P	168	147	186	157	176	170	165	168		171
L	169	148	187	158	177	171	166	169		172
I	219	195	233	200	226	217	207	210	211	
Y	220	196	234	201	227	218	208	211	212	
R	368	365	376	305	425	348	325	332	323	
E	369	366	377	306	426	349	326	333	324	
K	370	367	378	307	427	350	327	334	325	
I	425	425	436	363	485	408	385	392	382	
Y	426	426	437	364	486	409	386	393	383	
T	427	427	438	365	487	410	387	394	384	

Table S6. Transmembrane identity of Zebrafish α-2 adrenoceptors with Human α-2 Adrenoceptors

	ADA2A_HUMAN	ADA2B_HUMAN	ADA2C_HUMAN
ADA2A_DANRE	73%		
ADA2B_DANRE		79%	
ADA2C_DANRE			89%
AA2DA_DANRE	72%		
AA2DB_DANRE	76%		

Table S7. TM and Loop Boundaries in Human and Zebrafish α- 2 adrenoceptors, Human Dopamine D3 Receptor (3PBL), Bovine Rhodopsin (1HZX) with total number of residues in brackets

	HUMAN			ZEBRAFISH						
	ADA2A	ADA2B	ADA2C	ADA2A	ADA2B	ADA2C	AA2DA	AA2DB	3PBL_A	1HZX_A
Helix -I	L39-T60 (22)	A16-T39 (24)	I62-T78 (17)	S26-T49 (24)	L38-T60 (24)	I46-T62 (17)	I41-T57 (17)	L38-T60 (23)	S35-K56 (22)	A44-Q64 (21)
ICL1	S61-A66 (6)	S40-A45 (6)	S79-A84 (6)	S50-A55 (6)	S69-G74 (6)	S63-P68 (6)	S58-A63 (6)	S61-A66 (6)	E57-T62 (6)	H65-R69 (5)
Helix-II	P67-V94 (28)	P46-L74 (29)	P85-M114 (30)	P56-L84 (29)	P75-A100 (26)	P69-L97 (29)	P64-I92 (28)	P67-V95 (29)	T63-V91 (29)	E70-G101 (32)
ECL1	M96-G102 (7)	L75-R81 (7)	A115-G120 (6)	M85-G91 (7)	N101-R110 (10)	M98-G104 (7)	M93-G99 (7)	N96-G102 (7)	T92-S99 (8)	Y102-V104 (3)
Helix-III	K103-T136 (34)	R82-S115 (34)	Q121-T154 (34)	G92-T125 (34)	S111-I143 (33)	E105-T138 (34)	S100-T133 (34)	S103-T136 (34)	R100-V133 (34)	F105-K141 (37)
ICL2	Q137-I150 (14)	R116-I129 (14)	Q155-T164 (10)	Q126-I139 (14)	S144-R153 (10)	Q139-T148 (10)	K134-T143 (10)	K137-T146 (10)	M134-S146 (13)	P142-F148 (7)
Helix-IV	K151-L169 (19)	K130-L148 (19)	P165-L190 (21)	K140-M161 (17)	T154-M180 (21)	P149-S167 (21)	P144-I167 (17)	P147-L169 (14)	C147-F172 (23)	G149-V173 (25)
ECL2	I170-Q193 (24)	I149-E169 (21)	V191-D206 (16)	K162-W176 (15)	N181-Y206 (26)	F168-D190 (23)	M168-D180 (13)	L170-N183 (14)	N173-N185 (13)	G174-P194 (21)
Helix-V	K194-R225 (32)	A170-K200 (31)	E207-K238 (32)	Y177-K205 (29)	S207-K231 (25)	H191-K222 (32)	E181-K212 (32)	E184-K215 (32)	P186-K216 (31)	H195-L226 (32)
ICL3	R226-	R201-	L239-	K206-	Q232-	T223-	Q213-	Q216-	Q217-	V227-T242 (16)
Helix-VI	N367-A398 (32)	T364-A395 (32)	A375-G406 (32)	N304-A335 (32)	N424-A455 (32)	E349-G378 (30)	M324-A355 (32)	E333-A362 (30)	L322-T353 (32)	T243-T277 (35)
ECL3	V399-P404 (6)	I369-P404 (9)	I407-P415 (9)	F336-P342 (7)	V456-P464 (9)	I379-P387 (9)	V356-P364 (9)	I363-P371 (9)	H354-S361 (8)	H278-G284 (7)
Helix-VII	R405-F429 (25)	H405-F429 (25)	G416-F440 (25)	E343-F367 (25)	E465-F489 (25)	D388-F412 (25)	E365-F389 (25)	D372-F396 (25)	P362-F386 (25)	P285-M309 (25)
ICL4	N430	N430	N441	N368	N490	N413	N390	N397	N387	N310
Helix-VIII	H431-C442 (12)	Q431-C442 (11)	Q442-I451 (10)	N369-C380 (12)	K491-L501 (11)	Q414-I423 (10)	K391-V400 (10)	R398-C409 (12)	I388-L398 (11)	K311-C322 (12)

