Two-dimensional crystallization of the mouse serotonin 5-HT_{3A} receptor

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Abstract

The mouse serotonin 5-HT_{3A} receptor is a homo-pentameric ligand-gated ion channel (pLGIC) mediating fast excitatory neurotransmission in the central nervous system. The molecular mechanism of ion permeation of 5-HT_{3A} receptors triggered by the neurotransmitter serotonin is not yet fully understood. The recent X-ray structure of the mouse serotonin 5-HT_{3A} receptor in complex with a stabilizing nanobody revealed for the first time the entire structure of a mammalian pLGIC in detergent. Structural information of the receptor in a lipid bilayer however is still limited primarily due to the lack of 2D crystals of the receptor in a lipid bilayer. Here we present our results on the formation and improvement of diffracting 2D crystals of the mouse 5-HT_{3A} by limited proteolysis and addition of conformational nanobodies.

Keywords 5-HT₃ receptor; 2D crystals; pentameric ligand-gated ion channel; nanobody; electron crystallography

1. Introduction

The serotonin 5-HT_{3A} receptor participates in signal transduction of the central and peripheral nervous system. It belongs to the family of pentameric ligand-gated ion channels (pLGICs), which are responsible for fast neuronal signaling and thus its malfunction is related to many neurological disorders (Nemecz et al., 2016). In the past ten years structures of different pLGIC have been solved in various conformations revealing their common quaternary arrangement (Tab. 1). The experimentally resolved structures of different pLGICs together with molecular modeling and molecular dynamics simulation allowed hypothesizing the activation mechanism of the pLGICs. However, the entire molecular mechanism involving ligand binding, subsequent channel opening and ion permeation remains still under debate (Nemecz et al., 2016; Yuan et al., 2016).

The recent X-ray structure of the mouse serotonin 5-HT_{3A} receptor in complex with the conformational nanobody VHH15 (PDB: 4PIR, (Hassaine et al., 2014)) revealed for the first time entire structure of a mammalian pLGIC including the intracellular domain (ICD). The ICD of pLGICs plays an important role in trafficking and clustering of receptors at the postsynaptic membrane (Thompson et al., 2010; Zuber and Unwin, 2013) and modulates gating kinetics (Bouzat et al., 1994) and channel conductance in 5-HT_{3A} receptor (Kelley et al., 2003; Peters et al., 2010). The inhibitory nanobody bound specifically between two adjacent subunits at the extracellular domain (ECD) covering the ligand binding pocket, but the expected closed state of the serotonin 5-HT_{3A} receptor could not be clearly identified. The pore diameter measured in the transmembrane region was between the expected values of open or closed channels, while the backbone conformation was comparable to an open channel (Hassaine et al., 2014). A recent molecular dynamics (MD) simulation of the 5-HT_{3A} receptor integrated in a lipid bilayer indicated that the crystal structure represents an non-conducting state where even

water molecules are excluded from the hydrophobic gate in the transmembrane region (Yuan et al., 2016). Although the MD simulations resolved the first structural transitions in the receptor after ligand binding, the entire process of receptor activation is not fully resolved.

Table 1 Overview of available structures and conformations of pentameric ligand-gated ion channels (pLGIC)

	Conformational state	Reference
Nicotinic Acetylcholine receptor (nAChR) (Torpedo)	closed	(Unwin, 2005)
	open	(Unwin and Fujiyoshi, 2012)
Ligand-gated ion channel of <i>Gloeobacter violaceus</i> (GLIC) (Bacterial)	closed	(Prevost et al., 2012)
	resting	(Sauguet et al., 2014)
	open	(Bocquet et al., 2009; Hilf and Dutzler, 2009)
Ligand-gated ion channel of <i>Erwinia chrysanthemi</i> (ELIC) (Bacterial)	closed	(Hilf and Dutzler, 2008)
Glutamate-gated chloride receptor (GluCl) (<i>C. elegans</i>)	closed/resting	(Althoff et al., 2014)
	open	(Hibbs and Gouaux, 2011)
γ -aminobutyric acid receptor A (GABA _A R) (human)	desensitized	(Miller and Aricescu, 2014)
Serotonin 3A receptor (5-HT _{3A} R) (mouse)	closed	(Hassaine et al., 2014)
Glycine receptor (GlyR) (zebrafish and human)	closed	(Du et al., 2015; Huang et al., 2015)
	open	
	modulated	

These open questions encouraged us to investigate the receptor structure in a more native-like environment. Lipid bilayers can provide lateral pressure on the receptor (Marsh, 2007) and their lipid composition can modulate the ion channel properties (Barrantes, 2015). Therefore we reconstituted purified receptors into lipid bilayers and analyzed the resulting 2D crystals by negative stain and cryo electron microscopy (EM).

