Molecular Modeling of Structures and Interaction of Human Corticotropin-Releasing Factor (CRF) Binding Protein and CRF Type-2 Receptor

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The corticotropin-releasing factor (CRF) system is a key mediator of the stress response and addictive behavior. The CRF system includes four peptides: The CRF system includes four peptides: CRF, urocortins I–III, CRF binding protein (CRF-BP) that binds CRF with high affinity, and two class B G-protein coupled receptors CRF_R and CRF_R. CRF-BP is a secreted protein without significant sequence homology to CRF receptors or to any other known class of protein. Recently, it has been described a potentiation role of CRF-BP over CRF signaling through CRF_R in addictive-related neuronal plasticity and behavior. In addition, it has been described that CRF-BP is capable to physically interact specifically with the α isoform of CRF_R and acts like an escort protein increasing the amount of the receptor in the plasma membrane. At present, there are no available structures for CRF-BP or for full-length CRFR. Knowing and studying the structure of these proteins could be beneficial in order to characterize the CRF-BP/CRF_αR interaction. In this work, we report the modeling of CRF-BP and of full-length CRF_αR and CRF_βR based on the recently solved crystal structures of the transmembrane domains of the human glucagon receptor and human CRF_R, in addition with the resolved N-terminal extracellular domain of CRFRs. These models were further studied using molecular dynamics simulations and protein–protein docking. The results predicted a higher possibility of interaction of CRF-BP with CRF_2αR than CRF_2βR and yielded the possible residues conforming the interacting interface. Thus, the present study provides a framework for further investigation of the CRF-BP/CRF_2αR interaction.

Keywords: corticotropin-releasing factor, corticotropin-releasing factor binding protein, corticotropin-releasing factor receptor, class B G-protein coupled receptor, molecular modeling, molecular dynamics, protein-protein docking

INTRODUCTION

Corticotropin-releasing factor (CRF) system plays pivotal roles in the regulation of physiological responses and adaptation to stress (1, 2), and in the interaction between stress and addictive behavior (3). CRF activates the hypothalamic–pituitary–adrenal axis (4, 5) and also acts as neurotransmitter in different brain regions (2, 6).

The CRF peptides comprised CRF and urocortins I–III (UCNI–III), mediate their actions through the activation of two G-protein coupled receptors (GPCRs) CRF type-1 (CRF_R) and CRF type-2
physically interacts with CRF2 in a variety of VTA nerve terminals, including projections from the
they share a high sequence homology (70%) differing preferen-
binge drinking by a CRF2R-mediated mechanism (18).
addition, it has been suggested that CRF-BP modulates ethanol
dynamics (MD) simulations and protein–protein docking. ECD and TM regions) and their analysis by means of molecular
interaction and determining the residues
involved are the logical next steps on the study of the escort-like
protein function and the facilitatory action of CRF-BP over CRF2R.
CRF2R belongs to class B1 subfamily of GPCRs. Obtaining the
crystal structures of full-length class B GPCRs remains dif-
ficult because of technical issues regarding receptor production,
purification, and stability (21, 22). Structures of the N-terminal
extracellular domain (ECD) of various class B GPCRs have been
determined by X-ray-crystallography and NMR (21) including
CRF4R (23) and CRF2R (24, 25). The structure of the trans-
membrane domain (TM) of the human glucagon receptor (26)
and CRF2R (27) have been reported, and more recently, the first
structure of a full-length glucagon receptor in complex with an
antibody and in its inactive conformation have been determined
using X-ray-crystallography (28). On the other hand, there are no
crystal structure or structural models reported for CRF-BP.
The present study aimed to search for the prediction of the residues
involved in the CRF-BP/CRF2R interaction and the characterization
of this interaction. Herein, we report the generation of comparative
models of CRF-BP, CRF2R, and CRF2R (including the
ECD and TM regions) and their analysis by means of molecular
dynamics (MD) simulations and protein–protein docking.