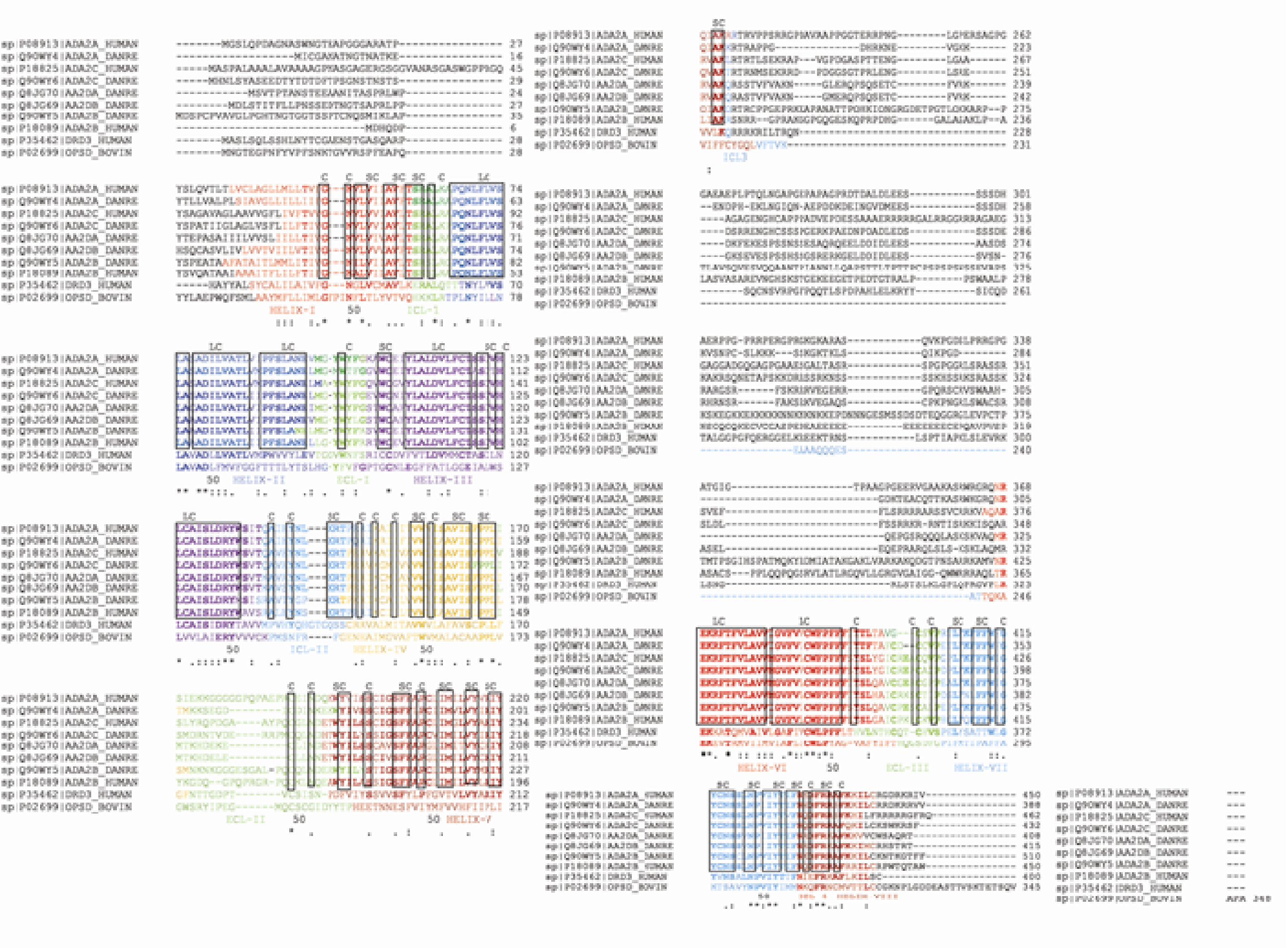

Figure S1. Clustal W alignment of Human and Zebrafish α-2 adrenoceptors, 3PBL and 1HZX.