In this crystallization note we describe how to obtain diffracting 2D crystals of the mouse serotonin 5-HT_{3A} receptor by limited proteolysis and how this affects crystal formation. Furthermore, we successfully improved the 2D crystal diffraction quality by addition of a conformational nanobody. Though nanobodies have been widely used as crystallization chaperon in 3D crystallization, no approach in 2D has been demonstrated so far.

2. Material and methods

2.1. Expression and purification of mouse 5-HT_{3A} receptor and nanobody VHH7

Mouse 5-HT_{3A} receptor was expressed and purified as previously described (Hassaine et al., 2014, 2013) with following modifications. Cells were disrupted using an Ultra-Turrax (T25 basic, IKA) on ice: 30 sec at each speed, 1 min at highest speed, 30 sec pause in between. Non-lysed cells were removed by centrifugation (500 x g, 10 min, 4°C, Eppendorf centrifuge 5810R) and the crude plasma membranes were collected by ultracentrifugation (235,000 x g, 1.5h, 4 °C, Ti45 rotor). After solubilization of the crude plasma membranes with 1% C₁₂E₉ (50 mM Tris pH 7.4, 500 mM NaCl) rotating for 2h at 4°C, non-solubilized material was removed by ultracentrifugation (370,000 x g, 1.5h, 4 °C, Ti70 rotor). The supernatant was filtered through a 0.45 µm filter and applied to a 1ml StrepTactin column (StrepTrap HP, GE Healthcare) equilibrated with 50 mM Tris pH 7.4, 125 mM NaCl, 0.01% C₁₂E₉ (running buffer). The column was washed with 25 column volumes of running buffer before eluting the protein with running buffer containing 5 mM d-desthiobiotin. The fractions eluting around 1.6 ml were pooled and concentrated to 2-3 mg/ml using a 100 kDa MWCO concentrator (Vivaspin). The concentrated, full-length receptor was treated with trypsin as previously described (Hassaine et al., 2014). The trypsinized sample was concentrated to ~300 µl and applied to a Superdex200 10/300 size-exclusion chromatography column (GE Healthcare) equilibrated with running buffer. The peak fractions around 10.5 ml were pooled and concentrated to 2-3 mg/ml. The trypsinized sample was further treated with chymotrypsin under the same condition as described for trypsin. The trypsin-chymotrypsin-treated receptors were directly used for 2D crystallization.

Nanobody VHH7 was expressed and purified as previously described (Hassaine et al., 2014).

2.2. 2D Crystallization

5-HT_{3A} receptors (full-length, trypsin-treated or trypsin-chymotrypsin-treated) purified in C₁₂E₉ were mixed with DOPC or DOPC/DOPG at a weight ratio of 4:1 (4 mg/ml in 0.02% C₁₂E₉; Avanti polar lipids) to obtain lipid-to-protein ratios of 0.2-0.5 (w/w) and then diluted to 1 mg/ml with crystallization buffer (50 mM HEPES 7.5, 125 mM NaCl, 3 mM NaN₃, 0.02% C₁₂E₉; final volume: 30 µl). The mixture was kept at 4 °C overnight and then dialyzed for 14 days at 22 °C against detergent free crystallization buffer using a 100 kDa MWCO Vivaspin500 concentrator.

2.3. Nanobody-receptor complex formation on 2D crystals

Suspensions of trypsin-chymotrypsin-treated 5-HT_{3A} receptor 2D crystals were mixed with purified nanobody VHH7 in a 1:5 molar ratio (1 mole receptor in 2D crystals estimated by the amount used in crystallization setups, 5 mole VHH7) and incubated for 2 h at 4 °C. The crystals were washed three times by centrifugation (10000 x g, 5 min, 22 °C, Eppendorf centrifuge 5424) and resuspension of the pellet in dialysis buffer (50 mM HEPES 7.5, 125 mM NaCl, 3 mM NaN₃; final volume 20 µl).