MATERIALS AND METHODS
Molecular Modeling of Human CRF,
CRF-BP, CRF2R, and CRF2R
The molecular models of CRF-BP, CRF2R, and CRF2R were
constructed using MODELER (29, 30), as implemented in the
Protein Modeling module of Discovery Studio v2.1 (Accelrys
Inc., San Diego, CA, USA). Human CRF-BP, CRF2R, and CRF2R
reference sequences were retrieved from the Uniprot database,
with accession numbers P24387, Q13324-1, and Q13324-2,
respectively (31). CRF was modeled using the crystal structure
of human CRF inactive analog (PDB: 1GO9) containing a D-Phe
residue at position 12 and alpha-aminoisobutyric acid in position
15 (32).

For CRF-BP, top scoring models produced by threading-based
approaches identified by Muster and Phyre2 servers (33, 34) were
retrieved, aligned, and used as starting templates to generate a
human CRF-BP model. Fragments from gastric intrinsic factor
receptor cubilin (PDB: 3KQ4) (35) and neuropilin (PDB: 2QQL)
(36) were used to construct the model. Secondary structure
elements restraints such as α-helices and β-sheets as predicted
by PSI-SV server (37) were included, as well as experimentally
determined disulfide bridges (38) during modeling (Figure S1A in
 Supplementary Material).

For CRF receptors modeling, we used the crystal structure of
CRF1R (PDB: 4K5Y) (27), the N-terminal ECD of human CRF2R
in complex with UCNI (PDB: 3N96) (23), and murine CRF2R in
complex with Astressin analog peptide (PDB: 2JND) (24) as tem-
plates. In addition, the crystal structure of the transmembrane
bundle of glucagon receptor (PDB: 4L6R) was used as guide
to model the N and C-terminal portions absent from the available
CRF-R crystal structure (26) (Figures S1B,C in Supplementary
Material). For each protein model, a set of 100 models were con-
structed and the best model according to Modeler internal PDF
score was subjected to a molecular minimization protocol using
the CHARMM22 force field available within Discovery Studio
(39, 40). The protocol consisted of 5,000 steps of steepest descent
method, followed by 10,000 steps of conjugate gradient method
to reach a final root-mean-square (RMS) gradient of 0.001 kcal/
mol/Å².

The overall quality of the final models was assessed by
Ramachandran plot using the RAMPAGE server and quality
model assessment with ProSA (protein structure analysis) server,
respectively (41, 42). ProSA web was used to check and compare
the obtained protein structural models with those experimen-
tally determined by X-ray crystallography or NMR (42). The
ProSA z-score indicates overall model quality and measures the
deviation of the total energy of the structure with respect to an
energy distribution derived from random conformations. The
APBS software was used to calculate the spatial distribution of
electrostatic potential on protein atoms using a two-dielectric
implicit solvent model and the finite difference method to solve
the Poisson–Boltzmann Equation (43). The dielectric constant
used was 4 for proteins and 80 for the solvent.