The conserved residues between Human and Zebrafish α-2 adrenoceptors are enclosed in a box. Conserved residues are labeled as C, Short Conserved as SC, Long Conserved motifs as LC; Helices and Loops are coloured differently, the most conserved residue in each transmembrane helix region is designated the number 50 according to the Ballesteros-Weinstein convention

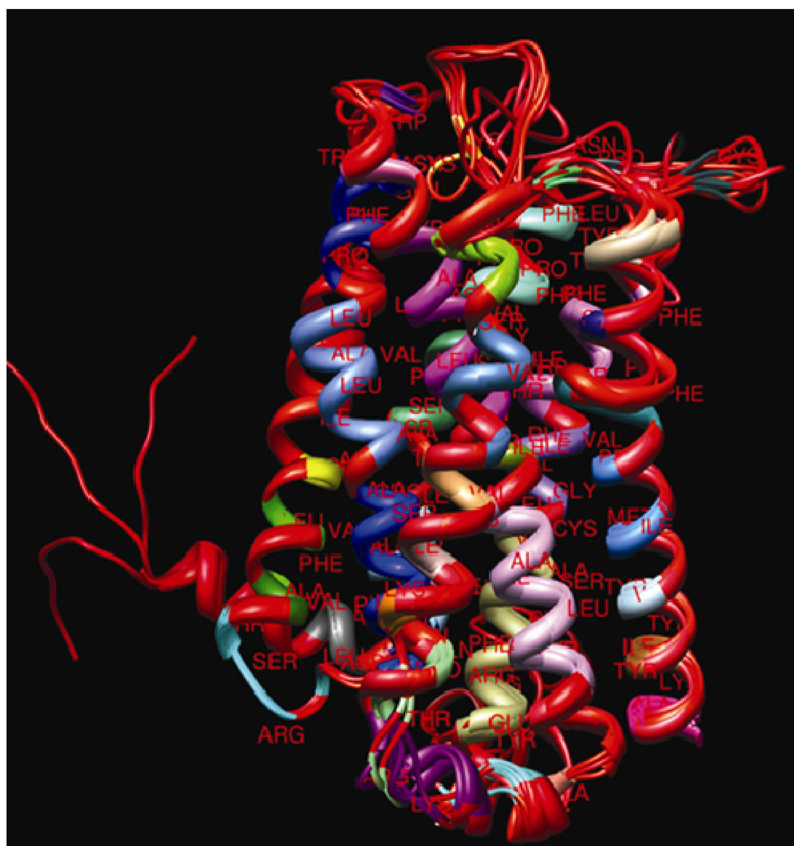

Figure S2. Conserved residues/motifs in the structural models of Human and Zebrafish α-2 adrenoceptors based on 3PBL (residue details available in Figure S1)

Colour scheme: Helix-I: GN- Yellow, LV-Green, AV- Forest Green, T-Cyan;
ICL1: SR-Cyan, L-Light Sea Green;
Helix –II: PQN- Blue, LFLVS-Blue, LA-Blue, ADI- Cornflower Blue, LVATL-Cornflower Blue, PFS-Medium Blue, LANE-Medium Blue;
ECL1: W- Purple;
Helix- III: WC- Hot Pink, YLALDVLFCT- Magenta, SI-Olive Drab, H, LCAIS, LDRY-Plum;
ICL2: A-Salmon, Y- Turquoise, KRT-Dark Magenta;
Helix- IV: P, R-Light Green, K- Orange, I-Rosy Brown, VW- Sandy Brown, I-Steel Blue, AVIS-Steel Blue, PPL-Chartreuse;
ECL2: C- Goldenrod, N-Spring Green;
Helix V: WY- Tan, S-Dark Slate Blue, SFF- Dark Cyan, P- Dodger Blue, IM- Dodger Blue, VY- Light Blue, IY-Sienna, AK- Deep Pink;
Helix VI: EKRFTFV, LAVV- Dark Khaki, GVFV- Medium Purple, CWFPFF, Y- Orchid;
ECL3: C, P- Dark Slate Grey;
Helix VII: LF, FFW- Aquamarine, GYCNS-Sea Green, NP- Firebrick, IYT, F-Sky Blue;
ICL4: N- Navy Blue; Helix VIII: DFR, F-Dim Gray