2.4. Negative stain and cryo-EM

3 µl of crystal suspension was applied to a glow discharged (5 mA, 15 sec, Cressington carbon coater 208carbon with glow discharge unit) continuous carbon coated copper grid (3 mm, 400 mesh, in-house coating) for 1 min. Excessive liquid was removed by blotting and the sample was stained using 3 µl of 2% uranyl acetate for 1 min. After removing excessive stain by blotting, the grid was air-dried. For cryo-electron microscopy, 3 µl of crystal suspension was loaded onto a glow discharged holey-carbon grid (Quantifoil, R2/1) coated with a continuous-carbon film and incubated for 1 min. Afterwards the grid was rapidly plunge frozen in liquid ethane using a FEI Vitrobot (100% humidity, blot force 1-3, blot time 2-4 s).

2.5. Image processing

Images were acquired under low dose conditions (Fujiyoshi, 2013) with an exposure time of 1 sec and an intensity of 10 e/A². Negatively stained samples were imaged at 74,000 times magnification (JEOL2200FS, 200kV) and cryo electron microscopy images at 50,000 times magnification (Philips CM200 FEG, 200 kV), both on a CCD camera (TVIPS F416, 4k x 4k). Images showing a diffraction pattern in the calculated Fourier transform were processed with the 2dx software package (Gipson et al., 2007; Stahlberg et al., 2015), following the standard procedures and protocols.

2.6. Generation of 3D model

The 2D projection map was loaded into COOT (Emsley et al., 2010) and two copies of the 5-HT_{3A}-VHH15 complex (extracted from PDB: 4PIR; assuming similar binding of VHH7) were manually arranged to fit into the determined density areas, with reasonable protein arrangement along the z-axis to reflect the lipid bilayer. In order to visualize a 2D crystal with classical 3D crystallographic tools, a very long c-axis of 1000 Å and symmetry P2₁2₁2₁ was assumed, leaving an a/b plane with P12₁1 symmetry.

3. Results and discussion

Limited proteolysis of the purified mouse serotonin 5-HT_{3A} receptor had improved the diffraction quality of 3D crystals (Hassaine et al., 2014) and thus we explored this approach to analyze the effect on 2D crystallization. We therefore tested three different protein samples: I) the intact mouse 5-HT_{3A} receptor including two Twin-Strep-tags per subunit for affinity purification (full-length), II) the truncated receptor obtained by limited proteolysis with trypsin (trypsin-treated) and III) subsequent treatment with chymotrypsin (trypsin-chymotrypsin-treated). All samples were pure and homogeneous as shown by SDS-PAGE (Fig. 1D) and negative stain EM images respectively (Fig. 1A-1C). The top and side views reflecting the shapes calculated from the 3D structure are shown in the right panel of Fig. 1A-1C.

Negative stain EM was used to compare the different protein samples reconstituted in DOPC lipids. While reconstitution of full-length receptor resulted in small tightly packed vesicles with 60-160 nm in diameter (Fig. 1E), size and packing improved for the trypsin-treated receptor to 100-225 nm (Fig. 1F). Shape and packing of the vesicles were comparable to recently published electron tomography data showing that all receptors facing the same direction (Kudryashev et al., 2016). The conical shape of the receptor limits the final size of the proteoliposomes. Additional limited proteolysis of trypsin-treated receptors with chymotrypsin enhanced the quality of the reconstitution, allowing the 5-HT_{3A} receptor to form larger (275-500 nm), crystalline single bilayer lipid patches (Fig. 1G). The Fourier transform (FT) of these patches showed low resolution diffraction patterns of ~50 Å (Fig. 1H). The size of the crystalline patches was further increased by changing to a lipid mixture of DOPC/DOPG (4:1, w/w) to 600-750 nm (Fig. 2A). However no improvement in protein packing could be achieved (Fig. 2B). Image processing resulted in 2D density maps showing two doughnut shaped densities per unit cell with parameters $a = 109.9 \pm 0.8$ Å, $b = 108.9 \pm 1.4$ Å, $\gamma = 90.6 \pm 1.3^\circ$ (Fig. 2C). These densities had the same dimensions as receptor top views calculated from the X-ray structure. The relative intensity of neighboring densities suggested a difference in level of staining between upper and lower membrane side (Chiu et al., 2007), thus the receptor molecules were predicted to be in a head-to-tail packing in the lipid membrane. This protein packing would explain the increased size of crystalline patches with trypsin-chymotrypsin-treated receptors, as the alternating orientation of receptors would relax the membrane curvature.