MD Simulations
The CRF1Rs and the CRF2R/CRF-BP complex were inserted
into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine POPC
lipid membrane considering the spatial arrangements of the
protein with respect to the hydrocarbon core of the lipid bilayer,
as obtained from the OPM database (44). For the CRF1R sys-
tems, a 150 Å × 150 Å × 120 Å box consisting of the protein,
lipids, classic TIP3P model for water molecules, and 150 mM
KCl was generated using the membrane builder module of CHARMM-GUI (45, 46). In a similar fashion, the CRF2αR/CRF-BP complex was embedded in a 140 Å × 140 Å × 160 Å box. MD simulations were carried out with the NAMD 2.9 simulation package (47), using the CHARMM36 force field parameters for proteins and lipids (48, 49). Periodic boundary conditions were imposed in all three directions and the Particle Mesh Ewald method was used to account for full long-range electrostatic interactions within the selected boundary condition within a relative tolerance of 1 × 10⁻⁶ (50). The final systems were composed of nearly 235,000 atoms for CRF2Rs, and nearly 255,000 atoms for the CRF2αR/CRF-BP complex. The simulations were started from different seeds, and three replicas of 100 ns for each CRF2 receptor were performed, while a single 100 ns simulation was performed for the CRF2αR/CRF-BP complex. A 12 Å cutoff was used to compute non-bonded interactions with a smooth switching function applied at a distance of 10 Å. To impose the thermal exchange with an external thermostat, the isobaric–isothermal ensemble (NPT) with constant number of particles N, pressure P, and temperature T was used. Constant temperature was maintained by coupling the system to a thermal bath whose temperature is maintained via Langevin dynamics with a friction coefficient of 1 ps⁻¹. Constant pressure was maintained using a Langevin piston at a nominal value of 1 atm (51). The SHAKE algorithm, with a tolerance of 1 × 10⁻⁸ Å, was applied to constrain the length of all covalent bonds involving hydrogen, thus allowing the use of a 2 fs integration time step along with the r-RESPA integrator, which allows a multiple time step scheme where bonded, short-range non-bonded, and long-range electrostatic terms are calculated every 2, 2, and 4 fs, respectively. By plotting Cα-root-mean-square deviation (RMSD) and RMS fluctuation (RMSF) along the MD simulation, we assessed the structural equilibration reached by our models. To further characterize the structure of the CRF2Rs, three parameters were calculated: angle phi, defined between the hinge region (connecting the TM with the ECD) and the center of mass (COM) of the TM domain; angle theta, defined between the hinge region and the COM of the ECD; and the distance between the COM of both domains. Both angles helped define the orientation of the ECD with respect to the XY plane (parallel to the membrane plane) and the Z axis (perpendicular to the membrane) (52). Also, for the CRF2αR/CRF-BP complex, the total internal energy of the complex was calculated, as well as the total interaction energy between the CRF2αR and the CRF-BP in terms of its electrostatic and van der Waals components. These calculations were performed using the NAMD Energy analysis tool available in the Visual Molecular Dynamics v1.9.3 (VMD) software (53).

**FIGURE 1** | Molecular model of CRF-BP: (A) Secondary structure depiction of the obtained folding for human CRF-BP. The residues forming part of disulfide bridges are shown with the carbon atoms in magenta stick representation. (B) Electrostatic potential surface plotted onto the solvent accessible surface (+1 kT/e). The positive and negative electrostatic values are colored in blue and red, respectively.
Protein–Protein Docking and Protein–Protein Interactions (PPIs) Calculations

Protein–protein docking was performed using Hex v8.0 with default parameters (54). Briefly, for the generation of the top scoring solutions, we used an initial Steric Scan at $N = 16$, followed by a Final Search at $N = 25$, obtained by using just the steric contribution to the docking energy. We used the Shape only correlations, the 3D Fast Lite as FFT mode, with a grid dimension of 0.6 Å. These orientations are sorted by calculated energy, and a new set of trial orientations are generated for the top scoring. 10,000–20,000 orientations using the Scan Step and SubSteps were used to construct new distance samples in steps of ±(Scan Step 0.75 Å)/(Substeps 2) from the initial orientations, 1 Å resolution was used to scan the search space and a 0.5 Å resolution was used to perform the high-resolution scoring (55). A final minimization protocol for the top scoring solution complexes consisted of 20,000 steps of steepest descent method, followed by 10,000 steps of conjugate gradient method to reach a final RMS gradient of 0.001 kcal/mol Å² to obtain the final models. Protein interactions such as disulfide bonds, hydrophobic interactions, ionic interactions, hydrogen bonds, aromatic–aromatic interactions, aromatic–sulfur interactions, and cation–π interactions within a protein or between proteins in a complex were calculated using the PPI server (56).