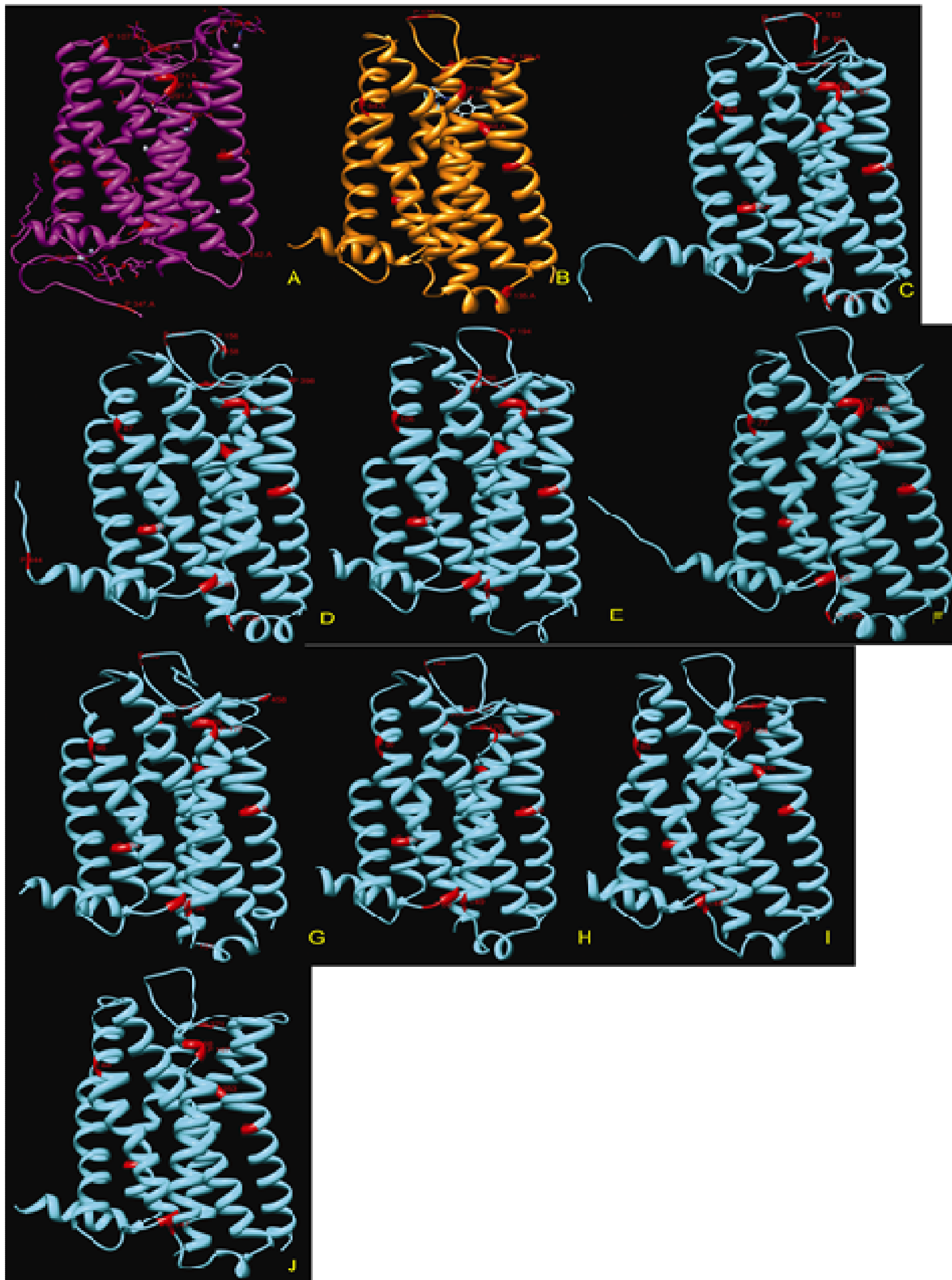

Figure S3. Conserved Proline residues (2.38, 2.59, 4.39, 4.59, 4.60, 5.50, 6.50, 7.50) in the transmembrane and loop regions. (A) 1HZX (B) 3PBL (C) ADA2A_Human (D) ADA2B_Human (E) ADA2C_Human and (F)ADA2A_Danre (G) ADA2B_Danre (H) ADA2C_Danre (I) AA2DA_Danre (J) AA2DB_Danre.