Figure 1 Effect of modifications to the mouse 5-HT_{3A} receptor. Negative stain electron microscopy images of mouse 5-HT_{3A} receptor. Protein samples in C₁₂E₉: (A) full-length receptor with double Twin-Strep-Tag, (B) trypsin treated receptor, (C) trypsin-chymotrypsin treated receptor; all samples showed homogeneous particle populations with the expected top and side views (inserts). (D) SDS-PAGE of the three protein samples: 1) full-length, 2) trypsin-treated, 3) trypsin-chymotrypsin-treated (monomeric (I) and dimeric (II) subunits, C-terminal fragment (*)). (E) Full-length receptors reconstituted in DOPC formed small (60-160 nm) tightly packed vesicles with no 2D order. (F) Trypsin-treated receptors formed bigger vesicles (100-225 nm). (G) Additional treatment of the receptor with chymotrypsin formed larger single bilayer lipid patches (275-500 nm) with increased 2D order, showing first diffraction spots in the FT (H). Scale bar: (A/B/C) 50 nm, (E/F/G) 100 nm. Box size: 242 Å

The conformational nanobody VHH7 was introduced to further improve 2D crystals of the mouse 5-HT_{3A} receptor. It is known that nanobodies can reduce sample heterogeneity by stabilizing a defined conformation of the target protein and that they can introduce new crystal contacts in 3D crystals. We tested two methods for 2D crystallization of 5-HT_{3A} receptor with nanobody: 1) co-crystallization, leading to smaller, less ordered crystals (data not shown), and 2) decoration of already formed single bilayer lipid patches with nanobody (Fig. 2D). The nanobody most likely reduced the structural flexibility of receptors resulting in higher resolution diffraction in the FT of negatively stained receptor images to ~33 Å (Fig. 2E). Unit cell parameters and 2D projection maps were not altered by nanobody decoration, as judged from the available low resolution information (Fig. 2F). Binding of the nanobody was confirmed by SDS-PAGE (Fig. 2G).

Figure 2 Improving 2D crystal diffraction quality by lipid mixture and nanobody. (A) Negative EM micrograph showing a single bilayer lipid patch of trypsin-chymotrypsin-treated receptors obtained under improved crystallization conditions using a lipid mixture (DOPC/DOPG = 4:1) resulted in further increased patch size (600-750 nm). (B) Corresponding FT showing a similar diffraction pattern as depicted in Figure 1H. (C) Projection map of 2x2 unit cells indicating a head-to-tail (grey-white) orientation of two receptors per unit cell ($a = 109.9 \pm 0.8$ Å, $b = 108.9 \pm 1.4$ Å, $\gamma = 90.6 \pm 1.3^\circ$). (D) The negative stain image of trypsin-chymotrypsin-treated receptor 2D crystals decorated with nanobody VHH7. (E) Decoration further improved crystal packing as showed by an improved diffraction pattern in the FT. (F) The resulting projection map was comparable to undecorated 2D crystals with similar unit cell parameters. (G) Binding of the nanobody (*) was confirmed by SDS-PAGE showing the corresponding band besides monomeric (I) and dimeric (II) subunits of the trypsin-chymotrypsin-treated receptor. Scale bar: 100 nm.

Cryo-EM of the best 2D crystals decorated with nanobody (Fig. 3A) did not result in improved resolution (Fig. 3B). The cryo 2D projection maps revealed slight rearrangements in protein packing compared to negative stain with similar unit cell parameters of $a = 109.7 \pm 1.2 \text{ \AA}$, $b = 107.8 \pm 1.5 \text{ \AA}$, $\gamma = 89.9 \pm 1.1^\circ$ (Fig. 3C), most likely due to absence of the dried uranyl acetate used in negative staining. Each receptor has two closer ($\sim 74 \text{ \AA}$) and two more distant ($\sim 80 \text{ \AA}$) neighbors. The suggested 2D plane group $P12_1$ (ALLSPACE, (Valpuesta et al., 1994)) is a further indication for the head-to-tail packing (Table A.1). This allowed generation of a 3D model to depict the protein arrangement within the lipid bilayer. Assuming full decoration of 5-HT_{3A} receptors with VHH7 results in clashes between nanobodies bound to receptors facing in the same direction (Fig. 3D). This could not be circumvented by any rotation of the receptors. The mentioned clashes could induce heterogeneous packing and therefore would limit the resolution to $\sim 33 \text{ \AA}$ (judging from the diffraction spots in the FT of cryo-EM images). In addition, the 2D crystals tend to form clusters on the grid, which could not be resolved by high or low salt washing, generating thick layers of ice and resulting in low hit rates of crystals in cryo-EM.