**RESULTS**

Corticotropin-Releasing Factor Binding Protein (CRF-BP) Modeling and Validation

We have previously demonstrated that CRF-BP and CRF$_{2\alpha}$R interact (20). In order to further characterize this interaction and predict which residues are forming the interacting interface, we reasoned that the prediction of structural models for CRF-BP and CRF$_{2\alpha}$R were necessary. There are no crystal structures or modeling for CRF-BP. As CRF-BP sequence is conserved among species but displays no significant sequence similarity to any other known
protein experimentally resolved; a threading approach was used to predict a model of the structure of CRF-BP. Fragments from neuropilin (PDB: 2QQL) (36) and gastric intrinsic factor receptor cubilin (PDB: 3KQ4) (35) were used as starting templates. The predicted structural model for CRF-BP fold consisted in two modules. The first module containing residues 50–180 which displayed two short alpha helices, six antiparallel beta-sheets, and the first pair of disulfide bridges C60–C81 and C104–C141. The second module comprised residues 180–245 of the protein, with a pair of beta-sheets and one alpha helix that contained a second pair of disulfide bridges C183–C205 and C237–C264 (Figure 1A). An electrostatic potential surface (EPS) was obtained for CRF-BP. In the protein, two acidic patches were observed (Figure 1B left, red colored) and when the protein was turned in 180°, two basic patches were observed (Figure 1B right, blue colored). In order to validate the predicted model, a Ramachandran plot distribution and a ProSA protein quality analysis were performed. Ramachandran statistics showed that more than 95% of the residues of the predicted model were in the allowed geometric regions for amino acids (Table 1; Figure S2A in Supplementary Material). Although some amino acids were positioned in the non-allowed regions, they were residues participating in protein turns. This result indicates that the obtained fold is feasible. ProSA protein quality analysis casted out a Z-score = −3.46 (Table 1), value that falls in the range of native structures (Figure S2B in Supplementary Material) (42). The obtained binding mode was in agreement with site-directed mutagenesis data from CRF-BP.

CRF-BP is a protein that binds CRF and UCNI with high affinity, and these interactions have been well characterized (58, 59). To further validate the CRF-BP structural model, protein–protein docking experiments between CRF-BP with CRF and UCNI were performed and the predicted residues involved in this interaction were obtained and compared with previously published data. The obtained binding modes predicted that the C-terminal domain of CRF and UCNI may bind over the positively charged surface at the N-terminal domain of CRF-BP (Figure 2A). The CRF-BP/CRF interaction comprises mainly CRF-BP residues R55, R56, C60, L61, D62, M63, L64, T71, F72, and T73, and CRF residues V18, M21, A22, E25, Q26, A28, and Q29 (Figure 2B). In addition, the CRF-BP/UCNI interaction comprises mainly CRF-BP E51, R55, R56, C60, L61, D62, M63, L64, S65, I86, and W116, and UCNI L18, L21, A22, S26, E29, E32, Q33, N34, I36, and D39 (Figure 2C). This obtained binding mode was in agreement with site-directed mutagenesis data from CRF-BP.
(12). Furthermore, it has been previously described that CRF-BP binds CRF as a dimer (59). Therefore, we also performed protein-protein docking experiments for two CRF-BP alone and with CRF in order to further validate our CRF-BP structural model. The results showed that the CRF-BP model was permissive for a symmetrical homodimerization arrangement (Figure 3A) and for interacting with CRF as a dimer (Figure 3B). Thus, all the aforementioned validation approach suggest that the predicted fold is feasible, of good quality, and in agreement to previously published data (12, 60). In addition, the obtained binding model for (CRF-BP/CRF)2 predicted that residues 19–38 of CRF are sandwiched by the CRF-BPs. In addition to already described interaction between CRF and a CRF-BP monomer, CRF may also interact with two patches within the C-terminal domain of CRF-BP (Figure 3C). CRF-BP residues 235–238 and 257–264 contacts CRF, with prediction of the residue E238 from CRF-BP displaying H-bond interactions with R23 and Q26 of CRF. CRF-BP D262 main chain carbonyl group also contact R23 of CRF. An additional H-bond interaction is predicted to occur between the side chain of T258 from CRF-BP and the main chain NH group of N34 from CRF.