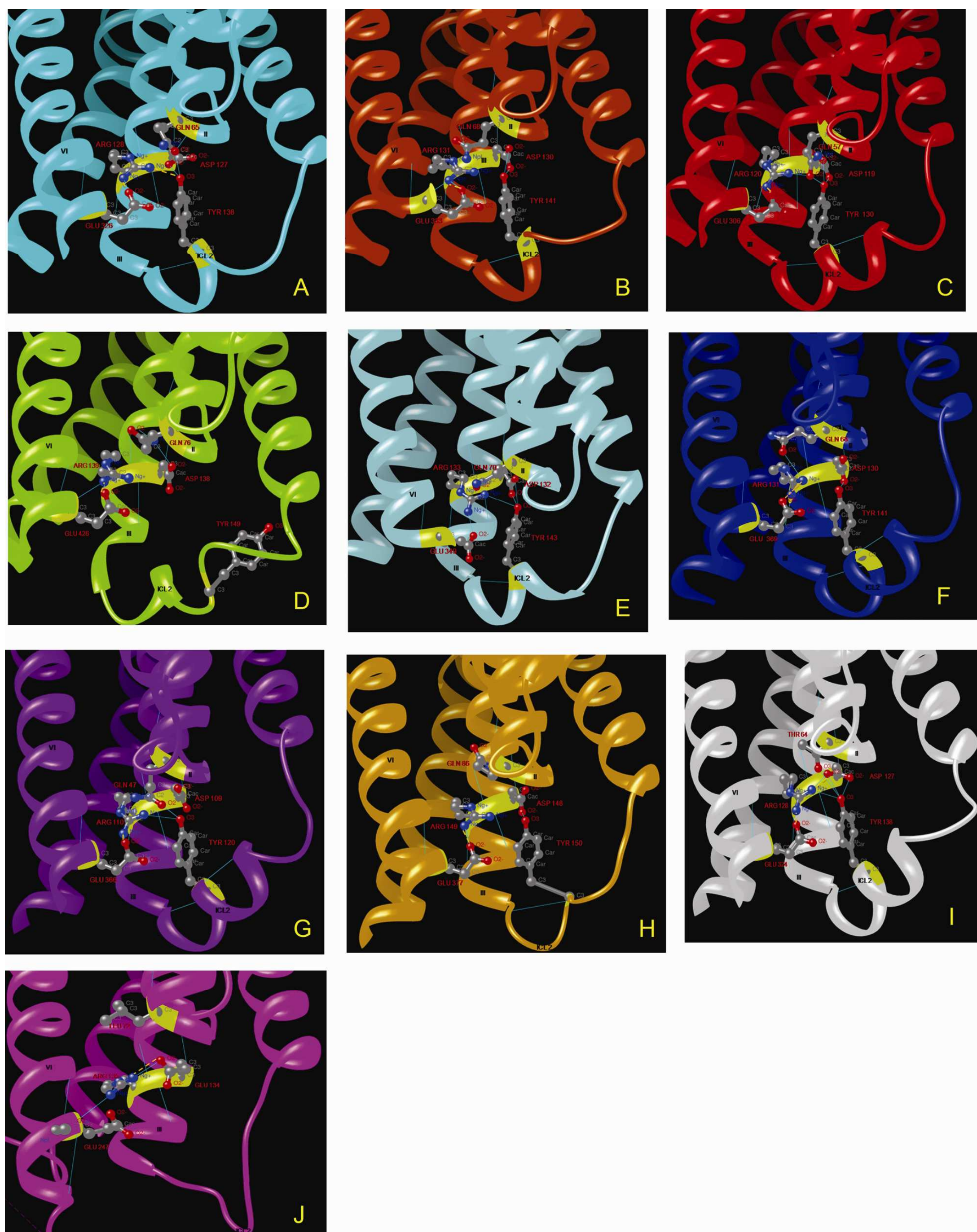

Figure S4. Ionic lock conformation. (A) AA2DA, (B) AA2DB, (C) ADA2A, (D) ADA2B, (E) ADA2C Zebrafish, (F) ADA2A, (G) ADA2B, (H) ADA2C Human, (I) 3PBL, (J) 1HZX. Ionic lock conformation, in the form of salt bridge (represented by orange dashed line) between R(3.50) and E(6.30) and polar interactions in the form of Hydrogen bonds (represented by blue line) between D(3.49), R(3.50), Y(ICL2).