Figure 3 Cryo-EM of the best 2D crystal of the mouse serotonin 5-HT_{3A} receptor revealing potential spatial hindrance for full nanobody-decorated receptors. (A) Cryo-EM image of a 2D-crystal of trypsin-chymotrypsin-treated 5-HT_{3A} decorated with nanobodies. (B) The corresponding FT shows sharp diffraction spots up to 30 Å. (C) 2D projection maps with 2x2 unit cells of protein density indicates formation of new crystal contacts through the nanobody resulting in reduced distance of the two receptors per unit cell ($a = 109.7 \pm 1.2 \text{ \AA}$, $b = 107.8 \pm 1.5 \text{ \AA}$, $\gamma = 89.9 \pm 1.1^\circ$). (D) 3D model of a head-to-tail packing of neighboring receptors fitting the 2D projection map was generated using the recent X-ray structure of 5HT_{3A} (purple and gray). The model showed spatial hindrance between fully formed receptor-nanobody complex facing the same direction (receptors: purple, nanobodies with clashes: cyan/blue and orange/red). The yellow nanobody has the potential to interact with the ICD of a receptor facing the opposite direction (gray). Scale bar: 100 nm.

In summary, a major breakthrough in 2D crystallization of the mouse serotonin 5-HT_{3A} receptor was achieved by modification of the receptor with limited proteolysis and addition of conformational nanobodies to reduce sample heterogeneity. This approach could be used as a common technique in cryo-EM to improve the diffraction quality of 2D crystal.

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Contribution

JR purified and crystallized the receptor and collected and processed all electron microscopy data. GH expressed the receptor and expressed and purified VHH7. MC supported cryo electron microscopy sample preparation and data collection at the Center for Cellular Imaging and NanoAnalytics (C-CINA) of the Biozentrum, University Basel. HS, HV, XL supported the project with scientific guidance and discussion, and preparation of the manuscript.

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4. Appendix

All 2D crystallization experiments were performed with receptors purified in C₁₂E₉, a very low-CMC detergent best preserving the ligand binding state of the mouse 5-HT_{3A} receptor (Hovius et al., 1998). We also tried purification of receptors in detergents known for favoring 2D crystallization: DDM, DM, LDAO, OG (Abeyrathne et al., 2012; Lasala et al., 2015), followed by trypsin digestion. The negative stain EM results of receptors transferred into different detergents are shown in Figure A1. Besides C₁₂E₉, DDM and DM could maintain sample homogeneity. In addition, DDM kept the receptor stable against trypsin digestion, like C₁₂E₉. However, no 2D crystals have been found with receptors purified in DDM.

Figure A.1 Negative stain electron microscopy images of trypsin-treated 5-HT_{3A} receptors transferred into different detergents: (A) C₁₂E₉ (B) DDM, (C) DM, (D) LDAO, (E) OG. (F) SDS-PAGE of full-length 5-HT_{3A} receptors 1) after trypsin treatment in different detergents: 2) C₁₂E₉ 3) DDM, 4) DM, 5) LDAO, 6) OG. Only C₁₂E₉ and DDM allowed a controlled limited proteolysis by trypsin (i.e. without complete protein degradation). Scale bar: 50 nm.

Table A.1 Phase residuals of all two-dimensional space groups calculated by ALLSPACE (Valpuesta et al., 1994).

SPACEGROUP		Phase residual vs other spots (90 random)	(No)	Phase residual vs theoretical (45 random)	(No)	Target residual based on statistics taking Friedel weight into account
1	p1	26.9	46	19.7	46	
2	p2	30.7*	23	15.4	46	39.4
3b	p12_b	71.6	16	28.8	8	30.0
3a	p12_a	27.6*	15	51.7	6	29.4
4b	p121_b	16.8*	16	16.2	8	30.0
4a	p121_a	76.9	15	29.0	6	29.4
5b	c12_b	71.6	16	28.8	8	30.0
5a	c12_a	27.6*	15	51.7	6	29.4
6	p222	61.9	54	38.6	46	32.2
7b	p2221b	61.1	54	15.6	46	32.2
7a	p2221a	25.8*	54	15.4	46	32.2
8	p22121	61.9	54	41.7	46	32.2
9	c222	61.9	54	38.6	46	32.2
10	p4	60.9	59	41.3	46	31.7
11	p422	57.4	125	41.0	46	29.2
12	p4212	56.0	125	41.3	46	29.2
13	p3	42.2	20	--	--	26.9
14	p312	53.6	52	20.5	8	27.8
15	p321	43.9	56	34.6	16	28.6
16	p6	44.3	63	16.3	46	31.4
17	p622	47.2	131	16.3	46	29.1

* = acceptable

Used reflections were better than IQ 5 and the maximum resolution was 15 Å