**Corticotropin-Releasing Factor Type-2 Alpha (CRF2αR) and Type-2 Beta (CRF2βR) Receptors Modeling and Validation**

There are still no full-length CRF receptor crystal structures available (21, 22). For CRF2αR and CRF2βR, only the ECD structures obtained by NMR are available, human CRF2αR-ECD in complex with UCNI, and murine CRF2βR-ECD in complex with Astressin analog peptide (PDBs: 3N96 and 2JND, respectively) (23, 24). Thus, the recently solved crystal structure of CRF-R, in addition to the CRF2αR and CRF2βR ECDs available structures, were used as templates to predict a model for the structure of CRF-Rs (Figure 4A). The crystal structure of the transmembrane bundle from glucagon receptor (PDB: 4L6R) was used to model the extended helix 1 in the N-terminal region (TM1stalk region), the intracellular loop 2 (IC2), and the helix 8 in the C-terminal region, which are absent from the CRF-R crystal structure (21, 61). Considering the already available structural information and guided by similar works in class B GPCRs, the N-terminal domain of CRF was located in a position able to interact with the J-domain on the CRF-R TM bundle (62, 63). The EPS obtained for CRF-Rs showed that the electrostatic potential is similar for both receptors, with minor differences in the N-terminal region. The CRF2αR (Figure 4C) showed a more extensive basic patch than CRF2βR (Figure 4B).

Molecular dynamics were performed in order to test the stability of the CRF-R models including the ECD and TM regions. The receptors were embedded in a pre-equilibrated POPC lipid bilayer and solvated using the Membrane Builder in the CHARMM-GUI web server (Figure S3 in Supplementary Material). Each system was subjected to a 100 ns of MD simulations with three replicas. The RMSD and RMSF were computed over the course of the simulation for the Ca atoms of the proteins to measure structural stability and qualitatively characterize the dynamics of the proteins.
The CRF2αR and CRF2βR MD trajectory analyses showed no significant changes in the RMSD values for the ECD (red lines) and TM (blue lines) regions (Figures 5A,D). However, the RMSD values calculated for the full-length receptors showed a significant change for CRF2αR and CRF2βR (Figures 5A,D, black lines). The changes obtained for CRF2αR can be attributed to translational and rotational movements of the ECD relative to the TM domain. The values obtained are coincident with three main different conformational states for CRF2αR: open-like, semi-closed, and closed-like (Figure 5B). The closed-like state was the one obtained at the end of the simulation, indicating that the receptor has a higher tendency for that conformation. In the case of CRF2βR, the values obtained are coincident with only one open-like conformational state, which varies the angle of extension (Figure 5E). RMSF describes the average fluctuation of each Cα atom of the amino acid residues in the proteins over the simulation time (Figures 5C,F). The general fluctuations of specific regions of the proteins are similar for both CRF2R. Within the TM region, the peaks of higher movement are coincident with the intra and extracellular loops. Moreover, in the ECD region, the peaks with higher movement are coincident with the loop that connects the α-helix with the β sheet bundle, and with the loop that connects the ECD with the TM region (stalk region).

Both CRF2αR and CRF2βR showed great variation in phi and theta angles (Figure 6). Average theta angles for CRF2αR are near to the 40°–50° range, while for CRF2βR the average value is close to 20° ± 10° (Figure 6B). According to the data described for the GCGR, an angle of less than 20° corresponds to the closed state of the receptor while values close to 40° are associated to an opened state (52). Upon analyzing the phi angle, CRF2αR reaches an average value of 65° ± 20°, while CRF2βR stays at higher values at 85° ± 20° (Figure 6D). Analog to angle theta, low values of

![Figure 5](image-url)
angle phi (~20°) have been associated with the closed conformation, which would mean that our simulations are either in the open conformation or in a semi-closed conformation. This is further supported by the distance between ECD and TM COMs, where both receptors reach similar values in the range of 55–60 Å (Figure 6C and Figure S4 in Supplementary Material), and this distance was associated with an open conformation. The results suggest that CRFβR is more stable and displays mainly only one conformational state and that CRFαR is less stable, reflected by more fluctuations within the Cα, and it has three main conformational states. In addition, in both receptors, the ECD region is the one with more fluctuations.

**Protein–Protein Docking for CRF-BP Binding to CRF₂Rs**

We previously demonstrated that CRF-BP physically interacts with the ECD of the α but not with the β CRF-R isomorph (20). In order to test our models, a protein–protein docking was performed to predict the potential binding mode for CRF-BP with the ECD region of CRF₂Rs. For the CRF-BP/CRF₂αR interaction, characterization (20) immunofluorescence co-localization analyses using the Santa Cruz Biotechnology N-20 anti CRF₂R antibody (residues W27-Q46 for CRF₂αR and 153-Q73 for CRF₂βR) were performed. We reasoned that, if the residues recognized by the antibody are available to bind the antibody they should not be participating on the CRF-BP/CRF₂R interaction, thus, these residues were excluded from the search space during the protein–protein docking assay. The best solution obtained for CRF-BP/CRF₂αR docking casted out a total energy value of −838.1 kJ/mol and for CRF-BP/CRF₂βR −685.9 kJ/mol (Table 2, Figure S5 in Supplementary Material). Both protein have similar number of residues (382 and 372) and molecular mass (44.53 and 43.74 kDa); therefore, the estimated binding energy values suggest that CRF-BP could bind to CRF₂αR and form a more stable complex compared to CRF₂βR.

Molecular dynamics simulations were performed to test the stability of the predicted CRF-BP-CRF₂αR model and qualitatively characterize the dynamics of the complex (Figure 7). The CRF-BP-CRF₂αR complex MD trajectory analyses showed no significant changes in the RMSD values for the CRF-BP N-terminal domain (blue lines). However, the RMSD values calculated for the full-length complex, CRF-BP and CRF-BP C-terminal domain display a significant variation (Figure 7A green, black, and red lines, respectively). The changes can be attributed to translational and rotational movements of
Table 2 | Protein–protein docking for CRBP binding to CRF2 subtype receptors.

| CRFαR-CRFPB | CRFβR-CRFPB |
|-------------|-------------|
| Cluster     | Solution    | Etotal (kJ/mol) | Cluster | Solution | Etotal (kJ/mol) |
| 1           | 1           | −838.1          | 1       | 1         | −685.9          |
| 2           | 2           | −827.2          | 2       | 2         | −672.2          |
| 3           | 6           | −783.2          | 3       | 4         | −661.7          |
| 4           | 7           | −756.6          | 4       | 5         | −642.0          |
| 5           | 9           | −735.6          | 5       | 6         | −629.8          |
| 6           | 10          | −734.4          | 6       | 7         | −627.3          |
| 7           | 11          | −702.4          | 7       | 8         | −617.4          |
| 8           | 13          | −700.9          | 8       | 9         | −608.0          |
| 9           | 13          | −698.9          | 9       | 10        | −597.0          |
| 10          | 14          | −698.1          | 10      | 11        | −581.2          |

The ECD relative to the TM domain and particularly the C-terminal domain of CRF-BP, in agreement to the higher RMSF of the C-terminal domain of CRF-BP (Figure 7B). The energy of interaction of the CRF-BP-CRFαR complex during the dynamics indicates that the complex is stable and that the main contribution comes from electrostatics rather than van der Waals interactions (Figure 7C). This phenomenon was observed along with loss of connections between C-terminal domain of CRF-BP and CRFαR, as shown by enhanced flexibility with respect to starting conformation. The interaction between CRF-BP and CRFαR, the amino acids present in the interacting interface previously determined using the Protein interaction server (56), were also measured during the CRF-BP-CRFαR complex dynamics. For CRF-BP/CRFαR interaction interface, hydrophobic interactions (Figure 8A), ionic interactions within 6 Å (Figure 8B), cation–π interaction within 6 Å (Figure 8C), aromatic–aromatic interactions within 4.5–7 Å (Figure 8D), protein–protein side chain hydrogen bonds (Figure 8E), and protein–protein main chain hydrogen bonds were characterized (Figure 8F). Interactions remain in a similar range through most part of the dynamics upon loss of contacts in the last part of the simulation time, in agreement with a higher RMSF of this zone in CRF-BP.

In order to explore the relative contribution of these residues in the binding affinity for CRF-BP/CRFαR, different point mutations of CRFαR, and mutant combinations, were generated and the binding energy values were determined and compared with the WT (Table 3). The deletion of the first 12 amino acids (12aa) has a high contribution to the loss of the binding energy values predicted, and the loss was even higher in the 12aa/Y95Q/S96G/Q97E mutants. These results suggest that the first 12aa, that conform the α-helix, are important contributors and have synergistically effects with Y95, S96, and Q97 for CRF-BP/CRFαR binding affinity.
**DISCUSSION**

In the present study, we predicted and validated the structural models for CRF-BP, CRF_αR, and CRF_βR. These models could be used for future investigation in order to further explore the CRF system. In addition, we were able to predict a higher possibility of interaction of CRF-BP with CRF_αR than CRF_βR. Even more, we predicted the residues that could be participating in the CRF-BP/CRF_αR interaction.

The predicted CRF-BP model displayed good Ramachandran plot distribution and ProSA protein quality assay. In addition, the predicted binding modes of CRF-BP with CRF and CRF-BP with UNC1 are consistent with published site-directed mutagenesis data and functional assays showing pivotal roles for CRF-BP R56 and D62 in the interaction with CRF and R56, M63, and L64 in the interaction with UNC1 (12). Even more, the obtained CRF-BP model was permissive for homodimerization and for interacting with CRF as a dimer, which is also in agreement with previously published data indicating that the CRF-BP generates a dimer form complex after binding to CRF (64). Furthermore, CRF-BP contains an alpha helix in the N-terminal region determined as the sorting signal for CRF-BP to entering the regulated secretory pathway (*Unpublished results*), the obtained CRF-BP model also presents this secondary structure. Even though we were able to obtain low-accuracy model (65) for CRF-BP because it shares less than 30% of sequence homology with the protein fragments used as templates, the aforementioned data all together demonstrated that the obtained CRF-BP model is feasible, of good quality and in agreement to previously published data. Thus, the model is suitable for further structural predictions and modeling of PPIs.

For CRF_Rs, we were able to obtain a high-accuracy model due to a high sequence homology with the template structures (65). Models were obtained using a comparative modeling approach and the crystal transmembrane structure of CRF_R (27) that shared a 70% sequence homology with CRF_Rs (9), and to
TABLE 3 | Protein–protein docking energies of CRF-BP to CRF₂R mutants.

| Protein | Mutant residues | Etot (kJ/mol) |
|---------|-----------------|--------------|
| H7      | E11 F68 V71 Y73 Y95 S96 Q97 |
| WT      |                 | −657.1       |
| MUT 1   | D K             | −672.3       |
| MUT 2.1 | L V L           | −686.6       |
| MUT 2.2 | L G L           | −690.7       |
| MUT 2.3 | L V E           | −690.7       |
| MUT 2.4 | L G E           | −660.5       |
| MUT 2.5 | Q V L           | −700.6       |
| MUT 2.6 | Q G L           | −700.6       |
| MUT 2.7 | Q V E           | −660.7       |
| MUT 2.8 | Q G E           | −639.2       |
| MUT 3.1 | N S L           | −655.3       |
| MUT 3.2 | N S Q           | −641.8       |
| MUT 3.3 | L S L           | −705.9       |
| MUT 3.4 | L S Q           | −629.9       |
| MUT 4   | D K Q G E       | −655.5       |
| MUT 5   | D K L S Q Q G E | −639.9       |
| MUT 6   | D K L S Q Q G E | −621.4       |
| MUT 7   | D K L S Q Q G E | −623.1       |

Deletion F68 V71 Y73 Y95 S96 Q97
| MUT 8 | 1–12 F88 V71 Y73 Y95 S96 Q97 |
| MUT 9 | 1–12 Q G E |
| MUT 10| 1–12 L S Q Q G E |
| MUT 11| 1–12 L S Q Q G E |

N-terminal ECD of human CRF₂αR (23), and murine CRF₂βR (24) obtained by NMR as templates.

The accuracy of the model is important to define the predictions and studies that can be performed with them. As our models are not obtained by NMR or X-ray, that could achieve even a 100% accuracy, limitations in their use to study catalytic mechanisms, and designing and improving ligands are needed to be considered, although, our models can be used in studies including, docking of small ligands, defining antibody epitopes, refining NMR structures, among others (65).

The RMSD values obtained for CRF₂R showed movements of the ECD related to the TM domain and suggest three different conformational states. Similar results have been observed for the glucagon receptor (52, 63), which are consistent with the behavior of the two domain model described for class B GPCRs (66).

Considering the conformational states obtained for the CRF₁Rs in the MD, the higher tendency for CRF₁βR and CRF₁αR is to be in the closed-like and semi-closed conformation, respectively. The differences could be explained by the differences in length and composition in the CRF₁αR and CRF₁βR N-terminal domain. It has been described that CRF₁αR and CRF₁βR differ in their N-terminal domain, due to alternative splicing: the first 34 amino acids of the CRF₁R α isoform are replaced by 54 different amino acids in the β isoform (67). In addition, the β isoform has a cleavable signal peptide while the α isoform has a non-cleavable pseudo signal peptide, resulting in the absence and presence of the N-terminal α-helix, respectively (68, 69). Considering that CRF₁αR is localized mainly intracellularly (70–73) and CRF-BP binds the N-terminal domain of CRF₁αR and acts as an escort protein increasing the levels of the receptor in the plasma membrane (20), an open-like conformational state tendency for CRF₁αR should be expected in order to be able to interact with CRF-BP. Even more, the CRF-BP/CRF₁αR MD suggests that CRF-BP stabilizes the receptor in the open-like state.

The protein–protein docking performed between CRF-BP with CRF₁αR, and CRF-BP with CRF₁βR showed higher affinity of CRF-BP for CRF₁βR than CRF₁αR, this is in agreement with our previous results showing an isoform specific interaction between CRF-BP and CRF₁αR (20). MD simulations of the predicted binding mode of CRF-BP to CRF₁αR indicates that interactions remain in a similar range through most part of the dynamics upon loss of contacts in the last part of the simulation time, in agreement with a higher RMSF of this zone in CRF-BP. This phenomenon was observed along with loss of connections between C-terminal domain of CRF-BP and CRF₁αR, as shown by enhanced flexibility with respect to starting conformation.

In addition, considering the aforementioned differences between CRF₁αR and CRF₁βR in their N-terminal region and the EPS obtained for both CRF₁R isoforms showing different charge patches in their N-terminal region it makes sense to predict an isoform specific CRF-BP/CRF₁R interaction dependent on the first 12aa in the N-terminal region. Even more, CRF-BP is a CRF₁αR escort protein, and there is evidence showing that the escort proteins RAMP1-3 bind the α-helix of the calcitonin receptor (74), further supporting the idea of CRF-BP binding the first amino acids, which conforms the α-helix of the CRF₁αR. Although, experimental approaches will be necessary to confirm the interacting interface.

It has been described that CRF-dependent neuronal plasticity in the VTA and stress-induced relapse to cocaine seeking behavior is dependent on CRF-BP and CRF₁R (16, 17). It would be interesting to determine if the interaction between CRF-BP and CRF₁R is necessary for these CRF-dependent effects. The structural models generated in the present study could be used for the design of specific peptides capable of blocking the CRF-BP/CRF₁R interaction and test the implication of this interaction on the CRF-dependent effects.

There is a large fraction of sequences whose structure is difficult to be determined experimentally, like GPCRs, thus, structure prediction is important to obtain structural information. In this regard, the models reported herein provide a structural framework to work on further hypotheses and open new avenues of research on the CRF system.

CONCLUSION

In summary, our results provide the first molecular models for CRF-BP and for full-length CRF₁αR and CRF₁βR. These molecular models allowed predicting the residues involved in the CRF-BP/CRF₁αR interaction. These results are the starting point for future studies of the effect of the CRF-BP/CRF₁αR interaction on stress-induced relapse to drug seeking behavior.
AUTHOR CONTRIBUTIONS

PS, KG, and CL conceived and designed research; CL and SG-M performed molecular modeling and simulations; PS, SG-M, CL, and KG analyzed data; PS, KG, and CL wrote the paper. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2018.00043/full#supplementary-material.

DATA SHEET S2 | PDB files coordinates for all proteins.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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